The relationship between vitamin D status in early life and the
developing immune phenotype: lessons from observational
studies and a randomised controlled trial

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STATEMENT OF CANDIDATE CONTRIBUTION

The work presented in this thesis was performed between August 2011 and June 2015 at the School of Paediatrics and Child Health, University of Western Australia under the supervision of Winthrop Professor Susan Prescott, Associate Professor Debra Palmer, Assistant Professor Paul Noakes, Associate Professor Deborah Strickland, Dr Kristina Rueter and Professor Meri Tulic.

All of the experimental work herein, including participant recruitment, questionnaire data and blood collection, blood processing, data management, flow cytometry and data analysis, was performed by the author with the exceptions of those acknowledged below.


Anderson Jones contributed to the conception and design of the study (60%), data and sample collection (90%), analysis and interpretation of data (90%) and manuscript preparation (80%).


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All the work described in this thesis is original and has not been previously submitted for a degree at this or any other university.

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Associate Professor Deborah Strickland of the Telethon Kids Institute, Perth, was instrumental in designing the flow cytometry antibody panel, training and running experiments, and data analysis. Additional training and intellectual input was provided by Dr Bree Foley and Professor Prue Hart, also of the Telethon Kids Institute, Perth.

ABSTRACT

Background: Allergic diseases have reached epidemic proportions in Westernized countries over the last 30-40 years, with similar trends currently emerging in developing areas. The rapid rise in prevalence and coincidence with industrialisation indicates that changes in environment and lifestyle must be responsible. Identifying which
environmental factors play a causal role in the development of allergic disease will be a crucial step in developing primary prevention strategies. Numerous causative factors have been postulated by researchers, among which are changes in sun exposure and dietary patterns that dispose to a reduced vitamin D status in the population.

There are multiple lines of evidence to support a role of vitamin D in allergic disease including geographical variation in prevalence (corresponding to UV exposure), *in vitro* studies demonstrating immunomodulatory properties of vitamin D, and cohort studies reporting associations between vitamin D status and the presence or development of allergic disease. Few of these studies, however, have utilized longitudinal study designs, combinations of immunological and clinical outcomes, or intervention in the form of randomized controlled trials. This PhD thesis aims to contribute to the scientific literature by investigating longitudinally the association between vitamin D status in early infancy the development of allergic disease, in addition to testing the hypothesis that increasing the vitamin D status of infants through supplementation will result in a phenotypic immune profile representing a reduced risk of allergic disease.

**Aims:** The specific aims of this thesis are to: 1) describe the 25-hydroxyvitamin D (25[OH]D) status of pregnant women in Perth, Western Australia, in order to quantify the proportion of this population with suboptimal 25(OH)D levels, whilst identifying intrinsic and extrinsic factors associated with 25(OH)D status (*Chapter 2*); 2) investigate the relationship between 25(OH)D status at birth (cord blood) and in infancy (6 months of age) in relation to risk of clinical allergic disease in infancy and childhood (*Chapters 3 and 4*); and 3) identify differences in infant immune phenotype between participants stratified by vitamin D supplementation, 25(OH)D status and UV exposure (*Chapter 6*).
Methods: Firstly, serum 25(OH)D was measured in n=165 pregnant women recruited for participation in an allergy prevention trial (Chapter 2). These results were analysed in relation to factors previously reported to be associated with 25(OH)D levels, including sun exposure, body mass index, skin pigmentation and vitamin D intake.

Secondly, in a subset of n=231 infants at risk of allergic disease (parental history of atopy), cord blood (CB) 25(OH)D was examined in relation to the development of symptomatic allergic disease by 12 months of age (Chapter 3). This was followed by examination of a subset of n=225 infants whereby 25(OH)D levels at birth and 6 months were analysed in relation to peripheral blood mononuclear cell (PBMC) function at 6 months (Chapter 4), and clinical outcomes at 6, 12 and 30 months of age.

Next, in order to isolate the effects of vitamin D from possible confounders a randomized controlled trial (RCT) was initiated (the Vitamin D in Allergy trial [VITAL]). This pilot trial aimed to investigate differences in the infant immune phenotype dependent on vitamin D supplementation stratification and 25(OH)D levels. Infants were randomized to receive either vitamin D (400 IU cholecalciferol) or a placebo product to 6 months of age. Mononuclear cells and blood plasma were collected at birth (CB) and 6 months of age (n=42). Flow cytometry, utilizing two novel, multicolor panels, was used to phenotypically characterize regulatory (T regulatory cells) and antigen presenting (dendritic cell) components of cord blood mononuclear cells and PBMC collected at 6 months of age. An additional observational component was incorporated in this study, whereby a subset of participants (n=31) were issued ultraviolet radiation dosimeters; the purpose of which was to quantify the contribution
of sun exposure to plasma 25(OH)D levels, and examine independent effects on the immune phenotype.

**Results:** The study of 25(OH)D levels in pregnant women (Chapter 2) revealed a substantial (49.6%) proportion of the women investigated had insufficient (<75 nmol/L) levels of 25(OH)D at the time of assessment. This included 15.1% with levels <50 nmol/L (deficient). These findings are important in light of growing evidence that vitamin D insufficiency in utero or at birth poses long term health risks for the offspring, including an increased risk of allergic disease. Circulating levels were largely influenced by ambient ultraviolet (UV) radiation and to a lesser but significant extent, vitamin D intake from supplements. This study provides evidence that low dose vitamin D supplementation reduces the risk of vitamin D deficiency in Australian pregnant women and should be considered as routine practice.

In Chapter 3 an inverse association was observed between CB 25(OH)D levels and the development of eczema by 12 months of age. The odds of eczema decreased linearly as CB 25(OH)D levels increased (13% per 10 nmol/L rise in 25(OH)D). This finding supports the hypothesis that vitamin D insufficiency in late pregnancy or early infancy poses a risk for the development of allergy in children, however was unable to identify mediating mechanisms for the observation.

The follow-up study to Chapter 3 confirmed a reduced risk of eczema at 12 months of age with greater CB 25(OH)D levels in an exclusively high risk (maternal atopy) population (Chapter 4). Furthermore, analysis found that the altered risk for eczema is detectable as young as 6 months of age. The study also provides evidence of underlying differences in infant immune function associated with 25(OH)D status. Specifically,
higher CB 25(OH)D levels were associated with reduced allergen-specific Th2 cytokine production (interleukin (IL)-5 and IL-13), while higher levels of 25(OH)D at 6 months of age were associated with stronger responses to innate stimuli (TLR ligands). Although Chapters 3 and 4 find significant associations between 25(OH)D status, immune function and allergy in a longitudinal cohort, the studies were limited by their observational design. This limitation was addressed by the VITAL randomized controlled trial, presented in Chapter 6.

Chapter 6 reports multiple phenotypical differences in regulatory T cell (Treg) and dendritic cell (DC) populations between participants stratified by vitamin D supplementation, and by circulating 25(OH)D levels. Supplementation with 400 IU vitamin D3/day resulted in higher 25(OH)D levels at 6 months of age, in comparison to the placebo group. Both vitamin D supplementation and 25(OH)D level at 6 months of age were associated with a profile of Treg previously noted to be highly suppressive (higher expression of HLA-DR on CD25⁺FoxP3⁺CD127⁻ Treg), and a lower percentage of circulating mature DC (fewer CCR7⁺ plasmacytoid DC [pDC], CD16⁺ conventional DC [cDC] and CD16⁻ cDC). An additional finding to come out of this study was the highly significant correlations between average daily UV exposure in the first 3 months of life and the immune phenotype at 6 months of age.

Infants exposed to higher amounts of UV radiation from 0 – 3 months of age had a significantly lower percentage of CCR7⁺ DC (all subsets), in addition to a lower percentage of pDC and CD1c⁺ cDC expressing the T cell co-stimulatory molecule CD86. Taken together, these results provide evidence that vitamin D supplementation and higher 25(OH)D levels in infancy support the development of an effectively suppressive Treg pool, and DC with reduced propensity for T cell activation. The results
also suggest similar associations for UV exposure in early infancy. Whether the effects of UV are independent of vitamin D synthesis will need to be confirmed by future studies.

**Conclusion:** The observational studies and RCT conducted for this thesis find that vitamin D deficiency and insufficiency is prevalent in infants and pregnant women in Perth, Western Australia, and that lower 25(OH)D levels during early infancy (birth to 6 months of age) may increase the risk of allergic disease. The analyses presented here indicate that higher 25(OH)D3 levels in infancy contribute to the normal maturation of the infant immune system, away from the Th2-skewed fetal immune phenotype. Importantly, appropriate immune maturation and the subsequent risk of allergic disease may be modulated through the improvement of vitamin D status in early life; either by supplementation or exposure to UV radiation. Future studies should examine infant vitamin D supplementation in relation to clinical outcomes, and further explore the relative contributions and mechanisms of vitamin D and UV radiation to the developing immune system.
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ABBREVIATIONS

25(OH)D: 25-hydroxyvitamin D
25(OH)D2: 25-hydroxyvitamin D2
25(OH)D3: 25-hydroxyvitamin D3
1,25(OH)2D: 1α,25-dihydroxyvitamin D
1,25(OH)2D2: 1α,25-dihydroxyvitamin D2
1,25(OH)2D3: 1α,25-dihydroxyvitamin D3
2-ME: 2- Mecaptoethanol
APC: Antigen Presenting Cell
BLG: Beta lactoglobulin
CB: Cord blood
CBMC: Cord blood mononuclear cell
CCR7: C-C chemokine receptor type 7
CD: Cluster of differentiation
cDC: Conventional dendritic cell
CIA: Chemiluminescent immunoassay
CI: Confidence interval
DBP: Vitamin D binding protein
DC: Dendritic cell
DMSO: Dimethyl sulphoxide
DOHaD: Developmental Origins of Health and Disease
FCS: Fetal calf serum
FoxP3: Forkhead Box P3
HDM: House dust mite
IFNγ: Interferon gamma
IgE: Immunoglobulin E
IL: Interleukin
LC/MS/MS: Liquid chromatography tandem mass spectroscopy
LPS: Lipopolysaccharide
LTA: Lipoteichoic acid
MC: Mononuclear cells
MFI: Mean fluorescence intensity
MHC: Major histocompatibility complex
OR: Odds ratio
OVA: Ovalbumin
PBS: Phosphate-buffered saline
pDC: Plasmacytoid dendritic cell
PHA: Phytohaemagglutinin
PolyI:C: Polyinosinic:polycytidylic acid
RPMI: Roswell Park Memorial Institute
RR: Risk ratio
SCORAD: Scoring atopic dermatitis
SPT: Skin Prick Test
TCR: T cell receptor
Th: T helper cell
TLR: Toll like receptor
TNFα: Tumour necrosis factor alpha
Treg: Regulatory T cell
UV: Ultraviolet
VDR: Vitamin D receptor
VITAL: Vitamin D in Allergy Study
Chapter 1

The role of vitamin D in the allergy epidemic: a review of the literature


Introduction – Changes in vitamin D status and our emerging understanding of its role in health and disease

Changes in vitamin D status over human history

The term “vitamin D” is commonly used to refer to a group of dietary and endogenously sourced secosteroid metabolites, best known for their role in calcium and phosphorus homeostasis and skeletal health. Humans obtain the majority of their vitamin D through endogenous production in the skin following exposure to ultraviolet (UV) B radiation, which produces vitamin D3 (cholecalciferol) (1), although small amounts of vitamin D3 and vitamin D2 (ergocalciferol) are also obtained through the diet. Naked-skinned hominids evolved in a UV-rich environment, and it is proposed that depigmentation evolved as a means to optimize vitamin D synthesis as hominids migrated out of Africa (2). Having spent the largest fraction of our evolutionary history as hunters and gatherers, followed by agrarian societies, humans maintained lifestyles of regular UV exposure, ensuring adequate vitamin D status. Over approximately the past 300-400 years, however, human society has experienced rapid changes to our environment and lifestyle, reducing our habitual UV exposure.

The spread of urbanization from the 17th century resulted in a shift towards indoor occupation and decreased outdoor activities, with a subsequent dramatic and overt rise in vitamin D deficiency and related diseases (reviewed in (3)). This is evidenced by endemic rickets – the vitamin D deficiency-related bone disease of children - during this period (3). The incidence of rickets peaked in the early 20th century following mass urban migration and industrialization (3). Around this same time the antirachitic properties of cod liver oil (a dietary source of vitamin D3) and UV radiation were being discovered, and soon widespread supplementation and food fortification had largely
eradicated rickets in developed countries (3). Today, while the incidence of rickets in developed countries is low, many experts believe that these same societies continue to experience a high prevalence of sub-optimal vitamin D status, with consequences that extend beyond skeletal health (4-6). However, before discussing the health consequences of deficiency it is useful to describe the metabolism, physiological actions and reference ranges of vitamin D.

**Vitamin D metabolism, actions and assessment of status**

Following ingestion or transport from the skin the calciferols are rapidly cleared from the lymphatics and blood by the liver (reviewed in (7)). Here they undergo hydroxylation by one or more cytochrome P450 25-hydroxylase enzymes to the major circulating form, 25-hydroxyvitamin D (25[OH]D) (7). Depending on whether the original form was D2 or D3 the resulting metabolite will be 25(OH)D2 or 25(OH)D3, respectively. (*Note – throughout this thesis the terms 25[OH]D and 25[OH]D3 are used in what may appear to be an interchangeable manner. This is on account of different styles of reporting by researchers or the specificity of various vitamin D assays. Certain assays report the total of 25[OH]D2 and 25[OH]D3 as 25[OH]D. In most cases 25[OH]D3 comprises the majority of total circulating 25[OH]D as this is the form produced endogenously. Where the metabolites are not specified or when speaking generally the term 25[OH]D will be employed). While 25(OH)D is the predominant circulating vitamin D metabolite it is largely inert, displaying minimal binding affinity for the vitamin D receptor (7). In order to be biologically active 25(OH)D must undergo further hydroxylation to 1α,25-dihydroxyvitamin D (1,25[OH]2D) (7).
This second hydroxylation step to produce 1,25(OH)$_2$D for circulation occurs at the kidneys and is under tight homeostatic control (7). Expression of the 1α-hydroxylase enzyme (CYP27B1) in the proximal tubules is regulated primarily by levels of parathyroid hormone (PTH), which in turn is responsive to circulating levels of calcium (7). In situations of low plasma calcium PTH is increased, stimulating transcription of 1α-hydroxylase and the production of 1,25(OH)$_2$D (7). This active vitamin D metabolite then acts to restore plasma calcium levels by increasing calcium and phosphate absorption from the intestines, decreasing renal calcium excretion and increasing bone resorption (reviewed in (8)). The increased plasma calcium and 1,25(OH)$_2$D initiate a negative feedback process whereby PTH and 1,25(OH)$_2$D production is suppressed, and 1,25(OH)$_2$D is inactivated, thus keeping plasma calcium within a narrow range (8). A second regulator of 1,25(OH)$_2$D is fibroblast growth factor 23 (FGF23) (7). FGF23 production is stimulated by 1,25(OH)$_2$D and subsequently acts to inhibit renal phosphate reabsorption, inhibit 1α-hydroxylase and induce the vitamin D breakdown enzyme 24-hydroxylase (CYP24A1) (7).

Suboptimal production of 1,25(OH)$_2$D in the setting of vitamin D deficiency (low 25[OH]D levels) results in decreased absorption of dietary calcium and phosphate from the intestines, and causes secondary hyperparathyroidism (9). The increased PTH levels mobilize calcium from the skeleton and decrease phosphate reabsorption in the renal tubules (9). Although this maintains plasma calcium levels in the normal range bone mineral density suffers, resulting in osteopenia and osteoporosis in adults, or impaired mineralization and skeletal deformities (rickets) in children (9). For many years vitamin D was associated exclusively with mineral metabolism and skeletal health, however, in recent decades it has become apparent that vitamin D acts on many more tissues than
was previously recognized. It is now known that receptors for 1,25(OH)₂D are found in tissues throughout the body, and that non-renal tissues possess 1α-hydroxylase enzymes (10, 11).

Aside from the classical target sites (bone, intestine, kidney) the vitamin D receptor (VDR) is present in a wide range of tissues including, but not limited to, cells of the cardiovascular system (12), skin (13) and immune system (14). VDR activation (by binding of 1,25[OH]₂D) in these tissues is associated with modulation of inflammatory processes, oxidative stress, cell proliferation, and regulation of immune function (12, 15). These functions provide some explanation for the epidemiological associations between vitamin D status and cardiometabolic conditions, cancers and immune disorders (reviewed in (16)).

Although 1,25(OH)₂D is the biologically active metabolite it has little utility as a measure of vitamin D status (9). 1,25(OH)₂D in circulation is under tight regulation, dependent on serum PTH, calcium and phosphate levels (9). 25(OH)D, on the other hand, is present at stable serum concentrations, and it is the substrate pool from which renal and non-renal tissues draw on for the production of 1,25(OH)₂D. As such, 25(OH)D is generally considered the best indicator of vitamin D status (9). Although opinions on “optimal” levels of 25(OH)D vary (4, 5), recent guidelines from the Endocrine Society define vitamin D deficiency as a 25(OH)D <50 nmol/L (<20 ng/ml), and vitamin D insufficiency as <75 nmol/L (<30 ng/ml) (9).
An area for concern is the recognition of deficiency and insufficiency in pregnant women globally (17-20) and the long-term consequences this may pose to the health of the offspring. The developing fetus depends entirely on its mother for supply of 25(OH)D and poor maternal status is reflected in the infant at birth (21). As described by the developmental origins of health and disease hypothesis (DOHaD), exposures in pregnancy and early life are crucial factors in determining organ structure and function in the offspring, including the phenotype and functional capacity of the immune system (22). It is the effects of vitamin D on the developing immune system, particularly as it relates to allergic disease that is of primary interest to this review. The following sections will examine the observational and experimental evidence related to early life (in utero through childhood) vitamin D status and allergic disease, in addition to exploring the multiple potential mechanisms through which vitamin D may exert its influence.

**Vitamin D status in pregnancy and allergy outcomes in the offspring**

Maternal dietary and environmental exposures during pregnancy have an influence on immune development and determine whether genetic susceptibility for allergic disease is actualized (23). It has, therefore, been an aim of research to identify factors that modulate the developing immune system to the detriment or benefit of the offspring. Exposures during the antenatal period that have been demonstrated to modulate immune development or alter the risk of allergic disease include tobacco smoke (24), microbes (25) and vitamin D (26-28). The latter has been investigated from several angles – estimates of dietary intakes, measurement of maternal and cord blood (CB) 25(OH)D levels – and in relation to multiple outcomes, and the findings and implications of this research is discussed below.
Observational studies of maternal vitamin D intake

Maternal, and subsequently fetal (21), 25(OH)D levels are determined by her UV exposure and exogenous vitamin D intake, with greater intakes being associated with higher circulating 25(OH)D levels (20, 29). Due to the association between exogenous intake and 25(OH)D level some researchers have used maternal vitamin D intake as a proxy for vitamin D exposure in utero, investigating the influence this has on allergic outcomes (26, 30-34). In order to estimate vitamin D intakes during pregnancy these researchers utilized food frequency questionnaires, with (26, 30-32) or without (33, 34) additional information on supplemental vitamin use. The studies include a range of outcomes in the offspring including eczema (26, 31, 33, 34), allergic rhinitis (31, 32), asthma (26, 31, 32, 34) and wheezing (26, 30, 33), and present somewhat differing results.

In those studies investigating eczema outcomes, for example, findings vary between a possible reduced risk with maternal intakes above the 25th percentile (≥172 international units [IU]/day) (OR [95% CI] 0.63 [0.41, 0.98]) (33), no association between intake levels and risk (26, 31), or an increased risk for the highest quartile of intake (median 344 IU/day) compared to the lowest quartile (median 128 IU/day) (OR 1.65 [1.09, 2.50]) (34). (For reference, the recommended daily allowance for vitamin D during pregnancy, put forward by the Institute of Medicine and the Endocrine Society, is 600 IU (9).) The inverse association between intake and eczema risk described by Miyake et al (33) may, however, be an anomalous finding. As per the studies of Camargo et al (26) and Erkkola et al. (31), there was no apparent dose-response relationship when analysed by quartiles of intake (p for trend 0.18), and the inverse association for intakes ≥172 IU/day was no longer significant after controlling for vitamin E intakes (33).
Additionally, this same group of researchers later reported a positive association between pregnancy vitamin D intakes and eczema risk (34).

Respiratory conditions such as wheezing and asthma, on the other hand, have tended to be inversely associated with maternal vitamin D intakes, with some exceptions. Camargo et al (26) and Devereux et al (30) both report that the offspring of mothers in the highest category of vitamin D intake (representing median intakes of 724 IU/day and 275 IU/day, respectively) had significantly lower odds of recurrent or persistent wheeze in the first three (OR 0.39 [0.25, 0.62]) to five (OR 0.33 [0.11, 0.98]) years of life, respectively. Yet despite the association with wheezing Devereux et al (30) did not find maternal vitamin D intakes to be related to asthma risk at five years of age; in agreement with the findings of Miyake et al (34). Two other groups, however, did find that higher intakes of vitamin D during pregnancy reduced the risk of asthma in the offspring at five (31) and seven (32) years of age. Erkkola et al (31) find that in multivariate models both vitamin D from foods (HR 0.80 [0.64, 0.99]), as well as total vitamin D intakes (food plus supplements) (HR 0.79 [0.64, 0.99]) are inversely associated with asthma. Similarly, Maslova et al (32) report a lower risk of asthma for those in the highest versus lowest quintile of intakes (RR 0.74 [0.56, 0.96]).

Direct comparison between these studies is problematic as different food frequency questionnaires were used throughout, and studies were conducted across diverse settings (the USA (26), Finland (31), Denmark (32), Scotland (30) and Japan (33, 34)). This is of relevance as the exogenous sources of vitamin D vary significantly between these countries. For example, in the USA the major dietary source of vitamin D is fortified milk, although in the population studied by Camargo et al (26) vitamin supplements
contributed more to the total vitamin D intake than did dietary sources (mean 319 IU/day and 225 IU/day, respectively). In contrast, fish is the greatest contributor of dietary vitamin D in Scotland, Finland and Japan (30, 31, 33). Miyake et al. (33) report that the use of vitamin supplements was uncommon in pregnant Japanese women (4.2%), and therefore this data was not included in their analysis, while in Finland supplements did contribute to total intakes, but to a lesser extent than food sources (mean [SD] intake from food and supplements 5.1 [2.6] IU/day and 1.35 [2.6] IU/day, respectively) (31). Although the researchers typically made effort to control for confounding by dietary variables we cannot ignore the fact that dietary sources of vitamin D (e.g. dairy (33, 34) and fish oil (35, 36)) have been independently associated with immunological and clinical outcomes in other studies.

The ideal strategy to facilitate accurate estimation of vitamin D intake and control for confounding variables is to conduct randomized controlled trials (RCT) of vitamin D supplementation. However, there remains a paucity of clinical trials in this field. At the time of writing, there is only one randomized controlled trial investigating maternal vitamin D supplementation in the context of allergy outcomes in the offspring (37). In this trial there were no significant difference between intervention and control groups in terms of wheezing, allergic disease, immunoglobulin E (IgE) an eosinophil count (37). However, the trial was severely limited by small sample size (n=180 divided into three groups) and a low dose protocol (800 IU/day or 200,000 IU single bolus) that was inadequate to achieve sufficient CB 25(OH)D levels (median [IQR] 26 nmol/L [17–45] for the daily dose and 25 nmol/L [18–34] for the bolus dose) (37). Results from two large antenatal vitamin D randomized controlled trials – the Vitamin D Antenatal Asthma Reduction Trial (VDAART) (NCT00920621) and the Vitamin D Supplementation During Pregnancy for Prevention of Asthma in Childhood
(ABCvitaminD) (NCT00856947) – are eagerly awaiting in the coming years. These trials utilize higher dose regimens (2400 IU – 4000 IU) and will be extremely valuable in determining the efficacy of antenatal vitamin D supplementation in the primary prevention of allergy. Until these results are available, however, further information on the relationship between pregnancy vitamin D status and allergic outcomes can be obtained from studies of circulating 25(OH)D levels which, as a direct biological measurement, represents a better marker of status than estimated dietary intakes.

**Observational studies of maternal and cord blood 25(OH)D status**

Two strategies have been employed to estimate the influence of fetal 25(OH)D exposure on health outcomes; the first by measurement of maternal 25(OH)D levels in pregnancy, and the second by measuring levels in CB at birth. 25(OH)D diffuses across the placental barrier so that CB 25(OH)D levels correlate strongly with those of the mother (21). Subsequently, maternal vitamin D deficiency is reflected in the neonate (38). There are multiple examples of both approaches in the published literature which, taken together, suggest a possible protective association for CB, but not maternal, 25(OH)D levels.

Investigating the association between maternal 25(OH)D and the development of allergy in the offspring are four large cohort studies (n=1724 (39), n=5515 (40), n=860 (41) and n=1672 (42)); each reporting no association between maternal 25(OH)D and allergic or respiratory outcomes in the offspring. These studies investigated multiple outcomes including wheeze, asthma, sensitization and eczema, with duration of follow-up ranging 36 months – 8 years of age. Furthermore, collectively the studies have investigated the influence of vitamin D status at various stages of pregnancy (median
12.6 weeks (39), 18 weeks (42), 34 weeks (41), or any stage (40)), indicating no effect of timing of exposure on allergic outcomes. Contrary to these results, one observational study does find a positive association between pregnancy 25(OH)D status and allergy in the offspring (43); however results of this study are limited by diagnostic criteria and loss to follow up. Therefore, the large sample size, duration of follow-up, range of outcomes and consistency of findings of the large epidemiological studies strongly suggests that maternal 25(OH)D status is not associated with the risk of allergic outcomes in the general population.

Whereas maternal 25(OH)D levels during pregnancy do not appear to be significantly associated with allergic outcomes, studies of CB 25(OH)D have yielded fairly consistent findings in terms of a reduced risk of wheeze and allergic skin disease, but conflicting findings for other outcomes (results from CB studies are summarized in Table 1.1). Those reporting an increased risk include a German birth cohort study, wherein children in the highest quartile of CB 25(OH)D3 had increased odds of food allergy in the second year of life (4.65 [1.50, 14.48]) (44). Interestingly, this finding was based on only 8 cases of food allergy out of the n=272 participants, and relied on parent report rather than objective clinical diagnosis (45).

Weisse et al (44) are not the only group to report an increased risk of allergic conditions with higher CB 25(OH)D. In a longitudinal study from birth to five years age Rothers et al (46) examined the association between CB 25(OH)D and allergen sensitization (skin prick test, total and specific IgE), allergic rhinitis and asthma. The authors report a non-linear relationship between 25(OH)D and total and allergen specific IgE, these markers being significantly higher when CB 25(OH)D levels were <50 nmol/L or >100 nmol/L.
In addition, the adjusted OR of being skin prick test positive at 5 years of age was increased where CB 25(OH)D levels were >100 nmol/L (3.4 [1.0, 11.4]) (46). CB 25(OH)D3, however, was not significantly associated with the development of asthma or allergic rhinitis up to 5 years of age, although the authors do note a non-significant trend for allergic rhinitis (adjusted OR 2.4 [0.8, 7.3]) (46).

| Table 1.1. Association between cord blood 25(OH)D and outcomes in the offspring |
|---|---|---|
| **Sample size and outcomes** | **Association with higher 25(OH)D** | **Ref.** |
| N=882-823 children followed up at 3 months and 5 years for outcomes of respiratory tract infections, cumulative wheeze, and asthma | • Respiratory tract infections (3 months) ↓<br>• Wheeze (5 years) ↓<br>• Cumulative wheeze (5 years) ↓<br>• Asthma ↔ | (47) |
| N=219 children followed up to 5 years for allergen sensitization, allergic rhinitis and asthma | • Sensitization ↑ (>100nmol/L)<br>• Allergic rhinitis ↔<br>• Asthma ↔ | (46) |
| N=239 children, questionnaires on asthma, wheeze, allergic rhinitis, and eczema conducted at 1, 2, 3 and 5 years of age | • Transient early wheeze ↓<br>• Eczema (1,2,3 and 5 years) ↓<br>• AR ↔ | (48) |
| N=272 children followed up at 1 and 2 years of age for atopic eczema, food allergy, total and five food specific IgE | • Food allergy ↑<br>• Eczema ↔<br>• Total and specific IgE ↔ | (44) |
| N=257 children at hereditary risk followed up at 7 years (and various time points between depending on outcome) for asthma, respiratory infections, allergic rhinitis and eczema | • Lung symptoms (significant cough or wheeze or dyspnea) ↓<br>• Asthma ↔<br>• AR ↔<br>• Eczema ↔ | (49) |
| N=270 children at hereditary risk followed up at 1 and 3 years of age for eczema, food allergy, asthma, allergic rhinitis and allergen sensitization | • Eczema (1 and 3 years) ↓<br>• Food allergy (1 year) ↓<br>• Sensitization ↔<br>• AR ↔<br>• Asthma ↔ | (50) |

The lack of association between CB 25(OH)D level and the risk of asthma or allergic rhinitis is supported by a later study, where researchers find an inverse association between CB 25(OH)D and early (first three years of life) transient wheeze (OR 0.67
The association with early wheeze did not translate to other respiratory outcomes such as later or persistent wheeze, asthma or allergic rhinitis (48). This study did, however, find that CB 25(OH)D was inversely associated with the risk of eczema by one, two, three and five years of age, and when analysed by early (OR 0.73 [0.62, 0.90]) or late (OR 0.75 [0.60, 0.94]) onset (48). Most recently, an Australian study found similar results regarding CB 25(OH)D and the risk of eczema (50). In this study it was observed that a 10 nmol/L increase in CB 25(OH)D reduced the risk of eczema at one year of age by 12% (RR 0.88 [0.81-0.96]) and the cumulative risk by three years of age by 8% (RR 0.92 [0.86-0.97]). Furthermore, the risk of IgE-mediated food allergy at one year of age, and eczema with sensitization (atopic eczema) by 3 years (RR per 10 nmol/L rise in CB 25(OH)D 0.83 [0.72-0.96]) were also significantly reduced with increasing 25(OH)D levels.

Thus, from the findings discussed here it could reasonably be concluded that maternal 25(OH)D levels in pregnancy (at all stages measured) are not a major determinant of the risk of allergic disease in the offspring. Studies of CB 25(OH)D, on the other hand, have demonstrated some degree of consistency as regards inverse trends for the risk of wheezing and eczema in children up to five years of age. This is a somewhat surprising result given the positive correlation between maternal and CB 25(OH)D levels, and the reason for these apparently conflicting findings is not established. A possible explanation is that vitamin D status in early infancy (CB levels largely determining vitamin D status for the first 6 – 8 weeks of life (51)) and childhood is important for appropriate immune maturation in response to the multitude of environmental and bacterial antigens encountered postnatally; an hypothesis put forward by independent researchers (52, 53).
Vitamin D exposure in infancy and childhood in relation to allergic outcomes

Although there is little doubt that the antenatal period has a significant influence on the developing immune system, postnatal exposures play a crucial role in the maturation of immune function from the placental-controlled Th2 fetal phenotype (reviewed in (54)). Possibly the best recognized maturation stimuli is microbial exposure; popularized in the “hygiene hypothesis”, first described by Strachan in 1989 (55). Strachan noted that the incidence of eczema and hayfever was inversely associated with the number of older siblings in the household, suggesting that unhygienic contact with siblings might confer protection against allergic conditions (55). Despite the popularity of the hygiene hypothesis others believe it does not adequately explain the increase in the prevalence of allergic and autoimmune disorders (53). Other immunomodulating factors are also likely to influence the risk of allergic disease in children, and vitamin D has become a prominent candidate. There now exists a substantial literature base examining the association between vitamin D exposure in childhood and adolescence and the risk of allergic disease, including ecological data (56-63), observational studies utilizing biological measures (64-69), and supplementation trials (70-76). This section will discuss the evidence for and against a role for vitamin D in the development and severity of allergic conditions based on these analyses.

Ecological studies

As previously discussed, exposure to UVB solar radiation is the predominant source of vitamin D in humans. Ground surface UVB radiation levels vary significantly with season and latitude, subsequently impacting the capacity for vitamin D synthesis (77). Therefore, if vitamin D status were a risk factor for allergic disease one might predict variation in the prevalence rates with latitude. Indeed, a search of the literature identifies
multiple studies reporting variations in the prevalence of asthma and allergy between populations residing at different latitudes. This review will predominantly discuss studies reporting on pediatric allergic disease; however, it should be mentioned that several of the large ecological studies investigate either a wide range of age groups or exclusively adults (56, 61, 78-80), some of which report an increased risk of asthma at lower latitudes (79, 80).

A strategy employed by a number of researchers to quantify the prevalence of allergic conditions is to examine data on the prescription rate of adrenaline autoinjectors (EpiPen ®) (56, 58) or hypoallergenic infant formulae (59) in different regions. Camargo et al (56) collected data on the total number of EpiPen prescriptions per state in the USA, finding a significant northeast bias in both EpiPen (adult, \( \geq 30 \text{kg} \)) and EpiPen Jr (child, 15-30kg) prescriptions. Compared to southern states with 2 to 3 prescriptions per 1000 persons, the northeast had four times this rate: 8 to 12 per 1000 persons (56). The higher prescription rate in this region remained significant after controlling for multiple factors including the number of medical specialists (allergists, pediatricians, emergency and primary care physicians), sex, ethnicity, education and household income (56). Following this study, Mullins et al (58) reported similar geographical trends in EpiPen prescriptions in Australia. Prescription rates (adjusted for age, sex, ethnicity, household income, number of allergists, pediatricians and medical practitioners) were significantly lower at lower latitudes in both the 0-4 years age range (\( \beta \) coefficient [95% CI]) (\( \beta = -51.9 \ [-71.0, -32.9] \)) and 5-15 years age range (\( \beta = -54.8 \ [-73.6, -36.0] \)) (58). In a separate publication Mullins et al (59) also report that prescription rates of hypoallergenic infant formula (n=36,165 prescriptions) display a north-south trend, being greater at southern latitudes (multivariable model \( \beta = -147.98 \ [-281.83, -14.14] \)). In these three studies the authors suggest that the findings may be due
to variations in sun exposure and vitamin D status (56, 58, 59). Although controlling for multiple social variables, these analyses are not adjusted for other climatic factors, which have also been suggested to be associated with allergy prevalence (79).

Data on emergency department visit or admission for allergic reactions in the USA (61) and Chile (57) have also been analysed for geographical patterns. The trend for emergency department visits for allergic reactions in the USA was reflective of the findings for EpiPen prescriptions, being greater in the northeast than southern parts of the country (61). The difference was even more clearly delineated when the analysis was restricted to food-allergic reactions, which disproportionately affect the younger age group (<10 years) (61). This association between latitude and food allergy has since been replicated in southern hemisphere countries by separate teams of researchers (57, 60). In Chile, a country spanning 39° of latitude (~4330 km), Hoyos-Bachiloglu et al (57) report a strong north-south increasing gradient in angioedema and food-induced anaphylaxis admissions in children (β 0.018 [0.006, 0.030] and 0.01 [0.003, 0.016], respectively, n=403). Solar radiation (MJ/m²/day) was also significantly associated with admissions in children, whereby regions with lower radiation had higher rates of angioedema and food-induced anaphylaxis (β -0.044 [-0.075, -0.012] and -0.024 [-0.04, -0.009], respectively) (57). Furthermore, when examining latitude and solar radiation in stepwise linear regression only solar radiation remained in the model, providing support for the hypothesis that differences in vitamin D exposure may be contributing to geographical variations in allergic disease (57).

Geographical variation in childhood food allergy is also reported in the Australian population (60). Data from the Longitudinal Study of Australian Children suggests that the odds of peanut allergy in the two cohorts investigated (4-5 years of age, n=3260, and
8-9 years of age, n=3198) is significantly greater in the southern (Victoria and Tasmania) states of the eastern seaboard than in the north (Queensland) (60). Additionally, the geographical associations for eczema in these cohorts were even stronger, with the odds being increased in both central (adjusted OR [95% CI] 1.75 [1.34, 2.29] in the 4-5 years of age cohort and 1.60 [1.14, 2.26] in the 8-9 years cohort) and southern (adjusted OR 2.58 [2.00, 3.32] in the 4-5 years cohort and 1.90 [1.36, 2.67] in the 8-9 years cohort) regions of the country. In agreement with the Australian findings, this same pattern in eczema prevalence in relation to latitude and solar radiation is also detected in large European (β 0.29 [0.07, 0.50] in 6-7 year olds and 0.16 [0.01, 0.31] in 13-14 year olds) (63) and American (OR 0.77 [0.67, 0.87] for states with high mean annual UV index) surveys (62). It should be noted, however, that while increasing latitude and decreasing solar radiation appears to be associated with a higher eczema prevalence, there are reports that eczema control may be worse for some patients in environments of higher temperature, humidity and solar radiation (81, 82). This may be a consequence of seasonal aeroallergens (pollens) (82) or increased sweating, which can promote Th2 and Th17 inflammation and downregulate filaggrin expression (associated with skin hydration) (81).

The strength of these ecological studies lies in the large, population-based samples with little likelihood of reverse causality. However, many are limited by broad geographical classifications (state of residence, as compared to county or city), self-reported outcomes and lack of biological measures. Although associations between latitude, solar radiation and allergic outcomes are supportive of a role for vitamin D in the prevalence of allergic disease, many other environmental factors also vary with latitude including, but not limited to, temperature, humidity and pollen levels. In addition, there is data to suggest that residing in a sun rich environment does not guarantee adequate vitamin D
status of the population (17, 83). As a result, inferring an association between vitamin D and allergic disease cannot be done from ecological data alone. It is useful, therefore, to look at whether the observed ecological trends translate into associations between serum 25(OH)D in children and allergic disease.

*Observational studies of 25(OH)D status*

Several cross-sectional studies investigating 25(OH)D status and allergic conditions in children and adolescents have been published, reporting inverse associations across a broad scope of geographical and cultural regions (64, 65, 68, 84). The largest of these studies analysed the 2005-2006 National Health and Nutrition Examination Survey (USA) data on n=3136 children and adolescents (mean [SE] age 12.5 [0.2] years) (68). In this analysis there was an increased risk of allergic symptoms (defined by the authors as allergic rhinitis, eczema or pet avoidance) in those with lower 25(OH)D levels (OR 1.79 [1.10, 2.93] for levels <37.5 nmol/L compared with ≥75 nmol/L) (68). This was despite no significant association between 25(OH)D and allergic rhinitis, eczema or pet avoidance as independent outcomes (68). Additionally, sIgE levels to 11 of 17 allergens tested were found to be greater in children and adolescents with 25(OH)D levels <37.5 nmol/L; the strongest associations being for peanut (2.39 [1.29, 4.45]), ragweed (1.83 [1.20, 2.80]) and oak (4.75 [1.53, 4.94]) (68). These findings regarding sensitization are similar to those seen in asthmatic Costa Rican children (median [IQR] 8.7 [7.6, 10.5] years of age, n=616), where serum 25(OH)D3 was inversely associated with total and dust mite-specific IgE (β -0.47 [-0.86, -0.08], and β -1.17 [-2.10, -0.24], respectively) (84), as well as in allergic Korean children (aged 3 – 24 months, n=266) where 25(OH)D status was inversely associated with the degree of sensitization (65).
Looking beyond sensitization there is some indication that vitamin D levels in young children are associated with clinical allergic disease, including eczema (65) and food allergies (64). The aforementioned study by Baek et al (65) found that, in addition to the association with sensitization, 25(OH)D is associated with eczema severity. Serum 25(OH)D was weakly inversely correlated with severity ($r = -0.196$, $p = 0.043$), explaining 11% of variation after controlling for sensitization ($R^2 = 0.112$, $p = 0.031$).

While there have been reports that a higher vitamin D status in early life (CB) is associated with an increased risk of food allergy (44), a recent Australian paper has described a reduced risk of challenge-proven food allergy in 14 – 18 month old infants with higher 25(OH)D levels (64). In infants of Australian-born parents the odds of an egg or peanut allergy were significantly greater where 25(OH)D levels were $\leq 50$ nmol/L (3.79 [1.19, 12.08], and 11.51 [2.01, 65.79], respectively) (64). Why the parents’ country of birth was significant was not explored by the authors, although it is reasonable to speculate that genetic differences associated with ethnicity, or epigenetic changes that occur with a change in environment may play a role.

Although cross-sectional studies such as those described above are useful in generating hypotheses they are of limited utility in identifying cause-effect relationships. The relationship between 25(OH)D and allergic airway disease, for example, may be subject to reverse causality, where allergic individuals may avoid outdoor activity, and subsequently UV exposure, due to impaired exercise capacity or allergen avoidance. Additionally, recent findings that 25(OH)D is a negative acute phase reactant suggest that low 25(OH)D may be a result, rather than a cause, of chronic inflammatory conditions (85). For such reasons longitudinal studies, particularly those commencing prior to the onset of allergic inflammation, are considered superior for identifying possible causal relationships.
Some longitudinal data do exist on the relationship between vitamin D status and allergic disease in children. Hollams et al (66) investigated the association between 25(OH)D levels and asthma, atopy, allergic rhinitis and bronchial hyperresponsiveness in children at 6 years of age, with follow-up at 14 years of age (66). At both the 6 year and 14 year time point current 25(OH)D status was inversely associated with bronchial hyperresponsiveness and sensitization, in addition to atopy (total IgE \( \geq 300 \) kU/L or sIgE \( \geq 0.35 \) kU/L) at 14 years (66). In longitudinal analysis a greater 25(OH)D status at 6 years of age was protective of atopy (OR 0.14 [0.04, 0.47]), asthma (0.11 [0.02, 0.84]), allergic rhinitis (0.17 [0.05, 0.59]) and dust mite sensitization (0.18 [0.05, 0.60]) at 14 years of age (66). While the longitudinal design of this study is a strength, the authors do note that they were unable to address the effects of vitamin D exposure in early life and throughout childhood, which may be relevant to the developing immune system (66). However, work by another group using data from a birth cohort have reported significant associations between early life 25(OH)D levels (birth to 3 years of age) and sensitization to food allergens in a longitudinal design (86).

Liu et al (86) measured 25(OH)D levels in samples of CB and peripheral blood at various time points between 1 and 3 years of age, in conjunction with sIgE to eight food allergens in 460 study participants. They report that while 25(OH)D levels at any single time point were not independently associated with food allergen sensitization, having persistent low 25(OH)D levels, which the authors define as CB \(< 27.5 \) nmol/L and postnatal levels \(< 75 \) nmol/L, significantly increased the odds for sensitization (2.04 [1.02, 4.04]) (86). Findings from the studies by Liu et al (86) and Hollams et al (66) are indicative of a protective effect of vitamin D in childhood on the development of allergic outcomes, suggesting that measures to improve vitamin D status during this period (i.e. supplementation) may be effective in primary prevention.
A number of longitudinal observational studies have specifically examined infant vitamin D supplementation in the context of allergic disease. This data has the advantage of focusing on the early life period and may therefore provide insight into the etiological role of vitamin D in the development of allergic disease. Despite the observed protective association of serum 25(OH)D and allergy (66, 86), it has been noted that the implementation of vitamin D supplementation from the early 20th century - leading to the widespread use in children and virtual eradication of rickets - coincided with the rise in allergic disease (87). Supporting this, several studies found vitamin D supplementation to be associated with an increased risk of atopic dermatitis (88) and allergic rhinitis (89).

In a Finnish birth cohort study Hyppönen et al (89) report that regular vitamin D supplementation in the first year of life was associated with a higher risk of allergic rhinitis (OR 1.33 [1.1, 1.6], n=7648) or atopy determined by skin prick test (1.33 [1.1, 1.64], n=5007) at 31 years of age. At the time of data collection (mid-1960’s) the dose of vitamin D given to infants was substantially greater than currently recommended (2000 IU/day compared with 400 IU/day recommended by the American Academy of Pediatrics (90)). This may be significant as other work conducted by Hyppönen et al (91) has found a non-linear association between 25(OH)D level and IgE, with IgE being elevated in situations of both low (<25 nmol/L) and high (>135 nmol/L) 25(OH)D levels. A possible interpretation of the birth cohort findings is that the doses administered to the population at the time were detrimentally high, hence it does not necessarily follow that supplementation at the current recommended dose poses an increased risk of allergic disease (89).
However, other authors investigating infant intakes at lower doses (closer to AAP recommendations) have also shown that atopic manifestations (particularly atopic dermatitis) were more prevalent in children with “high” (>524 IU/day) versus “low” (≤524 IU/day) intakes of vitamin D, albeit in a substantially smaller sample (n=123) (88). The increase in relative risk of atopic dermatitis (AD) was 18% per 40 IU/day increase in vitamin D intake, and a high vitamin D intake was a stronger predictor of AD than was family history of atopy. Unfortunately, neither of these studies have serological measures of 25(OH)D in order to support the association between vitamin D status and allergic disease risk, which leaves us with further ambiguous data regarding the role of vitamin D in the etiology of these conditions. While these observational studies of early life supplementation appear to provide evidence against infant supplementation for allergic outcomes, recent randomized controlled trials have found that supplementation may be beneficial as an adjunct or stand-alone treatment for conditions including asthma and eczema (70, 71, 73, 75, 76).

Randomized controlled trials

There is currently very limited published data from allergy-focused randomized controlled trials of vitamin D in children or breastfeeding mothers. Furthermore, none of the published trials have been designed to assess allergy prevention, instead focusing on treatment (Table 1.2). While these trials describe different populations, dosing regimens and outcomes, examination of the findings is warranted to assess whether trial data supports the observational reports of association between vitamin D and allergic disease.
Table 1.2. Vitamin D supplementation RCTs in children

<table>
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<th>Sample size and outcomes</th>
<th>Effect of supplementation</th>
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| N=164 mothers of breastfed infants with facial eczema randomized to 800 IU/day D3 or placebo for 6 weeks, from infant age 1 month. Primary outcome was eczema severity at 3 months of age (SCORAD); secondary outcomes were eczema, food allergy and wheeze up to 2 years of age. | • SCORAD at 3 months ↔  
• Food allergy by 2 years ↑  
• Any allergy by 2 years ↑ | (74) |
| N= 430 (mean age 10.2 years), placebo or 1200 IU D3/day for 15-17 weeks. Primary outcome was prevention of influenza A; number of asthma exacerbations were analysed as secondary outcome | • Asthma exacerbations ↓ | (75) |
| N= 48 (mean age 11.5 years), placebo or 500 IU D3/day for 6 months. Outcome was number of viral-induced asthma exacerbations. | • Asthma exacerbations ↓ | (73) |
| N=100 (mean age 9.5 years), placebo or 60,000 IU D3/ month for 6 months. Outcomes were asthma severity, number of exacerbations, peak expiratory flow rate (PEFR), steroid dose, level of control and emergency visits | • Asthma severity ↓  
• Asthma exacerbations ↓  
• PEFR ↑  
• Steroid dose ↓  
• Asthma control ↑  
• Emergency visits ↓ | (76) |
| N=11 (median age 7 years), placebo or 1000 IU D2/day for 1 month. Outcome was Investigator Global Assessment (IGA) of eczema severity. | • Eczema severity (IGA) ↓ | (71) |
| N=107 (median age 9 years), placebo or 1000 IU D3/day. Outcomes were Eczema Area and Severity Index (EASI), Investigator Global Assessment (IGA) | • Eczema severity (EASI + IGA)↓ | (70) |

Starting with the earliest of the postnatal trials in terms of infant age, Norizoe et al (74) supplemented breastfeeding mothers of infants affected by facial eczema. Mothers (n=164) were randomized to either 800 IU/day of cholecalciferol (D3) or a placebo product for 6 weeks (74). The intervention commenced when infants were one month of age, and were reviewed at three months of age for examination and scoring of eczema severity using the SCORAD analysis (SCORing Atopic Dermatitis) (74). Of the n=164 enrolled, n=18 (11%) attended the three month of age appointment, and n=119 (72.5%) and n=79 (48.2%) completed questionnaires at one and two years of age, respectively.
While the authors report no effect of supplementation on SCORAD in those infants attending the three month of age appointment, infants of the vitamin D-supplemented mothers had an increased risk of parent reported food allergy (RR 3.42 [1.02, 11.77]) or any allergic condition (food allergy, eczema or wheeze: RR 2.49 [1.16, 5.34]) by two years of age (74). However, this trial is subject to major limitations in terms of design and implementation, severely impacting the reliability of the findings.

In terms of direct supplementation in children, three trials in recent years have investigated vitamin D supplementation as an adjunct to standard asthma treatment, with positive results (73, 75, 76). The first of these investigated the efficacy of vitamin D supplementation (1200 IU) in the prevention of influenza A infection in children (mean age 10.2 years) (75). Viral-induced asthma exacerbations were examined as a secondary outcome, with the authors reporting 2 cases of asthma exacerbation in the intervention group, compared with 12 cases in the control group (RR 0.17 [0.04, 0.73]). The second and smallest of the three, examined vitamin D (800 IU) as an adjunct to budesonide therapy in newly diagnosed asthmatics (n=48) (73). Over a 6 month follow-up there were significantly fewer asthma exacerbations in the group receiving vitamin D in conjunction with budesonide (4 cases versus 11 in the control group, p = 0.029). The third trial conducted in n=100 children (mean [SD] age 9.6 [2.2] years), randomized participants to receive placebo or 60,000 IU D3/month, in addition to standard therapy for six months (76). Outcomes examined were asthma severity, peak expiratory flow rate, steroid dosage, number of exacerbations and emergency visits (76). There were significant improvements noted in all outcomes for the vitamin D group compared with the placebo group, strongly supporting a role for vitamin D supplementation as an adjunct in asthma management (76). The authors cite congruence between their findings and those of observational studies reporting on serum 25(OH)D levels and lung
function, steroid use, asthma control and emergency visits, suggesting that the previously reported associations are replicable through supplementation.

Interestingly, in contrast to these findings, a study of similar duration (28 weeks) in adults (n=408) did not find significant clinical benefit of vitamin D supplementation as an adjunct in asthma treatment (92). Whether this is due to differences in the age, and therefore disease progression, of the study population, ethnic and genetic variation (Indian versus predominantly White or African), or other factors is not clear from the available data. The authors of both studies recommend further trials to address issues such as sample size and duration of intervention (76, 92).

A similar pattern of results is reported for supplementation trials in eczema, with null results from one adult trial (93), but positive results in two small trials in children (70, 71). The first indication that vitamin D supplementation may be efficacious in treating eczema came from a small pilot study involving 11 children (median age 7 years) with predominantly mild (10/11), winter-related eczema (71). Five of the children received 1000 IU/day of ergocalciferol (D2) for one month, while the remaining six children received a placebo product. Investigator global assessment (IGA), performed by a pediatric dermatologist, improved in 4/5 children receiving vitamin D and was unchanged in the fifth child. In comparison, IGA improved in only one child in the placebo group, while four were unchanged and one worsened. Due to the small sample size the true clinical significance of vitamin D supplementation cannot be determined from this data alone, but based on this promising pilot data some of the investigators pursued a larger trial of similar design (70).
In this second, larger trial, n=107 Mongolian children (median age 9 years) with winter-related eczema were randomized to 1000 IU D3/day (n=58) or placebo (n=49) for one month (70). Consistent with results of the pilot trial the vitamin D group experienced significantly greater improvement in IGA, in addition to better improvement as assessed by Eczema Area and Severity Index (difference between change in means being -3.2; 95% CI -0.9, -5.5), and parental assessment of improvement (70). While the researchers did not measure serum 25(OH)D in the participants, they note that in a concurrent study of n=579 similarly aged schoolchildren in the same region 98% had levels <50 nmol/L (mean of 20 nmol/L), and thus extrapolate that the children in the RCT most likely had low 25(OH)D levels (70). These results suggest that low vitamin D may be important in the etiology of winter-related eczema, and that improving circulating 25(OH)D levels through supplementation is efficacious in improving symptoms. Whether these results can be extrapolated to non-winter-related eczema phenotypes or other allergic manifestations is yet to be determined, but based on the positive results from this and other RCTs in children further trials could be justified.

Having examined the associations between vitamin D (intake, serum levels and proxy markers such as latitude) and the development and severity of clinical outcomes, this review will now turn to some of the potential mechanisms by which vitamin D may alter the course of allergic disease. An understanding of the potential mechanisms is important as it directs the design and analysis of future research projects. As one of the better described and biologically plausible roles, the review will first discuss the immunomodulating properties of vitamin D in certain cell subsets, before more briefly discussing organ structure and function, environment-gene interactions and non-vitamin D actions of UV radiation.
**Immunomodulatory function of vitamin D and the implications for allergic disease**

Allergy, by definition, is a “hypersensitivity reaction initiated by immunological mechanisms” (94). Reactions may be antibody-mediated (typically immunoglobulin E [IgE]), characterized by the rapid onset of symptoms such as anaphylaxis, or cell-mediated which are typically delayed (hours or days) in relation to timing of the exposure. Antibody- and cell-mediated reactions are not mutually exclusive and frequently occur together, as is the case with atopic eczema/dermatitis syndrome. Yet despite different clinical manifestations between individuals, or within individuals over time (i.e. the allergic march (95)), the underlying immune mechanisms share enough similarities that specific patterns in immune development and responses can be identified to describe allergic and non-allergic phenotypes (96). Therefore, identifying factors which may beneficially modulate immune function is a key step in developing allergy prevention strategies.

A role for vitamin D in the immune system was first recognized through the identification of the vitamin D receptor (VDR) on monocytes and activated lymphocytes in the early 1980’s (14). Tsoukas et al (97) went on to describe immunoregulatory properties of vitamin D in cell culture, where the addition of 1,25(OH)₂D₃ inhibited IL-2 production by T cells activated with mitogen (PHA). This was an important finding as IL-2 is a potent growth factor, stimulating proliferation of T helper cells (98). Since these discoveries there has been much work on the role of vitamin D in the function of immune cell subsets, with implications for allergic disease. The following sections will describe the effects of vitamin D on T and antigen presenting cell subsets, both independently and in interaction.
**CD4⁺ T Helper cells**

T cells ("T" designating “thymus” – the organ of origin) are one of the major types of lymphocytes and are responsible for cell mediated adaptive immunity. T cells are identified by the pan T cell marker cluster of differentiation (CD) 3, and can be further classified into numerous sub-groups, one of which are CD4⁺ helper T (Th) cells. CD4⁺ Th cells differentiate from their naïve precursor cells in response to signals from antigen presenting cells (APC). Following the activation of APCs through pattern-recognition receptors such as toll-like receptors (TLR), antigen peptides are displayed on major histocompatibility complex (MHC) II, which can then bind to T cells via the T cell receptor (TCR) (99). This process, in combination with stabilization through adhesion molecules (e.g. CD80 and CD28) and cytokine signaling activates the T cells, commencing differentiation into helper subsets (99).

CD4⁺ Th cells play a central role in adaptive immunity by activating B cells to make antibody, recruiting neutrophils, basophils and eosinophils to sites of infection and inflammation, and enhancing the activity of macrophages and cytotoxic T cells (100). These various functions are possible because of the wide range of cytokines and chemokines produced by Th cells, which differentiate from naïve precursors into phenotypically distinct subsets (100). The earliest recognition of distinct subsets of Th cells was by Mosmann and Coffman (101) who, in 1986, identified two different types of Th cell based on their cytokine production profile. The authors dubbed these subsets Th1 and Th2, and for the next decade and a half the Th1/Th2 dichotomy would dominate allergy and autoimmune research (102). The Th1 subset is generally characterized by production of IL-2 and IFN-γ, while Th2 cells are those that produce IL-4, IL-5, and IL-13 (102). The Th2 subset is strongly associated with allergic disease
due to the roles of IL-4 and IL-13 in inducing IgE from B cells (103), and IL-5 in the maturation and release of eosinophils (reviewed in (104)), while autoimmune conditions are typically considered Th1-dominant. This dichotomous view of the Th subsets in allergic and autoimmune disease is now viewed as overly simplistic, following the discovery of other T cell subsets such as Th17 and regulatory T cells, and development of a greater recognition of the complexity of the interactions (105, 106). However, while the Th1/Th2 balance may not entirely explain the pathogenesis of immunological disease, the established role of these subsets provides a useful starting point for mechanistic research.

As mentioned previously, early experiments of vitamin D on T cell function found that the active metabolite, 1,25(OH)$_2$D$_3$, inhibited IL-2 production and T cell proliferation in response to PHA (14, 97, 107). Work by Reichel et al (108) and Rausch-Fan et al (109) provided support that 1,25(OH)$_2$D$_3$ acts directly on the Th1 subset, discovering that 1,25(OH)$_2$D$_3$ inhibits production of IFN$\gamma$. On the surface this tipping of the Th1/Th2 scales would not appear to be beneficial as regards allergic disease. However, while the inhibition of Th1 cells by 1,25(OH)$_2$D$_3$ is a consistent, robust finding, this does not appear to translate directly to an increase in pro-inflammatory Th2 cytokine production.

In both the aforementioned paper by Rausch-Fan et al (109), which utilized adult PBMC, and a paper by Pichler et al (110) published in the same year using CBMC, it was observed that culturing PHA-activated mononuclear cells with 1,25(OH)$_2$D$_3$ did not alter the percentage of IL-4-producing T cells, analyzed by flow cytometry. However, when CBMC were cultured under Th2-polarizing conditions, the presence of 1,25(OH)$_2$D$_3$ almost completely inhibited the induction of IL-4- and IL-13-producing
CD4⁺ cells; instead inducing an IL-6-producing phenotype (110). Although IL-6 produced by dendritic cells is associated with the proliferation of Th2 and Th17 cells in allergic asthma (111) Pichler et al (110) suggest that the synergistic induction of IL-6-producing T cells, with inhibition of classical IL-4-producing T cells, may actually be associated with reduced antibody-mediated allergic responses. This hypothesis was based on reports that while IL-4 induces B cell class switching to IgE, IL-6 induces B cell switching to an IgG or IgA class (112, 113). It was the conclusion of the authors, therefore, that in predominantly naïve cells (CBMC) vitamin D has a balanced effect on cytokine production, inhibiting both Th1 and Th2 cytokines (110). What is also apparent from the work of Pichler et al (110) is the influence that the immediate surrounding environment (cytokines and other stimuli) exerts on the differentiation and function of the Th subsets. Therefore, in order to develop a more complete understanding of the development and maintenance of an allergic immune phenotype we must expand our analysis to other cells that influence the cytokine milieu.

**Regulatory T cells**

**Function and phenotyping**

The ability of the immune system to promote inflammation is part of our defense against invading pathogens; however, the ability to control this inflammation and prevent inappropriate reactions against self- or innocuous exogenous-antigens is also necessary to prevent tissue damage and disease. Tolerance of self-antigens and environmental molecules was historically viewed as simply a lack of immune response. We now know that rather than being a passive state, tolerance is an active process under the control of various sets of “regulatory” cells.
While multiple subsets of T (114) and B (115) cells with regulatory function have been identified, this review will focus on the major subsets of regulatory T cells (Treg), as these are the best described in terms of allergic disease. Various combinations of surface and intracellular markers have been used for phenotyping Tregs, with an increasing number of subsets being described. First described in the 1980s and 1990s using combinations of non-specific markers CD4 and CD25 or CD45RB, phenotyping was greatly advanced by the discovery of the transcription factor forkhead box P3 (FoxP3) in the early 2000s (reviewed in (116)). Recently, several groups showed that the lack of cell surface CD127 can be a useful alternative to CD25 for the delineation and purification of human Treg cells; FoxP3 expression and suppressive ability being enriched in CD4^+ T cells that express low levels of CD127 (117). Thus, the benchmark Treg phenotype is CD4^+CD25^+FoxP3^+CD127^-.

The high expression of CD25 (IL-2 receptor α-chain) is indicative of the dependence of Treg on IL-2 for differentiation and function (116). Being unable to synthesize IL-2 themselves, Treg respond to IL-2 produced by conventional T cells on exposure to antigen, resulting in a negative feedback loop (116). The mechanisms through which Treg control immune responses are yet to be conclusively determined, although putative mechanisms have been described by Caridade et al. (118) in their recent review on the subject. Treg may control immune activity through one or multiple of the following mechanisms: the production of inhibitory cytokines IL-10, TGF-β and IL-35; cytolysis of effector T cells by granzyme B and perforin; competition for IL-2 or other APC signals or cytokines; depriving naive/effector cell of essential amino acids by stimulating APC to produce enzyme that consume the same; or lastly, the direct engagement of APC by Treg could result in weakened or abrogated signaling to naïve/effector cells. The different suppressive mechanisms utilized by Treg are likely to
be reflective of heterogeneity within the Treg population, with functionally distinct subsets of Treg being identified based on memory (119) and activation (120, 121) status. A notable example of this is the description of functionally distinct populations of Treg identified on the basis of HLA-DR (MHC II) expression (120). Treg that express HLA-DR represent a mature population that inhibit T cell proliferation and activity via an early contact-dependent mechanism, associated with high FoxP3 expression (120). HLA-DR- cells, on the other hand, initially enhance secretion of IL-4 and the suppressive cytokine IL-10, before later initiating FoxP3-associated, contact-dependent suppression (120).

Association with allergic disease

Treg number and function has been associated with clinical allergic disease in a remarkably consistent manner. Cross sectional studies have revealed that children with allergic disease have a lower proportion of Treg in circulation (122-124), dermatitic skin lesions (122) and bronchoalveolar lavage fluid (BALF) (125). Some of these also report associations between Treg and disease severity, such as inverse correlations with eczema severity (122) and allergen-specific IgE (123), and positive correlations between the proportion of Treg and forced expiratory volume (FEV1) (125). In addition to lower numbers of Treg, there is also evidence that Treg may be functionally impaired in allergic disease, showing a reduced ability to suppress T cell proliferation and Th2 cytokine and chemokine production (125). However, being cross-sectional studies these results cannot be taken to imply causation. As the observed associations between Treg and allergic disease may be a result of the established nature of the disease it is helpful to look at immune profiles in individuals pre-symptomatically.
As allergic disease is more common in the offspring of atopic parents numerous researchers have compared immune phenotype at birth based on the status of hereditary risk. In cases of parental history of allergy Treg numbers in CB appears to be reduced (126, 127), and the Treg of high risk neonates have a lower capacity to regulate inflammatory responses (127-129). Furthermore, an impaired number of Treg at birth has been independently associated with the development of allergic conditions in early childhood (126, 130). Hinz et al. (126) find that the odds of physician-diagnosed eczema (1.55 [1.00, 2.41]) or food allergen sensitization (1.55 [1.06, 2.25]) in the first year of life was significantly greater in children with a lower number of CB Treg, after controlling for parental history of allergy, gender and maternal exposure to tobacco smoke (n=345). This association is also reported by Herberth et al. (130) who find that a lower number of CB Treg is associated with an increased odds of eczema at one, two and three years of age (2.15 [1.05, 4.40], n=606; 2.02 [1.08, 3.77], n=546; and 2.02 [1.12, 3.67], n=514, respectively). Due to the strong associations between Treg and the development of allergic disease, identification of factors which influence the number and function of Treg is important for the development of preventative strategies. Interestingly, in addition to clinical outcomes vitamin D has been associated with Treg measures, suggesting that the reduced risk of allergic inflammation observed with higher vitamin D levels may be related to the modulation of Treg.

Investigations in asthmatic children has found that circulating 25(OH)D levels are positively correlated with number of peripheral (131) or frequency of BALF (132) Treg. Additionally, both serum 25(OH)D levels (133) and the in vitro addition of 1,25(OH)₂D (134) have been shown to be positively correlated with suppression of T cell proliferation by Treg. The former study, however, reported no significant correlation between vitamin D and Treg frequency or number in multiple sclerosis patients (133).
Conversely, Khoo and colleagues (135) have found that in healthy individuals seasonal changes in vitamin D status do not influence total Treg numbers, although Treg as a percentage of CD4+ cells is lower in summer as a result of an increased CD4+ compartment. However, the aforementioned studies are conducted in children or adults, and as such do not inform on the relevance of vitamin D on the establishment of an appropriately functioning Treg pool.

There are a limited number of papers addressing the relationship between vitamin D and Treg in CB, with those available reporting no (136), borderline significant (137) or negative (44) correlations. Although previous studies have associated CB Treg number with allergic disease risk (126, 127, 130), the functional capacity of Treg is also of relevance to immune disorders (125, 129, 133). Of the studies examining CB Treg in relation to vitamin D, only Chi et al (137) assessed functional capacity. These authors find no impairment in suppressive activity despite inverse correlations between CB 25(OH)D and Treg number (137). On account of the many reported interactions between Treg, vitamin D and allergic disease, further examination of the influence of vitamin D on the developing infant Treg pool (number and function) would be useful in understanding the role of vitamin D in allergic disease.

**Antigen presenting cells**

While all nucleated cells are capable of presenting antigen to CD8+ T cells, the term antigen presenting cell (APC) by convention refers to specialized cells that present antigen to CD4+ T cells (138). This definition covers dendritic cells (DC), macrophages, B cells (138) and monocytes (139), with DC being the most effective due to their ability to activate naive, as well as previously activated CD4+ T cells (138). Upon entry into
the body (through the respiratory tract, gastrointestinal tract or skin) antigens are captured by APC, proteolysed, and peptides expressed on MHC for presentation to effector T cells (138). The subsequent immune responses differ depending on the APC involved but includes differentiation and proliferation of effector T cells (DC), and B cell activation and antibody production (B cells) (138).

Given the important role APC play in immune responses it is unsurprising that differences in APC populations are been reported to be involved mechanistically in allergic disease (140-143). APC are of crucial importance to the Th1/Th2 (and Th17) paradigm of autoimmune and allergic disease, as the cytokines and stimulatory molecules they express in response to antigens are capable of polarizing T cells into various effector or regulatory subsets (99, 144). Therefore, factors which may influence APC function is of great interest to those working in allergy research.

As has been found with other cell types of the immune system, APC too possess the vitamin D receptor (145-147). What is more, DC (148), macrophages (149) and monocytes (150) are capable of producing 1,25(OH)₂D₃ from 25(OH)D₃, which can then exert it’s effects in the local environment. As the most effective of the APC, the relevance of DC in allergic disease will be examined in the following section, with particular attention given to the influence 25(OH)D₃ status may have on the development and function of these cells. While it is acknowledged that DC function in a broader context than initiation or regulation of allergic disease, detailed discussion of such roles is beyond the scope of this review.
Dendritic cells

Subset phenotyping

Dendritic cells, while part of the mononuclear phagocyte system along with monocytes and macrophages, differ phenotypically and functionally from the other members of the system; not least in their capacity to migrate from tissue (reviewed in (151)). DC are the predominant APC involved in the recognition and presentation of allergens, which in allergic individuals results in a cascade of events leading to IgE production and mast cell triggering (152). Due to the initiating role in this series of events, allergen recognition and presentation by DC is considered an attractive target in the study of allergic disease, although it is becoming evident that the capacity to induce and stimulate adaptive responses varies between DC subsets (153).

Comparative phenotypic analysis has identified a number of DC subsets, the most notable of the blood subsets being plasmacytoid DC (pDC) and conventional, or myeloid, DC (cDC) (154). All DC have in common the expression of MHC II and lack of lineage markers such as CD3, CD19 or CD56, while certain positive markers have been described to differentiate between pDC and cDC (154). pDC for example, are identified by their expression of CD123, CD303 and CD304, with cDC expressing CD11c (151). cDC are further categorized as CD11c+, CD141+ or CD16+ (154).

Role in allergic disease

The most distinct functional aspect of pDC is their rapid and substantial production of Type-I interferon to TLR7 and 9 ligands (e.g viral RNA), in addition to production of TNFα and IL-12 (155). pDC are efficient primers of CD4+ T cells, inducing Th1
polarization (155), although may also induce Th2 responses given the appropriate stimuli (156). pDC have also been shown to possess strong tolerogenic capacity through the induction of Treg differentiation, CD8+ T cell deletion and CD4+ T cell anergy (141, 155), making them of interest to investigations on the development and maintenance of tolerance.

In a study by Kool et al. (141) the authors investigated the mechanism underlying the suppression of asthma in mice following treatment with the hematopoietic growth factor Fms-like tyrosine kinase receptor-3 ligand (Flt3L). They found that Flt3L treatment enhanced the number of pDC recruited to the lung, reducing Th2-associated eosinophilic inflammation (141). The authors confirmed the role of pDC in the inflammatory response by demonstrating that selective removal or adoptive transfer of pDC during allergen challenge resulted in enhancement or suppression of inflammation, respectively (141).

The importance of liver pDC for the development of oral tolerance has also been shown in mice, where pDC induce anergy or deletion of antigen-specific T cells (157). Once again, depletion of pDC in vivo led to OVA-induced allergic responses (157). It should be noted, however, that findings tend to be tissue specific, and analysis of pDC in peripheral blood of allergic individuals has yielded varying results (140, 143, 158, 159). In support of the hypothesis that having a greater (or at least appropriate) number of pDC is beneficial in terms of tolerance and prevention of allergy three studies in children find that circulating pDC are inversely related to asthma (140, 142, 143). This is in comparison to findings in adults or older children with existing disease where greater numbers of circulating pDC have been reported (158, 160). It could be argued,
however, that research in infants, such as the study by Upham et al (143) are of greater utility in determining immune changes which antedate the development of allergic disease, in contrast to adult studies where differences may be a result rather than a cause of the pathology. It is logical, therefore, to explore the immune profile of individuals pre- and post-clinical manifestation, and identify factors which may favorably influence phenotype and function.

cDC, meanwhile, appear to be potent inducers of Th2 differentiation and allergic inflammation, as has been extensively reviewed by Lambrecht (161). Experiments have demonstrated that the injection of allergen-pulsed mature cDC, but not pDC or immature cDC, into the trachea of naïve mice or rats results in sensitization and airway inflammation to allergen challenge (161). Furthermore, systemic and localized airway removal of cDC cures all feature of asthma, including airway eosinophilia, bronchial hyper-reactivity, and a decrease in Th2 cytokines (161). Within the broader cDC population there is evidence that the CD1+ subset in particular may be the major contributor to allergic inflammation. In comparison to pDC and monocyte-derived DC, CD1c+ cells drove the greatest increase in T cell proliferation and Th2 cytokine production to allergen challenge (153). Importantly for the prospect of treating and preventing allergic disease, recent evidence suggests that DC maturation and propensity to induce Treg is influenced by the availability of vitamin D metabolites, presenting the possibility for therapeutic intervention.
Figure 1.1. The biologically inactive vitamin D metabolite 25(OH)D3 is hydroxylated to 1,25(OH)2D3 within the DC by the enzyme CYP27B1. 1,25(OH)2D3 acts as an autocrine agent, reducing the expression of costimulatory molecules and inflammatory cytokines by the DC, as well as a paracrine agent, suppressing the differentiation of naïve CD4+ T cells to Th cells and migration to secondary lymphoid tissue, whilst inducing suppressive Treg.

While it is has long been recognized that the active metabolite of vitamin D, 1,25(OH)2D3, can directly alter immune cell function, this particular metabolite has limited clinical utility due to its calcemic effects. The major circulating metabolite 25(OH)D3, however, has minimal binding affinity for the vitamin D receptor (VDR) through which 1,25(OH)2D3 exerts its effects, and thus is minimally calcemic (162). Recently published papers have found that, in culture, 25(OH)D3 will inhibit monocyte-derived DC maturation (148, 162) and induce the development of IL-10-producing Treg (162, 163) to an extent comparable with 1,25(OH)2D3. These effects are reported to be
dependent on the ability of DC to convert 25(OH)D3 to 1,25(OH)2D3, as blocking the 1α-hydroxylase enzyme CYP27B1 abolished the inhibitive and suppressive effects of 25(OH)D3 (162). These findings imply that provision of adequate 25(OH)D3 levels, for example through preventing/correcting vitamin D deficiency with supplementation, may facilitate a tolerogenic, anti-inflammatory immune profile. While the clinical research on this topic is limited one 12 week placebo controlled vitamin D supplementation trial in 57 adult participants found that vitamin D supplementation increased the percentage of Treg without changing the percentage of either pDC or cDC (164). This increase in Treg is compatible with the theory that DC will produce 1,25(OH)2D3 from inactive substrate in order to modulate VDR-expressing lymphocytes.

The analysis of experimental and clinical data is supportive of the hypothesis that vitamin D may modulate the risk of allergic disease through its effects on the number and function of Treg and DC. While the provision of vitamin D in vitro induces tolerogenic immune phenotypes, the efficacy of vitamin D supplementation in vivo remains underexplored. It would appear that preventative interventions would be best aimed at early life based on the evidence of antecedent immune profiles predicting the development of clinical symptoms, and emerging research is likely to address this topic. However, the role of vitamin D in the etiology of allergic disease may go beyond immunoregulation, with a number of studies describing a requirement for vitamin D in lung and epithelial structure and function.
Organ Structure and Function

Lung tissue

Asthma is one of the most prevalent allergic conditions and has been frequently associated with low vitamin D levels (66, 84, 165, 166). Asthma is characterized by airway inflammation and hyperresponsiveness, with elasticity and resistance of the tissues also being affected (167). There is emerging evidence that vitamin D plays a role in asthma not only by modulating inflammatory processes, but also in the developing structure and function of lung tissue (168). Murine studies reveal that exposure to vitamin D deficiency in utero is significantly associated with decreased lung volume, tissue volume and increased airway resistance (168). The researchers propose that, as reduced lung volume is a risk factor for lung function and respiratory illness in humans, vitamin D deficiency during critical periods of lung development could increase the risk of these conditions. The effect of vitamin D deficiency on airway development in mice is supported by a second study which finds increased pulmonary and tracheal resistance following vitamin D deficiency in the perinatal period (169). Further, the authors provide evidence of inhibited surfactant phospholipid synthesis with vitamin D deficiency (169), which may contribute to poor pulmonary function and altered innate and adaptive immunity (170).

While these studies were conducted in animal models there is also evidence that vitamin D deficiency is associated with altered lung function in humans (166, 171). Specifically, Black and Scragg (171) report a significant association between serum 25(OH)D, forced expiratory volume in one second (FEV1) and forced vital capacity (FVC). This finding remained significant after adjusting for potential confounding factors such as body size, gender, smoking history and physical activity (171). Similar findings were subsequently
reported in a Chinese population of newly diagnosed asthmatic adults (166). Forced expiratory volume, FEV1 as a percent of predicted, and FVC were all significantly positively correlated with serum 25(OH)D concentration ($p < 0.05$). The studies of Black and Scragg (171) and Li et al (166) were of a cross-sectional design and conducted in adult populations, thus limiting their utility in identifying potential causal links between vitamin D deficiency and respiratory disease. However, building on their work in animal models, research by Zosky et al (28) have identified significant associations between 25(OH)D levels in mid pregnancy and respiratory conditions in human offspring in a birth cohort study.

In this study clinical measures of lung capacity and function (FVC and FEV1) at six years of age were positively correlated with maternal 25(OH)D levels measured at 16-20 weeks gestation (28). While associations between lung function and maternal 25(OH)D were strongest in girls and persisted up to 14 years of age, in boys maternal vitamin D deficiency significantly increased the odds of asthma at six years of age (OR 3.03 [1.02, 9.02]) (28). Worth noting in this study is that maternal vitamin D was not associated with atopic status in the offspring, with the authors suggesting that vitamin D deficiency influences the risk of asthma and lung function deficits through effects on tissue growth and development, independent of immunological mechanisms (28).

**The skin**

As with the airways, the skin too is exposed to environmental allergens and may become involved symptomatically in allergic conditions. Indeed eczema, or atopic dermatitis, is commonly the earliest manifestation in the so-called “atopic march”, presenting early in childhood and preceding asthma and allergic rhinitis (172). A
Growing body of evidence has associated vitamin D status with allergic skin conditions including eczema, with both immunological and structural factors being implicated (173, 174).

Eczema is characterized by significant disruption of the skin barrier function (175), lesional infiltration of Th2 cells and impaired innate immunity (176). Although immune abnormalities are a common finding in eczema it has been argued that dysfunctional skin barrier function precedes the development of the condition (177). Evidence for this view comes from findings that over half of all children with eczema in the first two years of life do not have signs of IgE sensitization, rather becoming sensitized during the course of the disease (178). The disrupted skin barrier function results in increased transepidermal water losses and xerosis, a propensity for viral and microbial colonization, abnormal pH, and increased permeability to environmental allergens (175). The epidermal penetration of high-molecular-weight allergens (e.g. pollens and food) can then drive APC to enhance Th2 polarization (177), resulting in the characteristic Th2-dominant inflammation observed in eczema lesions.

The role of vitamin D in regulating skin homeostasis has been extensively reviewed elsewhere and a detailed description is beyond the scope of this review (173). Briefly, 1,25(OH)\(_2\)D inhibits proliferation and stimulates differentiation of keratinocytes, and induces multiple proteins associated with normal skin physiology, thus contributing to the formation of the permeability barrier (173). In the absence of the VDR or 1,25(OH)\(_2\)D the basal layers hyperproliferate, disrupting the function of differentiated layers and permeability barrier formation (173). The importance of vitamin D to skin
homeostasis is supported by the fact that keratinocytes not only possess the VDR but also 25-hydroxylase and 1α-hydroxylase enzymes (173).

However, while vitamin D supplementation, 25(OH)D status and UV radiation have been inversely associated with eczema severity or prevalence (62, 65, 70), it is not clear to what extent circulating 25(OH)D, local production of 1,25(OH)₂D or UV radiation contribute to maintaining skin homeostasis (173). Vitamin D deficiency alone is not sufficient to cause eczema or other allergic manifestations, as evidenced by the absence of association between rickets or kidney disease and atopy (52). It has, however, been suggested that vitamin D deficiency may act synergistically with other insults (i.e. infections) to disrupt skin barrier function, potentially through impaired production of antimicrobial peptides (179). In fact this “multi-hit” model is similar to that proposed by others to describe the synergistic effects of vitamin D status, the establishment of the microbiome and gastrointestinal infections on the development of allergic disease (52, 180).

The gut microbiome and intestinal barrier function

The gut has evolved a highly specialized immune network to cope with vast exposure to an array of foreign antigens from microbial flora and dietary products at its luminal surface, and is a crucial player in the development of immune tolerance (181). This network of gut-associated lymphoid tissue is the largest immune “organ” of the body and comprises distinct tissues (such as the Peyer’s patches and mesenteric lymph nodes) as well as a vast array of migrating cells that passage through the local tissues (such as DC, B cells, effector T cells and a range of regulatory populations) (181). These all act together to provide a finely balanced environment which affords immune defense
against pathogens, while at the same time suppressing unwanted inflammation against harmless commensals and allergens (181). As oral tolerance is arguably one the most critical functions of the gut-associated lymphoid tissue, there is growing interest in the developmental events in the gut that could contribute to the inappropriate immune responses associated with rising rates of food allergy. As immune cells including Treg and DC are influenced by vitamin D, there is speculation that vitamin D has the potential to directly influence immune function and tolerance in the local gut microenvironment.

Weiss (180) proposes that vitamin D may modulate signal trafficking between bacterial antigens from the gut and Treg and DC populations, influencing immune function and the development of tolerance. A similar hypothesis has also been put forward by Vassallo and Camargo (52), whereby the authors propose a “multi-hit” model involving vitamin D deficiency, altered microbial colonization, gastrointestinal infection and disruption of the intestinal barrier. The authors speculate that vitamin D deficiency compromises innate and adaptive immune function, including the production of antimicrobial peptides and the modulation of host-microbe signaling pathways, resulting in dysbiosis and susceptibility to gastrointestinal infections (52). Vitamin D deficiency may also compromise mucosal barrier function due to the role of 1,25(OH)₂D in regulation of tight junction proteins (52). The synergistic effect of gastrointestinal infection and impaired barrier integrity would increase the exposure to food proteins, thus contributing to the development of sensitization (52).

This hypothesis has support from animal models, where CYP27B1 knockout and VDR knockout mice display dysbiosis compared to wild type controls, and are extremely
susceptible to dextran sodium sulfate-induced colitis (182). Treatment of CYP27B1 knockout mice with 1,25(OH)\(_2\)D reduced Helicobacteraceae numbers and suppressed colitis symptoms including weight loss and blood loss (182). The authors conclude that the observed effects of 1,25(OH)\(_2\)D on the microbiome must be through indirect effects on the host immune responses, as prokaryotes do not express the VDR (182). However, while the vitamin D/microbiome hypothesis is biologically plausible a substantial amount of research is still required to determine whether modulation of vitamin D status in humans has a significant impact on the modulation of the gut microbiome, and subsequent risk of allergic disease. Proponents of the hypothesis call for studies into the relationship between early life 25(OH)D status, the microbiome and gastrointestinal infections, and ultimately RCTs in pregnancy and infancy (52).

While the exact physiological mechanisms underlying the aforementioned immunological and structural observations continue to be explored, there is growing evidence that genetic variability modulates metabolism of, and sensitivity to, vitamin D. It is recognized that 1,25(OH)\(_2\)D may have a role in regulating activity of a vast number of genes; directly and through epigenetic changes, including at sites directly related to immune cell transcription (183). The existence of genetic variations that alter the metabolism and actions of vitamin D may help to explain some of the inconsistency observed between studies may help in identifying at-risk individuals.
Nuclear Actions, Genetic Polymorphisms and Environmental Interactions

Nuclear Actions of Vitamin D

The vitamin D receptor (VDR), encoded by the VDR gene (VDR) (184), is a member of the nuclear receptor family subgroup NR1I (185). The VDR mediates its actions by first binding with its ligand (1,25[(OH)₂]D) then forming a heterodimer with the retinoid X receptor (RXR) (186). This heterodimer binds to the VDR response element (VDRE) and initiates recruitment of nuclear proteins into the transcriptional complex (186). The VDR is found on tissues throughout the body including immune cells, and has effects on apoptosis and cell differentiation (184). The DNA-bound VDR/RXR heterodimers can also down regulate transcription; as previously described in relation to T cell cytokine production (185). It is, therefore, quite feasible that VDR polymorphisms could significantly impact immune regulation by altering the differentiation and proliferation response (186).

Genetic variants of the Vitamin D Receptor

Mutation to the VDR gene is responsible for conditions such as the rare monogenetic disease 1,25-dihydroxivitamin D resistant rickets, however many more subtle genetic polymorphisms are frequent in the population (186). A recently published systematic review and meta-analysis of case-control studies has described the association between a number of VDR restriction fragment length polymorphisms and the risk of asthma (187). This analysis found a significant association between TaqI and BsmI polymorphisms, and a marginal association for SNP FokI and asthma risk. Additionally, within the studies analysed certain TaqI, BsmI and ApaI alleles were overrepresented in atopic and sensitized individuals (184), with two other VDR polymorphisms (rs2107301 and rs1540339) associated with eosinophil count (188). Overall, the effect size of VDR
polymorphisms is modest, and findings are not entirely consistent between studies. This may be for various reasons including sample size and ethnicity of the populations studied, but is also likely reflective of the fact that asthma and allergy are complex diseases involving multiple genetic and environmental factors (187). Even when considering vitamin D as an isolated environmental factor there are numerous other genes involved in the vitamin D metabolism pathway that have been associated with the risk of allergic disease (188). For example, Pillai et al (188) also describe significant associations between CYP24A1, CYP2R1 and the odds of asthma or clinical measures (spirometry), and there has been recent interest in the role of vitamin D binding protein in vitamin D status and allergy (189-193).

*Genetic variants of vitamin D binding protein*

The major function of vitamin D binding protein (DBP) is transport of 25(OH)D and 1,25(OH)_2D in circulation; however, analogous to the metabolites it transports, continued research since its discovery has revealed multiple implications for this protein in both skeletal and non-skeletal health (190). Of particular interest is the variation in binding affinity that occurs as a result of polymorphisms and the resulting availability of vitamin D to immune cells (191).

DBP is highly polymorphic (three common polymorphisms yielding 6 allelic combinations, plus >120 rare variants) and displays racial and geographic trends (190). The GC SNPs most commonly investigated are rs7041 and rs4588 (189). rs4588 polymorphisms, whereby adenine is substituted for cytosine to produce the protein referred to as Gc2, are reportedly strongly inversely associated with circulating DBP and 25(OH)D levels in young children (189), although others have also reported rs7041
polymorphisms (coding Gc1F and Gc1S) to be associated with 25(OH)D levels (193). Interestingly, while the former did not find rs7041 SNPs to be associated with circulating 25(OH)D levels (189), researchers from the same ground have reported the risk of asthma is greatest in Gc1F/1F diplotypes, compared with Gc1S carriers (192). A possible explanation for this finding is related to the binding affinity of the different DBP versions and, subsequently, availability of vitamin D metabolites for tissues.

The amino acid changes in DBP that occur between genotypes appear to influence the affinity of DBP for vitamin D metabolites, with the strongest binding occurring with Gc1F allele (191). It is hypothesized that the amount of free (unbound) vitamin D is the measure of most relevance to extra-renal, vitamin D-dependent physiological processes, as metabolites that are strongly bound to DBP (i.e. Gc1F versions) are largely unavailable for utilization by tissues such as monocytes and macrophages (191). The major limitation in this area of research is the difficulty of measuring free versus bound vitamin D using current technology. For this reason mathematical models have been employed to estimate free levels based on concentration and genotype-defined variations in binding affinity (191). Chun et al (191) have used such a model to estimate the effects of DBP genotype on the induction of the vitamin D target gene cathelicidin in monocytes. Supporting the “free hormone hypothesis”, the authors predict stronger monocyte responses associated with low affinity forms of DBP (191). While representing a promising hypothesis it remains early days for this area of research and further work is needed to explore associations between DBP, immune function and allergic disease. It must also be noted that, like other genetic correlates, the role of DBP in the development of allergic disease is likely to be influenced by a multitude of gene-gene and gene-environment interactions.
Gene-Environment Interactions

A likely reason that genetic association studies have been largely conflicting and failed to identify specific ‘causal’ genes is that the polymorphisms alone do not necessarily cause disease, but require interaction with environmental exposures (194). Some relationships between genotype and disease will only be seen in conditions of “high” exposure to an environmental factor of interest, whereas others may only be seen in conditions of “low” exposure. Thus, failure to examine the relationship in the context of exposure could lead to an inaccurate interpretations or missing potential relationships. Similarly, the potential effects of environmental exposures on health outcomes could be missed if variations in genetic predisposition are not taken into account. These effects are likely to explain inconsistencies between studies looking at the links between genetics and asthma phenotype. Functional consequences of polymorphisms may, therefore, vary with the availability of vitamin D to tissues and become apparent, or exaggerated, in an environment of vitamin D deficiency, such as observed in the case of several infectious diseases (195, 196). Similar complex interactions are now recognized between vitamin D status and other genes involved in immune responses related to allergic disease (67).

Lui et al (67) investigated the effects of vitamin D deficiency at birth (cord blood) as a risk factor for food sensitization, both independently and in interaction with a variety of genetic polymorphisms, in a study involving 649 children enrolled in a prospective birth cohort. While vitamin D deficiency alone was not significantly associated with sensitization in childhood, a significant interaction was noted between vitamin D deficiency and the C allele of the rs2243250 SNP of the IL-4 gene in relation to sensitization (OR 1.79 [1.15, 2.77]) (67). IL-4 plays a central role in IgE regulation and
IL-4 polymorphisms have been associated with asthma by other researchers (197), although risk was in the opposite direction to the findings for sensitization by Lui et al. (67).

All of these observations further highlight that allergic disease has complex multifactorial etiology, with numerous (and diverse) predisposing genes, and a wide range of interacting environmental factors (194). Genetic variations between study groups may partially explain conflicting findings on the relationship between vitamin D and allergic disease. Future research is likely to benefit from reduced costs and greater availability of gene analysis technology, and should help to provide a better mechanistic explanation of disease pathogenesis and identification of at-risk populations.

**Non-vitamin D actions of UV radiation**

Another facet to the vitamin D-allergy hypothesis is the fact that UV radiation, the major source of vitamin D, has immunomodulating effects independent of the production of vitamin D. UV-induced immunosuppression has been reported in mice (198) and humans (199), being associated with beneficial consequences such as protection from asthma and multiple sclerosis, but also with undesirable outcomes including skin cancer and attenuated vaccine responses (200). The local and systemic immunomodulating effect of UV radiation presents an alternative interpretation of ecological studies that report associations between latitude and allergic disorders, and raises the question of whether vitamin D status is to some degree merely acting as a biomarker for UV exposure.
Recently, Gorman et al (201) demonstrated not only that UV radiation is suppressive of Th1/Th17 contact hypersensitivity and allergen-induced Th2 inflammation, but these effects occur independently of changes in vitamin D status. Whilst unraveling the independent effects of vitamin D and UV exposure in humans is highly challenging – a result of the strong correlation between the two and the dominance of UV exposure as a source of vitamin D - a phenomenon in mice has enabled these researchers to investigate the variables independently. Gorman et al (201) noted that vitamin D deficient male, but not female, mice have reduced levels of the vitamin D precursor molecule 7-dehydrocholesterol in the skin, and subsequently fail to produce vitamin D in response to UVB exposure. Due to this phenomenon the effects of UV radiation on immunity can be examined and compared within a species without the use of gene knock-out or other interfering techniques.

In agreement with previous research (202), this set of experiments finds that UV radiation suppressed Th1/17-driven contact hypersensitivity equally in male and female mice (201). This was attributed to a reduced capacity for CD11c+ dendritic cells (cDC) derived from the bone marrow of UV irradiated mice to prime Th1/17 cells (201). In the model of allergic airway disease the researchers identified a reduced number eosinophils and IL-5 (Th2-specific responses) in the bronchoalveolar lavage fluid of UV-irradiated mice of both sexes (201). Several mechanisms have been suggested for the immunosuppressive effects of UV radiation including the production of soluble mediators such as prostaglandin E2 (PGE2), which can regulate DC maturation, migration and cytokine production (203), and the induction of CD4+CD25+FoxP3+ Treg (204). However, while these mechanisms may be relevant following erythema doses of UV radiation, experiments using lower doses of radiation, such as would more commonly be encountered in daily life, suggest other factors may be at play (202).
In contrast to experimental protocols employing erythemal doses of UV radiation, Rana et al (202) find that exposure to ~0.3 of a minimal erythemal dose for three consecutive days resulted in significantly suppressed contact hypersensitivity responses, without detectable differences in lymph node Treg populations. Rather, the authors report that UV irradiation inhibited the expansion of CD4$^+$ and CD8$^+$ effector T cells following hapten sensitization (202). To investigate the requirement for PGE2 in the immunosuppressive pathway, the authors applied indomethacin, a cyclooxygenase inhibitor, prior to sensitization (202). Indomethacin did not prevent UV-irradiation from inhibiting the induction of effector T cells, indicating that suppression of T cell activity is not dependent on PGE2 (202).

The potential for UV radiation to impact immune function and allergic disease is a fact often overlooked by vitamin D/allergy researchers. It does, however, present a plausible explanation for geographical trends in immunological disorders, as well as discrepancies between studies of vitamin D intake versus 25(OH)D status (for example (34) and (48)). As the scientific community embarks on larger and better designed studies of the role of vitamin D in the development of allergic disease it would be advisable to give consideration to the potential for confounding by UV radiation. This will be of particular importance in describing immunological outcomes as the differing mechanisms of vitamin D and UV may obfuscate the true effects of the intervention.

**Conclusions and Future Directions**

The dramatic and significant increase in allergic disease over the 20th and early 21st centuries has generally been attributed to environmental and lifestyle changes that occur with industrialization (205). An important consequence of urbanization and
industrialization has been a reduction in sun exposure and, subsequently, vitamin D deficiency (3). Vitamin D deficiency at subclinical levels is recognized to be prevalent in populations globally, and it is hypothesized that this is a contributing factor in the allergy epidemic (206). This review of the literature has examined that hypothesis at a mechanistic level, and in terms of clinical manifestation.

Observational studies provide evidence that suboptimal vitamin D levels at birth (determined by CB 25[OH]D measures) are prevalent in many populations and may increase the risk of wheeze and eczema in the offspring (47-50). The associations between 25(OH)D and outcomes in the offspring appear to be stronger for CB than maternal measures throughout pregnancy (39-42), possibly indicating a requirement for adequate vitamin D in establishing a tolerogenic immune profile in the early postnatal period (52, 180). However, whether measures aimed at improving status at birth (antenatal supplementation) or throughout infancy (postnatal supplementation) will be efficacious in the prevention of allergic disease is yet to be determined.

Mechanistically, there is clear evidence that vitamin D has immunoregulatory properties, and suboptimal supply of vitamin D to immune tissues may result in an inflammatory and allergic phenotype. In particular, vitamin D is associated with the cytokine production profiles of T cells (e.g. Th1/Th2 balance) (110), and the number or function of Treg (131, 134) and DC (148, 163). This review also identifies ecological and experimental data suggestive of a role for UV radiation in the development of allergic disease; potentially confounding associations between 25(OH)D levels and immune function or allergy outcomes. The available data also suggest that genetic variation within and between populations could be relevant to the influence of vitamin
D status on allergic outcomes. Single nucleotide polymorphisms of the vitamin D receptor (187), vitamin D binding protein (189), and non-vitamin D-pathway genes such as IL-4 (67), all may influence the interaction between vitamin D and immune function; subsequently limiting the generalizability of results.

Thus, despite the presence of inconsistency in the literature the evidence to date is supportive of further research into the role of vitamin D in the development of allergic disease. Important issues that remain to be addressed include 1) the current vitamin D status of pregnant women and infants in the Australian context – a country with one of the highest rates of allergic disease despite high levels of ambient UV radiation -, 2) associations between vitamin D levels in infants and their subsequent immune phenotype and risk of allergic disease, and 3) whether vitamin D supplementation effectively replicates the beneficial immunological associations previously reported for 25(OH)D. It is these questions that form the basis of the observational studies and experiments conducted for this thesis.
Chapter 2

Vitamin D deficiency and insufficiency is prevalent in pregnant women in Perth, Western Australia

Jones AP, Rueter K, Siafarikas A, Lim EM, Prescott SL, Palmer DJ.

Manuscript in preparation for submission
Introduction

Vitamin D is best recognised for its role in calcium metabolism and bone health although recent research has unveiled associations between low vitamin D levels and cancer, immunoregulation and cardiovascular disease (reviewed in (207)). Studies from multiple countries report widespread vitamin D deficiency, including in pregnant women (38, 208, 209). This is of concern as low 25(OH)D (the major circulating vitamin D metabolite) levels in pregnancy have been associated with an increased risk of adverse pregnancy outcomes including gestational diabetes, pre-eclampsia, small for gestational age infants and low birthweight infants (210). In addition, low maternal 25(OH)D in pregnancy has been linked to later consequences for the offspring in terms of bone development (211), respiratory tract infections (47), and allergic conditions such as eczema (48) and asthma (28). Based on such findings vitamin D has re-emerged as a public health issue with ongoing research into the prevalence and consequences of deficiency.

As the major source of vitamin D is endogenous synthesis initiated by exposure to ultraviolet (UV) radiation, it has been commonly assumed that vitamin D deficiency is largely restricted to populations living far from the equator. Yet recent studies have found that even in locations with high UV radiation vitamin D deficiency remains endemic (83, 212). Within the past decade a number of publications have reported prevalent vitamin D deficiency in pregnant women residing in Sydney, New South Wales, Goulburn Valley, Victoria and Adelaide, South Australia; localities at more southerly latitudes than Perth, Western Australia and with high proportions of dark skinned or veiled women (18, 213, 214). However, there is a lack of data on the vitamin D status of pregnant women in Perth, Western Australia.
Recent publications utilizing data from a Western Australian birth cohort study (the Raine Study, recruited in 1989-1990) indicate that vitamin D deficiency was highly prevalent (36%) in a population of predominantly white Caucasian pregnant women in Perth (215). In the absence of contemporaneous data it is uncertain whether the vitamin D status of the population has changed in the past 25 years. In addition to the lack of current data, none of the aforementioned Australian studies have examined the contribution of exogenous (supplemental) vitamin D to circulating 25(OH)D levels. Thus, in the present study we aim to describe the vitamin D status of pregnant women in Perth, Western Australia, in relation to factors including exogenous intake, body mass index (BMI), skin pigmentation and sun exposure (216).

Methods

Subjects

Participants were recruited from maternity hospitals in Perth, Western Australia, for involvement in a childhood allergy prevention research trial (ACTRN12606000281594) between 31st August 2012 and 30th August 2014. Criteria for exclusion were cigarette smoking during pregnancy, diagnosis of an autoimmune disease, or polycytesis. Appointments were conducted at 36-40 weeks gestation where questionnaire data, height and weight measurements, and a blood sample were collected. All participants provided written, informed consent. The study was approved by the Princess Margaret Hospital for Children Ethics Committee and Research Governance Office (1959/EP), Joondalup Health Campus Human Research Ethics Committee (1224), Sir Charles Gairdner Hospital Human Research Ethics Committee (2012-070), St John of God Health Care Ethics Committee (561), and the South Metropolitan Area Health Service Human Research Ethics Committee.
**Blood collection and 25(OH)D analysis**

Blood was collected from the cubital fossa into a serum clot activator tube (Vacuette, Z Serum Clot activator, Greiner Bio-One GmbH, Kremsmünster, Austria) and processed immediately after the appointment. Serum samples were analysed for total 25(OH)D by a competitive chemiluminescent immunoassay, automated on the Abbott Architect i2000 (Abbott Laboratories, Abbott Park, Illinois), operated by PathWest Laboratory Medicine, Western Australia. The assay uses 60 µl of serum to determine 25(OH)D concentration. Internal quality control data indicates the imprecision of the assay (coefficient of variation) as follows: 11.4% at 22 nmol/L; 5.2% at 48 nmol/L; 4.5% at 68 nmol/L; and 4.0% at 90 nmol/L.

**Intrinsic factors**

As the amount of vitamin D produced in response to UV exposure is determined by skin pigmentation (217) we determined skin type using a six category Fitzpatrick Skin Type questionnaire (218). Skin types I through VI are described as follows: I - Fair skinned Caucasians, burn very easily, never tan; II - Fair skinned Caucasians, burn easily, tan slowly and with difficulty; III - Medium skinned Caucasians, burn rarely, tan relatively easily; IV - Darker skinned Caucasians, virtually never burn, tan readily, e.g. Mediterranean ancestry; V - Asian or Indian skin; and VI - Afro-Caribbean or Black skin (219). Body weight and height were measured on the day of the appointment, and pre-pregnancy weight self-reported by participants.
**Exogenous vitamin D intake**

Vitamin D intake during the third trimester of pregnancy was ascertained through questions on the brand, dose and frequency of ingestion of multivitamin (containing vitamin D) and specific vitamin D supplements. Supplemental vitamin D intake is reported in international units (IU), where 1 IU is equivalent to 0.025 µg. Intake of vitamin D from food sources was not collected as for most Australians diet contributes <100 IU/day (220).

**Ultraviolet radiation and sun exposure habits**

To estimate the contribution of sun exposure to 25(OH)D status participants were asked about their sun protection habits and duration of sun exposure using a questionnaire adapted from a previously published example (221). Questions were asked on the use of sunscreen and hats when outdoors over the past two months. Body surface area exposed was reported as: face and hands; face, hands and arms; or face, hands, arms and legs.

UV Index (UVI) data was obtained from the Australian Radiation Protection and Nuclear Safety Agency to determine the relevance of seasonal changes in UVI to 25(OH)D levels. This data source has been used by others for modelling vitamin D synthesis (223).

**Statistical analysis**

All statistical analysis was conducted using SPSS v20 (IBM Corporation, Chicago, IL). A p value <0.05 was considered significant. Figures were generated in GraphPad Prism.
v 6.05 (GraphPad Software, La Jolla, CA). Season-adjusted 25(OH)D level was calculated following the example of Jenab et al. (224). Firstly, to account for differing numbers of observations per month an overall mean was calculated with weighting for the number of samples collected per month. Secondly, an unstandardised residual was calculated using a linear regression model with serum 25(OH)D as the dependent variable and month of collection as a categorical independent variable. Lastly, the unstandardised residual was added to the overall weighted mean serum 25(OH)D value to create the standardised value.

Correlative associations between 25(OH)D level and vitamin D intake, BMI and duration of sun exposure were analysed by linear regression, with unstandardised coefficients ($\beta$) (95% confidence intervals [95% CI]) and $R^2$ reported. Vitamin D intake was adjusted for body weight by dividing dose (IU/day) by kg of body weight. Raw and adjusted vitamin D intakes were correlated with season-adjusted 25(OH)D. BMI was calculated based on pre-pregnancy weight and reported as kg/m$^2$.

Independent sample’s t-tests were used for the comparison of means between two groups with parametric data, and one-way ANOVA where there were three or more groups. Mean and standard deviation (SD) are presented for the distribution of parametric data, and 95% confidence intervals (95% CI) for comparative analyses. For non-parametric data Mann-Whitney U tests and Kruskal-Wallis non-parametric one-way ANOVA tests were used and median and interquartile ranges (IQR) reported.
For categorical analysis of 25(OH)D status participants were classified as vitamin D deficient (<50 nmol/L), insufficient (50 - <75 nmol/L) or sufficient (≥75 nmol/L), following published guidelines (9). Categories for the analysis of pre-pregnancy BMI were <25, 25 - <30, and ≥30. Chi square (χ²) analysis was employed when comparing two or more categorical variables. Odds ratios (95% CI) for binary outcomes were calculated using binary logistic regression analysis.

Results

Population characteristics

Key demographic characteristics are presented in Table 2.1. One hundred sixty five (n = 165) participants were included in the analysis. Participants were predominantly of White Caucasian ethnicity (n = 137/165, 83%), as reflected by the high proportion of participants classified between I and III by the Fitzpatrick Skin Type questionnaire (Table 2.2). Our population showed a low prevalence of the classical risk factors such as dark skin (Fitzpatrick types V and VI), obesity, or lifestyle factors that preclude sun exposure (225).

<table>
<thead>
<tr>
<th>Table 2.1. Characteristics of antenatal study population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean (SD) (n=165)</td>
</tr>
<tr>
<td>Gestation (weeks), median (IQR) (n=164)</td>
</tr>
<tr>
<td>Gravidity, median (IQR) (n=162)</td>
</tr>
<tr>
<td>Completed tertiary (university) education, n (%)</td>
</tr>
<tr>
<td>Private hospital antenatal care, n (%)</td>
</tr>
<tr>
<td>Pre-pregnancy BMI, median (IQR) (n=157)</td>
</tr>
</tbody>
</table>

25(OH)D status of the study population

The 25(OH)D levels ranged from 19 nmol/L to 185 nmol/L with a mean (SD) season-adjusted 25(OH)D level of 74.5 (22.9) nmol/L. A serum 25(OH)D level of <50 nmol/L
was recorded in 25/165 (15.1%) of participants. A further 57/165 (34.5%) fell in the insufficient range (50 - <75 nmol/L). Sociodemographic factors were not associated with season-adjusted 25(OH)D levels, with the exception of private versus public antenatal care (Table 2.2).

**Table 2.2.** Season-adjusted 25(OH)D level by socio-demographic factors and Fitzpatrick Skin Type

<table>
<thead>
<tr>
<th>Sociodemographic factor (t-test)</th>
<th>Mean (95% CI) 25(OH)D (nmol/L)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gravidity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n=119)</td>
<td>75.2 (71.1 – 79.3)</td>
<td>0.411</td>
</tr>
<tr>
<td>&gt;1 (n=43)</td>
<td>71.9 (65.0 – 78.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Tertiary education</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (n=111)</td>
<td>76.0 (71.6 – 80.3)</td>
<td>0.246</td>
</tr>
<tr>
<td>No (n=54)</td>
<td>71.5 (65.4 – 77.7)</td>
<td></td>
</tr>
<tr>
<td><strong>Hospital</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Private (n=115)</td>
<td>77.2 (72.8 – 81.6)</td>
<td>0.021</td>
</tr>
<tr>
<td>Public (n=50)</td>
<td>68.3 (62.8 – 73.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Fitzpatrick Skin Type</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I (n=10)</td>
<td>77.7 (64.9 – 90.4)</td>
<td>0.258</td>
</tr>
<tr>
<td>Type II (n=51)</td>
<td>74.1 (68.7 – 79.6)</td>
<td></td>
</tr>
<tr>
<td>Type III (n=65)</td>
<td>71.9 (66.5 – 77.3)</td>
<td></td>
</tr>
<tr>
<td>Type IV (n=30)</td>
<td>79.8 (68.7 – 90.9)</td>
<td></td>
</tr>
<tr>
<td>Type V (n=2, both values presented)</td>
<td>41.4, 91.5</td>
<td></td>
</tr>
<tr>
<td>Type VI (n=1, single value presented)</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

**Intrinsic factors**

No difference in season-adjusted 25(OH)D levels were observed by Fitzpatrick Skin Type (Table 2.2) or ethnicity (data not shown). Comparing season-adjusted 25(OH)D levels of white Caucasians to all other participants yielded similar results (mean [95% CI] 74.9 [71.0-78.8] and 72.8 [63.9-81.6] nmol/L, respectively).

Pre-pregnancy BMI was not associated with season-adjusted 25(OH)D when analysed as a continuous or categorical. Season-adjusted 25(OH)D was, however, weakly inversely correlated with current weight (β = -0.238 [95% CI -0.471, -0.005], p = 0.046).
**Exogenous intake**

The majority of women, 138/163 (85%), reported taking a multivitamin supplement during the third trimester of pregnancy. Vitamin D was an ingredient in most of the multivitamin formulations used (129/137, 94%) (1 participant was unable to recall the brand used in order to calculate vitamin D intake). Specific vitamin D supplements were taken by 51/163 (31.3%) women, 41/51 (80.4%) of whom simultaneously took a vitamin D-containing multivitamin. The median (IQR) daily intake for those taking supplemental vitamin D was 500 (950) IU.

![Figure 2.1](image.png)

**Figure 2.1** Correlation between supplemental vitamin D intake and season-adjusted 25(OH)D level.

Vitamin D intake from supplements was positively correlated with season-adjusted 25(OH)D level ($\beta = 0.006$ [95% CI 0.001, 0.011], $p = 0.011$) (**Figure 2.1**). This correlation was strengthened by adjusting for body weight ($\beta = 0.622$ [95% CI 0.229, 1.016], $p = 0.002$), explaining approximately 6% of the variation in 25(OH)D levels ($R^2$...
Participants who took supplemental vitamin D in the form of a multivitamin, or both a multi- and single-vitamin product, had significantly higher season-adjusted 25(OH)D levels than unsupplemented women (Figure 2.2.), and women not taking supplemental vitamin D were at increased risk of deficiency (OR 3.62; 95% CI 1.3 - 10.2, p = 0.01).

**Figure 2.2.** Differences in mean (SD) season-adjusted 25(OH)D levels between participants taking no supplemental vitamin D (A), a vitamin D-containing multivitamin (B), a specific vitamin D supplement (C), or simultaneous use of both a vitamin D-containing multivitamin and a specific vitamin D supplement (D).

**Ultraviolet radiation and sun exposure factors**

Serum 25(OH)D levels were analysed in relation to average UVI over the 7, 30, 45, 60, 90 and 120 days preceding blood draw. Average UVI over the preceding 90 days (approximating one season) represented the strongest correlation with serum 25(OH)D (β = 3.011 [95% CI 1.852, 4.170], p <0.0001), and explained 14% of the variation in
25(OH)D levels ($R^2 = 0.139$). This association remained highly significant after controlling for supplemental vitamin D intake and current body weight ($\beta = 2.676$ [95% CI 1.542, 3.810], $p < 0.0001$).

The relevance of UVI to 25(OH)D levels can also be seen when compared to mean levels by month of blood draw (Figure 2.3). 24/25 (96.0%) participants with deficient 25(OH)D levels were recorded during winter and spring (June through November), representing an odds ratio of 16.5 (95% CI 2.2, 125.3, $p = 0.007$).

**Figure 2.3.** Seasonal variation in UV index (green line, mean [SD]) and 25(OH)D levels (bars, mean [SD]) in Perth, Western Australia over the study period.

The average weekly UVI over the 2013-2014 summer months of December-February was noted to be significantly greater than during the corresponding 2012-13 period (mean [95% CI] 13.1 [12.8-13.4] and 10.6 [10.1-11.1], respectively, $p <0.001$) (Figure
The higher UVI over summer 2013-2014 coincided with higher 25(OH)D levels in the winter of 2014 compared to the previous year (mean [95% CI] 79.5 [67.7-91.3] nmol/L and 60.6 [54.5-66.6] nmol/L, respectively, p = 0.002), confirming the correlation between 90 day average UVI and 25(OH)D levels.

25(OH)D levels in relation to sun exposure practices are presented in Table 2.3. Interestingly, while certain sun exposure practices were significantly associated with 25(OH)D levels (body surface area exposed and the use of sunscreen), these factors were also strongly seasonal. When adjusted for season sun exposure practices were no longer significantly associated with 25(OH)D levels.
Table 2.3. Variation in 25(OH)D level by sun exposure and protection factors, and in practices by seasonal

<table>
<thead>
<tr>
<th>Analysis of sun exposure and protection practices by season ($\chi^2$)</th>
<th>Mean (95% CI) 25(OH)D (one-way ANOVA)</th>
<th>Unadjusted</th>
<th>P</th>
<th>Season- adjusted</th>
<th>P</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
<th>Spring</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body surface area, weekend</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Face and hands (n=46)</td>
<td>67.7 (60.6 – 74.9)</td>
<td>0.005</td>
<td>73.8 (66.6 – 81.0)</td>
<td>0.946</td>
<td>0</td>
<td>6</td>
<td>32</td>
<td>8</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Face, hands and arms (n=65)</td>
<td>72.0 (66.1 – 78.0)</td>
<td>0.093</td>
<td>74.0 (68.8 – 79.2)</td>
<td>0.501</td>
<td>7</td>
<td>9</td>
<td>34</td>
<td>15</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Face, hands, arms and legs (n=47)</td>
<td>84.0 (76.4 – 91.7)</td>
<td>0.093</td>
<td>75.2 (68.2 – 82.3)</td>
<td>0.501</td>
<td>20</td>
<td>11</td>
<td>6</td>
<td>10</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Body surface area, week day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Face and hands (n=58)</td>
<td>70.6 (63.1 – 78.1)</td>
<td>0.093</td>
<td>76.7 (69.6 – 83.9)</td>
<td>0.501</td>
<td>0</td>
<td>4</td>
<td>42</td>
<td>12</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Face, hands and arms (n=63)</td>
<td>73.3 (67.1 – 79.5)</td>
<td>0.093</td>
<td>74.0 (68.7 – 79.3)</td>
<td>0.501</td>
<td>9</td>
<td>11</td>
<td>27</td>
<td>16</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Face, hands, arms and legs (n=37)</td>
<td>82.0 (75.3 – 88.7)</td>
<td>0.333</td>
<td>71.1 (65.4 – 76.8)</td>
<td>0.333</td>
<td>18</td>
<td>11</td>
<td>3</td>
<td>5</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Use of sunscreen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never (n=63)</td>
<td>65.1 (59.3 – 70.9)</td>
<td>0.001</td>
<td>70.6 (65.4 – 75.8)</td>
<td>0.230</td>
<td>3</td>
<td>7</td>
<td>39</td>
<td>14</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Sometimes (n=61)</td>
<td>81.4 (74.5 – 88.3)</td>
<td>0.001</td>
<td>77.4 (71.1 – 83.7)</td>
<td>0.230</td>
<td>15</td>
<td>13</td>
<td>19</td>
<td>14</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Always (n=34)</td>
<td>78.7 (71.2 – 86.3)</td>
<td>0.001</td>
<td>75.7 (67.9 – 83.5)</td>
<td>0.00</td>
<td>9</td>
<td>6</td>
<td>14</td>
<td>15</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Usually wore a hat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (n=101)</td>
<td>72.9 (68.5 – 77.2)</td>
<td>0.333</td>
<td>73.4 (69.5 – 77.4)</td>
<td>0.514</td>
<td>16</td>
<td>19</td>
<td>46</td>
<td>20</td>
<td>0.716</td>
<td></td>
</tr>
<tr>
<td>Yes (n=57)</td>
<td>77.0 (68.9 – 85.0)</td>
<td>0.333</td>
<td>75.9 (68.8 – 83.0)</td>
<td>0.514</td>
<td>11</td>
<td>7</td>
<td>26</td>
<td>13</td>
<td>0.716</td>
<td></td>
</tr>
<tr>
<td>Minutes spent in direct sun (Linear regression coefficient; median [IQR], Kruskal-Wallis one-way ANOVA presented for seasonal breakdown)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weekday (n=155)</td>
<td>0.002</td>
<td>0.957</td>
<td>-0.047</td>
<td>0.194</td>
<td>65 (77)</td>
<td>57 (30)</td>
<td>45 (35)</td>
<td>45 (42)</td>
<td>0.106</td>
<td></td>
</tr>
<tr>
<td>Saturday (n=154)</td>
<td>-0.016</td>
<td>0.534</td>
<td>-0.029</td>
<td>0.209</td>
<td>45 (116)</td>
<td>62 (100)</td>
<td>60 (97)</td>
<td>40 (69)</td>
<td>0.250</td>
<td></td>
</tr>
<tr>
<td>Sunday (n=154)</td>
<td>-0.011</td>
<td>0.626</td>
<td>-0.006</td>
<td>0.780</td>
<td>37 (64)</td>
<td>30 (90)</td>
<td>60 (100)</td>
<td>50 (75)</td>
<td>0.233</td>
<td></td>
</tr>
</tbody>
</table>
Discussion

Despite a low-risk study population and sun-rich environment this study finds vitamin D deficiency and insufficiency to be commonplace in pregnant women in Perth, WA. Uniquely, this study examined the contribution of vitamin D supplementation to the 25(OH)D levels of pregnant Australian women, finding that supplementation was an important factor in preventing vitamin D deficiency during the third trimester of pregnancy. Supplemental vitamin D intake produced a modest, dose-dependent increase in 25(OH)D levels, and women who did not take supplemental vitamin D were significantly more likely to have levels below 50 nmol/L. Of interest was the finding that despite providing a greater dose, the use of a specific vitamin D supplement provided no additional benefit to 25(OH)D levels than vitamin D-containing multivitamins. However, we cannot exclude the possibility that those women taking a vitamin D supplement were doing so based on medical advice following a 25(OH)D level test result earlier in pregnancy. Current recommendations are for pregnant women at risk of vitamin D deficiency to be screened early in pregnancy (225); thus, deficiency may have been detected and treated prior to assessment for the present study.

In comparison to results from the Raine Study – a population of pregnant women recruited approximately 25 years ago – this study finds a substantially lower proportion of mothers with vitamin D deficiency (36% and 15.1%, respectively) (215). Both studies were conducted in predominantly white Caucasian populations and used similarly performing vitamin D assays (Immunodiagnostic Systems enzyme immunoassay and Abbott Architect chemiluminescent immunoassay (226)); indicating that there has been a genuine improvement in the vitamin D status of this demographic
in the period between the two studies. One possible explanation for this is the increased prevalence of the use of vitamin D supplements in pregnancy over this period.

At the time of the Raine Study vitamin D supplementation was rare, and thus data on this variable was not collected (215). In contrast, 85% of participants in the current study took some form of supplemental vitamin D; a vitamin D-containing multivitamin, a specific vitamin D supplement, or both. At a median (IQR) daily intake of 500 (950) IU, and a dose-response rate of 2.5 – 5.0 nmol/L per 100 IU of vitamin D (227), the use of vitamin D supplements reasonably explains the change in 25(OH)D status over time. Yet despite this temporal improvement in status 49.6% of the participants in this study were vitamin D deficient or insufficient; a concern given the numerous adverse outcomes for the mother and offspring associated with low 25(OH)D levels (28, 47, 48, 210, 211, 215). Therefore, the data presented here suggest that routine low dose vitamin D supplementation, as is found in antenatal multivitamin formulations, is beneficial in preventing deficiency, without causing elevated levels (>250 nmol/L) (225). Higher doses may be necessary during winter and spring when insufficiency and deficiency is most prevalent.

Self-reported sun exposure factors such as time spent in sunlight and protective measures against sun exposure showed poor correlation to 25(OH)D levels, in agreement with previous studies (reviewed in (228)). A large number of individual factors are known to effect personal UV exposure (e.g. fabrics and sunscreen application), in addition to recall bias, all of which can compound to result in large errors in estimated exposure. While there was an association between skin surface area
exposed and 25(OH)D level this relationship did not hold when adjusted for season, suggesting that style of dress is influenced by seasonal climatic conditions.

The objective measure of ambient UVI, on the other hand, was highly significantly correlated with serum 25(OH)D levels; an association also reflected in the greater risk of deficiency and insufficiency in winter and spring. An unexpected observation in this study was the difference in UVI between the summers of 2012-13 and 2013-14, with higher levels being recorded over the latter. The higher UVI in the summer of 2013-14 was associated with higher 25(OH)D levels in the population over the 2014 winter, further highlighting the cumulative and long lasting contribution of UV exposure to 25(OH)D levels.

In contrast to other findings (229) this study did not detect an association between skin pigmentation, as assessed by ethnicity or Fitzpatrick skin type questionnaire, and 25(OH)D levels. This may be due to the small number of dark-skinned participants (n = 2 Type V and n = 1 Type VI), and thus low statistical power for these particular analyses. As such, the direct implications of these results are largely limited to a white Caucasian population and extrapolation to other population groups should be done with caution. In addition to the homogeneity of the study population this study is limited by the reliance on an automated chemiluminescent immunoassay, rather than the “gold standard” liquid chromatography tandem mass spectroscopy (LC/MS/MS) method. The choice of assay in the current study was dictated by a requirement to utilise a National Association of Testing Authorities accredited laboratory, and one that was able to analyse samples shortly after collection to determine eligibility for the trial. Although
not scientifically optimal the use of this assay reflects current clinical practice and is consistent within the study.

The novel information provided by this study has direct implications for the antenatal care of pregnant women residing in Perth, Western Australia. The data presented here suggest that current sun exposure and supplementation practices are inadequate to achieve sufficient 25(OH)D levels in approximately half of pregnant women in this locality. Regular use of a vitamin D-containing antenatal supplement represents a safe and effective strategy to reduce the risk of deficiency (<50 nmol/L), although higher doses may be required in winter and spring, or to achieve more desirable levels (≥75 nmol/L). As vitamin D insufficiency in pregnancy is associated with adverse health outcomes for mother and offspring these findings are relevant to antenatal care providers working within the Australian context.
Chapter 3

Cord blood 25-hydroxyvitamin D3 and allergic disease during infancy

Jones AP, Palmer D, Zhang G, Prescott SL.
Pediatrics 2012; 130(5):e1128-35

Appendix 1
Introduction
Allergic diseases are now the most common chronic disorders of childhood, with a pressing need to define the causal pathways and better prevention strategies. In particular, the rates of food allergy and eczema (230) have continued to increase dramatically in children as part of what appears to be a “second wave” of the allergy epidemic (205). Progressively earlier presentations with disease clearly implicate very early environmental influences such as exposures in pregnancy (205). Although this is likely to be multifactorial, there has been growing speculation that vitamin D insufficiency in pregnancy may have adverse consequences for early immune development of the fetus (231).

Previous studies provide a persuasive basis for the hypothesis that vitamin D may protect against allergic disease (reviewed in (232)). Reduced maternal dietary vitamin D intake in pregnancy has been reported as a risk factor for respiratory conditions such as wheezing, asthma, and allergic rhinitis (30, 31); however these studies were largely based on questionnaire-derived data rather than biological measures. Other studies have examined ‘season of birth’ as a surrogate marker of vitamin D status through sunlight exposure and found significantly higher rates of food allergy in children born autumn and winter (compared to spring and summer), providing indirect evidence that seasonal variations in sunlight exposure may make an important contribution to early disease risk (233). In older children, there is also evidence of lower serum 25(OH)D3 associated with allergen sensitization, eczema severity, and asthma (68, 165, 234).

Here, we have examined cord blood (CB) serum 25(OH)D3 levels, to reflect both dietary and non-dietary derived vitamin D in pregnancy. To our knowledge, this is the first study to examine CB 25(OH)D3 levels as an indicator of fetal exposure to vitamin D in relation to infant eczema outcomes. Our prospective cohort study with well-
defined allergic outcomes, documented maternal dietary and supplement intakes in pregnancy and CB serum samples provided an ideal opportunity to examine this question in addition to other early allergic outcomes.

Methods

Study design and subjects

The mother-baby pairs included in this study were derived from a larger (n = 669) prospective birth cohort recruited between 2002 and 2009 for the investigation of dietary exposures in relation to infant allergy outcomes. Only non-smoking mothers with healthy, uncomplicated term pregnancies were recruited. The population used for this study was selected on the basis of 1) ≥ 2 frozen cord blood serum samples in storage (317/669) 2) allergic outcomes assessed at 12 months of age (259/317) and 3) at least one parent with a history of allergic disease (eczema, asthma or hay fever) (231/259). The latter criterion was included as infants with a family history of atopy have a greater risk of developing allergic disease (50 – 80%) than those with no family history (20%) (235), providing greater statistical power, and results from this population are the most relevant and transferable to allergy prevention strategies. The cohort was recruited in Perth, Western Australia, as approved by the Princess Margaret Hospital Ethics Committee. Mothers provided written, informed consent.

Assessment of antenatal vitamin D intake

In the last trimester of pregnancy mothers completed a validated semi-quantitative food frequency questionnaire developed and analysed by the Commonwealth Scientific and Industrial Research Organisation in Adelaide, Australia (236). This recorded the frequency of consumption of 212 individual foods, mixed foods and beverages from which vitamin D intake in international units (IU) could be calculated. Energy intake
cut-offs were applied to identify unreliable records (237). In addition to food and drink items, details of all dietary supplements taken in the last trimester of pregnancy (including doses, brands and frequency of consumption) were collected. Reported supplement intake was converted into daily vitamin D intake (IU) using composition information provided by the manufacturers.

*Collection and analysis of umbilical cord blood samples for 25(OH)D3 concentration*

At delivery blood was collected from the umbilical cord or placental vein, and an aliquot (7-9 ml) transferred to a serum clot activator tube, which were kept out of light and processed within 8 hours. Aliquots of serum were stored at -80°C and transported in dry ice for analysis by RMIT Drug Discovery Technologies (RDDT, Melbourne, Australia) using liquid chromatography-tandem mass spectrometry (Applied Biosystems 4000 Q Trap and Agilent LC-MS/MS). The lower limit of quantification was 4.69 ng/mL for 25(OH)D3 and the intra-assay precision had a coefficient of variation of < 5%.

For categorical analysis by CB 25(OH)D3 concentrations we used cut-offs of < 50 nmol/L, 50 – 74.99 nmol/L and ≥ 75 nmol/L, as described in the literature (6). While similar cut-offs have been used by others in relation to CB concentrations (46, 47) there is evidence to suggest that neonatal 25(OH)D3 concentrations are generally lower than CB concentrations (238). We therefore used these values as categorical descriptors of vitamin D status rather than diagnostic criteria.

*Assessment of allergic status*

The primary outcome measures in the infants at 12 months of age were eczema and allergen sensitization. Infants were defined as having eczema if they had a doctor’s
diagnosis of eczema, or evidence of typical skin lesions. The extent and severity of the eczema was determined by the standardized SCORAD severity index (239), measured on the day and as mother-reported worst ever episode. An objective SCORAD of < 15 was classified as mild, 15-40 as moderate and > 40 severe. Allergen sensitization was assessed by skin prick testing (SPT) using common allergen extracts (whole egg, cow’s milk, peanut, house dust mite [Dermatophagoides pteronyssinus], cat, rye grass pollen, mould mix; Hollister-Stier Laboratories, Spokane, WA, USA). A wheal diameter of ≥ 3 mm was considered positive. The secondary outcome was IgE-mediated food allergy, which was defined as a history of immediate symptoms (typically within 60 minutes) following contact with and/or ingestion of food and a positive SPT to the implicated food. Information on respiratory symptoms (recurrent wheeze) and physician diagnosed asthma were also collected, but these were not analysed due to limitations in diagnosis at this age.

Statistical analysis

In this high risk population we estimated over 40% of infants would have vitamin D deficiency (209, 240) and ~40% infants at age one who would have either of food allergy, eczema or atopy (defined by skin prick test). Based on these assumptions, a sample size of 230 was estimated to detect an odd ratio of 2.5 with a power > 0.8 in the vitamin D deficiency group, at a significance of 0.05. The distribution of CB 25(OH)D3 concentration represented approximate normality after adjusting an outlying value. Because all results between the natural and adjusted data set were unaffected, parametric tests were performed using adjusted data. Objective SCORAD data and maternal vitamin D intake from diet or supplements were not normally distributed. Means were compared using the Mann-Whitney U-test. Differences in CB 25(OH)D3 concentration by month of birth were analysed by one-way analysis of variance and
Bonferroni post-hoc test. Logistic regression was used to estimate the risk of allergic outcomes by CB 25(OH)D3 status while adjusting for confounders. We included recognised confounders of vitamin D status and allergic disease in our analyses, specifically season of birth, pets in the home, infant gender, maternal age, maternal education, and ethnicity. All statistics were performed using SPSS software (version 19 for IBM, SPSS Inc., Chicago, Il, USA).

Results

Population characteristics

The characteristics of the study population (n=231) are shown in Table 3.1. The maternal population was predominately of white Caucasian ethnicity and tertiary educated. A history of maternal allergic disease was reported for 86.1% of the infants and 51.9% of the infants had both parents with a history of allergic disease.

<table>
<thead>
<tr>
<th>Maternal Characteristics</th>
<th>Study population n (%) unless otherwise stated</th>
<th>Total Cohort n (%) unless otherwise stated</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Caucasian</td>
<td>184 (79.7)</td>
<td>321 (73.5)</td>
<td>0.674</td>
</tr>
<tr>
<td>- Asian</td>
<td>9 (3.9)</td>
<td>16 (3.7)</td>
<td></td>
</tr>
<tr>
<td>- Other</td>
<td>6 (2.6)</td>
<td>16 (3.7)</td>
<td></td>
</tr>
<tr>
<td>- Not reported</td>
<td>32 (13.9)</td>
<td>84 (19.2)</td>
<td></td>
</tr>
<tr>
<td>Age, years (SD)</td>
<td>33.4 (4.5)</td>
<td>32.6 (4.6)</td>
<td>0.042</td>
</tr>
<tr>
<td>Bachelor degree or higher</td>
<td>88 (59.0)</td>
<td>154 (55.4)</td>
<td>0.466</td>
</tr>
<tr>
<td>Average daily supplemental vitamin D intake, IU (SD)</td>
<td>200 (248)</td>
<td>176 (228)</td>
<td>0.315</td>
</tr>
<tr>
<td>Average daily dietary vitamin D intake, IU (SD)</td>
<td>125 (98)</td>
<td>119 (86)</td>
<td>0.500</td>
</tr>
<tr>
<td>Pets in the home</td>
<td>130 (57.3)</td>
<td>243 (61.4)</td>
<td>0.316</td>
</tr>
<tr>
<td>Neonalal Characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex - Female</td>
<td>112 (48.5)</td>
<td>201 (53.2)</td>
<td>0.261</td>
</tr>
<tr>
<td>Birth weight, g (SD)</td>
<td>3455.8 (420.3)</td>
<td>3456.4 (458.9)</td>
<td>0.986</td>
</tr>
<tr>
<td>Gestational age, weeks (SD)</td>
<td>39.1 (1.1)</td>
<td>39.3 (1.2)</td>
<td>0.100</td>
</tr>
<tr>
<td>Gravidity, median (IQR)</td>
<td>2.3 (1.5)</td>
<td>2.3 (1.3)</td>
<td>0.442</td>
</tr>
</tbody>
</table>
Cord blood 25(OH)D3 concentrations

The mean (SD) CB 25(OH)D3 concentration was 58.4 (24.1) nmol/L, with a range of 9.18 to 246.34 nmol/L. The distribution of CB 25(OH)D3 concentrations are displayed in Figure 3.1.

Figure 3.1. Distribution of CB 25(OH)D3 concentrations. Shading represents the 3 status categories referred to in our analysis: light bars <50 nmol/L (n=88); medium bars 50–74.9 nmol/L (n=87); and dark bars ≥75 nmol/L (n=56).

Season of birth effect

Concentrations of CB 25(OH)D3 varied significantly by month of birth, with the Australian summer/autumn months of January, February and March representing significantly higher levels than the winter/spring months of August, September and October (Figure 3.2). Summer births showed a significantly greater percentage of CB 25(OH)D3 concentrations ≥ 75 nmol/L in comparison to spring births (43.9% and 12.1%, respectively) in addition to a smaller proportion with concentrations <50 nmol/L (12.2% and 65.5%, respectively, p < 0.001) (Figure 3.3).
**Figure 3.2.** Mean (SE) CB 25(OH)D3 level by month of birth. Levels in January (*) were significantly greater than August and September; Levels in February (#) and March (^) were significantly greater than August, September, and October (p <0.05).

**Figure 3.3.** Change in the proportion of infants in each of the 3 status categories across seasons. The proportions of CB 25(OH)D3 concentrations <50 nmol/L and ≥75 nmol/L are significantly different between summer/autumn and winter/spring (p <0.001).
**Maternal Characteristics**

While the season of birth were not significantly different for Asian and Caucasian participants, CB 25(OH)D3 concentrations were significantly higher for those of Caucasian (59.39 [23.77] nmol/L, n=184) compared with Asian ethnicity (37.01 [18.36] nmol/L, n=9) (p = 0.006). There was no relationship between CB 25(OH)D3 concentrations and maternal age controlling for season of birth, nor was maternal education a significant predictor of vitamin D status.

**Maternal vitamin D intake from diet and supplements in pregnancy**

The reported intakes of vitamin D from background dietary sources suggest 85.1% of women in the main cohort had dietary vitamin D intakes that were less than the recommended dietary intake during pregnancy of 200 IU/day (241). Antenatal supplement use was reported by 212/231 (91.8%) mothers, however information on brand, type and frequency was only provided by 146/231 (63.2%). Vitamin D intake from diet and supplements is presented in Table 3.1.

**The relationship between maternal intake and cord blood 25(OH)D3 concentrations**

Maternal intake of vitamin D from supplements was significantly correlated with CB vitamin D status (rho = 0.244, p = 0.003), whereas the relationship between CB levels and background dietary intake was not significant (rho = -0.105, p = 0.173).

**Association between cord blood 25(OH)D3 and allergic outcomes in infants**

**Eczema**

Consistent with other similar populations at high risk of allergic disease, eczema was the most common expression of the allergic phenotype, affecting 34% of the infants in this study (242) (Table 3.2). Cord blood 25(OH)D3 concentrations were significantly (p
= 0.018) lower in infants who had developed eczema by 12 months of age (Figure 3.4). The risk of eczema declined significantly as CB 25(OH)D3 increased, with a 10 nmol/L rise in CB 25(OH)D3 reducing risk by 13.3% (OR 0.87 [95% CI 0.77, 0.98], p = 0.020). The association remained significant after adjustment for multiple confounding factors (Table 3.3). We found a significant negative dose-response trend across categories of vitamin D status (OR 0.63 [0.44, 0.90], p = 0.013) and an OR 2.66 for infants with CB 25(OH)D3 < 50 nmol/L compared to the reference group of ≥ 75 nmol/L (95% CI 1.24, 5.72, p = 0.012) (Figure 3.5). Mean CB 25(OH)D3 concentration was not significantly different between IgE- and non-IgE-associated eczema.

**Figure 3.4.** Mean CB 25(OH)D3 concentrations for infants that were positive (orange bars) or negative (grey bars) for allergic outcomes by 12 months of age. * p = 0.018.

Objective SCORAD measures were conducted on 65/78 infants with eczema (severity categories described in Table 3.2). The median (IQR) SCORAD score on the day of assessment and worst ever were 7.8 (14) and 18.1 (13), respectively. We found no correlation between CB 25(OH)D3 concentration and SCORAD on the day of
examination (rho = -0.018, p = 0.889) or worst ever (rho = 0.092, p = 0.467). There was no difference in SCORAD score between IgE- or non-IgE-associated eczema.

Table 3.2. Allergy characteristics of the 231 infants at 1 year of age

<table>
<thead>
<tr>
<th>Infant characteristics at 1 year</th>
<th>n (%) unless otherwise stated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any allergic disease</td>
<td>100 (43.3)</td>
</tr>
<tr>
<td>Eczema</td>
<td>78 (34.1)</td>
</tr>
<tr>
<td>Objective SCORAD at 12 months (median, IQR)</td>
<td>7.8 (14)</td>
</tr>
<tr>
<td>- mild: n (%) with SCORAD &lt;15</td>
<td>51 (78.5)</td>
</tr>
<tr>
<td>- moderate: n (%) with SCORAD 15 – 40</td>
<td>13 (20)</td>
</tr>
<tr>
<td>- severe: n (%) with SCORAD &gt;40</td>
<td>1 (0.8)</td>
</tr>
<tr>
<td>Objective SCORAD at worst in preceding 12 months (median, IQR)</td>
<td>18.1 (13)</td>
</tr>
<tr>
<td>- mild: n (%) with SCORAD &lt;15</td>
<td>20 (30.8)</td>
</tr>
<tr>
<td>- moderate: n (%) with SCORAD 15 – 40</td>
<td>42 (64.6)</td>
</tr>
<tr>
<td>- severe: n (%) with SCORAD &gt;40</td>
<td>3 (4.6)</td>
</tr>
<tr>
<td>IgE mediated Food allergy</td>
<td>24 (10.4)</td>
</tr>
<tr>
<td>Sensitised to one or more allergen (SPT+)</td>
<td>46 (21.2)</td>
</tr>
<tr>
<td>- Egg</td>
<td>36 (16.7)</td>
</tr>
<tr>
<td>- Peanut</td>
<td>15 (6.9)</td>
</tr>
<tr>
<td>- Milk</td>
<td>4 (1.8)</td>
</tr>
<tr>
<td>- HDM</td>
<td>7 (3.2)</td>
</tr>
<tr>
<td>- Cat</td>
<td>2 (0.9)</td>
</tr>
<tr>
<td>- Rye grass pollen</td>
<td>2 (0.9)</td>
</tr>
<tr>
<td>- Mould mix</td>
<td>1 (0.5)</td>
</tr>
</tbody>
</table>

Table 3.3. Raw and adjusted odds ratios for eczema in relation to a 10 nmol/L rise in CB 25(OH)D³ (*controlled for season of birth, infant gender, pet ownership, maternal age and maternal ethnicity)

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted (n=229)</td>
<td>0.867 (0.769, 0.978)</td>
<td>0.020</td>
</tr>
<tr>
<td>Season of birth (n=229)</td>
<td>0.875 (0.766, 0.999)</td>
<td>0.048</td>
</tr>
<tr>
<td>Infant gender (n=229)</td>
<td>0.868 (0.769, 0.978)</td>
<td>0.021</td>
</tr>
<tr>
<td>Pets in home (n=225)</td>
<td>0.867 (0.768, 0.978)</td>
<td>0.020</td>
</tr>
<tr>
<td>Maternal age (n=228)</td>
<td>0.866 (0.767, 0.977)</td>
<td>0.020</td>
</tr>
<tr>
<td>Maternal ethnicity (n=197)</td>
<td>0.858 (0.754, 0.977)</td>
<td>0.021</td>
</tr>
<tr>
<td>Multivar. model* (n=193)</td>
<td>0.857 (0.739, 0.995)</td>
<td>0.042</td>
</tr>
</tbody>
</table>
Allergen sensitization

Skin prick tests were conducted on 217/231 infants. Of these 21.2% were found to be sensitized to at least one allergen (see Table 3.2). Cord blood 25(OH)D3 concentration was not significantly associated with an increased risk for allergen sensitization (OR 1.0 [0.98, 1.01], p = 0.642) (Figure 3.5).

![Figure 3.5](https://via.placeholder.com/150)

**Figure 3.5.** Odds ratio for the development of allergic outcomes in infants with CB 25(OH)D3 <50 nmol/L, compared with reference category ≥75 nmol/L.

IgE-mediated food allergy

IgE-mediated food was present in 24/231 infants. Egg was the most common allergy affecting 6.5% of infants in this study, followed by milk and peanut allergy affecting 1.7% and 1.3%, respectively. Four of the 231 infants (1.7%) displayed allergy to more than one food. The risk of developing IgE-mediated food allergy was not related to CB 25(OH)D3 (OR 1.00 [0.99, 1.02], p = 0.584) (Figure 3.5).
Recurrent wheeze

There was no significant difference in mean CB 25(OH)D3 concentration between infants with or without recurrent wheeze (56.94 [24.44] nmol/L and 58.6 [24.1] nmol/L, respectively), nor was risk of this outcome related to CB 25(OH)D3 concentration (OR 1.00 [0.98, 1.01], p = 0.731).

Association between maternal vitamin D intake and allergic outcomes in infants

Maternal vitamin D intake from supplements was not different for infants with or without eczema (p = 0.571), allergen sensitization (p = 0.563) or IgE-mediated food allergy (p = 0.341). Supplemental intake (analyzed in increments of 50 IU) displayed no association with the risk of eczema (OR 1.02 [0.95, 1.11], p = 0.517) allergen sensitization (OR 0.98 [0.90, 1.07], p = 0.698) and IgE-mediated food allergy (OR 1.08 [0.97, 1.19], p = 0.169).

Discussion

There is an increasing body of evidence linking vitamin D status and immune function, raising important questions about the relationship between fetal vitamin D status and the rising predisposition for allergic disease in young infants. This is the first study to report that reduced CB 25(OH)D3 levels, as an indicator of vitamin D status in utero, are associated with an increased risk of eczema in the first 12 months of life. Interestingly, although 25(OH)D3 concentrations were significantly lower in infants with eczema there was no association between vitamin D status and allergen sensitization or presence of IgE-mediated food allergy in this cohort. In addition, we found that only 24.2% of participants had adequate vitamin D concentrations (≥ 75 nmol/L) despite the sunny and temperate climate experienced in Perth, Australia (although we recognize that CB 25(OH)D3 are generally lower than neonatal concentrations (238)). We did find marked
seasonal variation in CB vitamin D status; summer births displaying significantly greater concentrations.

Consistent with our findings Miyake et al. (33) found an association between lower maternal vitamin D consumption in pregnancy and increased risk of eczema in infants. These observations are also in keeping with a series of pregnancy studies which found that lower vitamin D intakes were associated with increased risk of other potential (respiratory based) indicators of an allergic phenotype including recurrent wheeze (26, 30, 39), subsequent asthma and allergic rhinitis (31). In addition, several studies using indirect measures of non-dietary vitamin D such as season of birth (surrogate for sunlight exposure) found that birth in winter months was associated with higher rates of subsequent eczema (243) and food allergy (233). Although these studies support a protective role for improving status, they cannot exclude confounding effects of other seasonal factors such as variations in humidity and viral infections. The confirmed seasonal variations in vitamin D levels observed in our study provide support for the hypothesis that vitamin D is independently associated with eczema. Contrary to our results, Gale et al. (43) found that maternal serum 25(OH)D concentrations >75 nmol/L were associated with an increased risk of visible eczema on examination at 9 months of age. The method of diagnosis may be relevant here as the risk of eczema was not significant when assessed using the modified UK Working Party’s diagnostic criteria for atopic dermatitis.

There are relatively few other studies utilising CB 25(OH)D3 to assess neonatal vitamin D in relation to allergic and immune outcomes. A recent longitudinal study conducted in New Zealand reported a protective association between CB 25(OH)D3 and the risk of wheezing and respiratory infection at 15 months of age, but consistent with our
findings, no effect on sensitisation (47). Also supporting our findings that CB 25(OH)D3 was not associated with IgE-mediated outcomes is a study by Liu et al. (67) that reports no overall association between vitamin D status and food-specific IgE levels (however, the authors did find that risk was increased for particular genotypes). The protective effects of vitamin D on eczema in our study, together with protective effects on wheezing in other studies (47) appear to be independent of IgE related features, raising questions about the potential mechanisms.

The role of vitamin D in both skin barrier function and local antimicrobial defence could contribute to protective effects at mucosal and cutaneous surfaces. In the skin, the CYP27B1 enzyme (possessed by keratinocytes and monocytes) is required to hydroxylate 25(OH)D3 to the active 1,25(OH)2D3 (244). This active form facilitates the production of the anti-microbial peptide cathelicidin (244). Notably, in subjects with atopic dermatitis 25(OH)D3 levels are positively correlated with serum cathelicidin and its production in both keratinocytes and neutrophils (245). It is possible that insufficient vitamin D levels contribute to the impaired barrier function characteristic in eczema (246), because diminished CYP27B1 and reduced production of 1,25(OH)2D3 result in hyperproliferation of the basal layers and disrupted barrier integrity (173), coupled with impaired antimicrobial activity (245). Cathelicidin levels in lesional skin of established eczema increase significantly in response to oral 25(OH)D3 supplementation (244), supporting a role of vitamin D in promoting antimicrobial functions and barrier integrity. As in the study by Kanda et al. (245) we did not find that serum vitamin D levels predicted the severity of disease (SCORAD).

A limitation of this study is that the high risk nature of the population may not be reflective of other populations. In the absence of serial blood collections throughout
pregnancy and infancy it is also not possible to determine if variations in vitamin D levels at different stages of development differentially influence eczema risk. Likewise, although infant vitamin D supplementation and food fortification is not standard practice in Australia we have not accounted for variations in infant vitamin D intake. Although we acknowledge that CB 25(OH)D3 concentrations reflect recent vitamin D status, mainly in the last trimester, this biological measure remains a more accurate indicator of vitamin D status than dietary intake or other surrogate measures.

The findings of this study provide new evidence that reduced fetal exposure to vitamin D increases the risk of eczema in infants by 12 months of age. This adds to the growing body of evidence that vitamin D status is important for many aspects of health and that interventions to improve vitamin D status in pregnancy may be an important part of preventive strategies. This will be more definitively assessed through randomised controlled trials to assess the effects of maternal and/or infant vitamin D supplementation on immune development and clinical outcomes in childhood.
Chapter 4

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

Jones AP, D’Vaz N, Meldrum S, Palmer D, Zhang G, Prescott SL.

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Appendix 2
Introduction

The global rise in allergic disease, particularly in very young children (247, 248) 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 (96, 249). Early immune function is also modified by a range of early life exposures including maternal microbial exposure in pregnancy (reviewed in (250)), maternal dietary patterns (35, 251) and maternal smoking (24). Recently, vitamin D status has also been proposed as a potential risk factor for the epidemic of allergic disease so early in life (206).

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 (48, 252), food allergy (64, 86), respiratory tract infections and wheeze (47, 66) in early childhood, while low levels in older children are reported to be predictive of asthma and atopy (66). This is consistent with immunological research demonstrating that the active vitamin D metabolite, 1α,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 (253)), and altered Th cytokine responses in vitro (110, 254). 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 1260600281594), as approved by the Princess Margaret Hospital Human Research Ethics Committee. Full details of the original study have been previously published (255). 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) (SPT) 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 (256). 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 4.1. 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 venipuncture 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 x 10^6.

**Figure 4.1.** Proportional Venn diagram representing the number of study participants with cord blood (CB) or 6 months of age 25(OH)D3 measurements, and cell culture data.

**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 (257). Sample type (plasma or serum) does not affect measured 25(OH)D concentration (258), 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 $1 \times 10^6$ cells/ml either alone (control) or with optimised concentrations of Toll-like receptor (TLR) ligands for a) TLR3 (Polyinosinic:polycytidyllic acid, 50 μg/ml, paired with CB 25(OH)D3 n=60, paired with 6 month 25(OH)D3 n=73), b) TLR2 (Lipoteichoic acid, 20 μg/ml, paired with CB 25(OH)D3 n=57, paired with 6 month 25(OH)D3 n=68), c) TLR9 (CpG oligonucleotide, 3 μg/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 μg/ml, $2 \times 10^6$ 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 whether their infant had shown typical signs of eczema (dry, red, itchy skin) (259) at all three time points, and SCORAD (Scoring Atopic Dermatitis) (239) 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 (260), 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)(261). 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 (262), 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 (263) (Figure 1), and Prism v 6 (GraphPad Software Inc.) (Figures 2 through 5).

**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 4.1. This subset of infants did not differ significantly from the main cohort population.

### Table 4.1. Comparison of characteristics between those participants with 25(OH)D3 and the total cohort

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

25(OH)D3 levels at birth and 6 months

25(OH)D3 was substantially higher at 6 months of age than at birth (CB) - with respective means (95% confidence intervals) 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 4.2. Fish oil supplementation had no significant impact on 25(OH)D3 levels (p = 0.418).

**Figure 4.2.** Mean (SD) cord blood (light shaded bars) and 6 months of age peripheral blood (dark shaded bars) 25(OH)D3 by month of collection.

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 4.3). 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 IFNγ (rho = -0.43, p = 0.008) (Figure 4.3) and TNFα (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 (IFNγ rho = -0.111, p = 0.320 [Figure 4.3] and TNFα 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).
Figure 4.3. Scatter plots of cytokine production to house dust mite (HDM) and ovalbumin (OVA) stimulation, in relation to 25(OH)D3 levels in cord blood (CB). ** = \( p \leq 0.01 \).
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 TNFα (rho = 0.23, p = 0.027) and IL-17A (rho = 0.24, p = 0.023) to OVA. There were similar trends for TNFα 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 4.2).
Table 4.2. Correlations between 25(OH)D3 and cytokine production to TLR ligands

<table>
<thead>
<tr>
<th>TLR</th>
<th>Cytokine</th>
<th>Spearman’s rank correlation coefficient (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CB 25(OH)D3</td>
</tr>
<tr>
<td>TLR2</td>
<td>IL-1β</td>
<td>0.058 (0.668)</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>0.047 (0.727)</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>-0.144 (0.286)</td>
</tr>
<tr>
<td></td>
<td>IL-12</td>
<td>0.005 (0.975)</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
<td>-0.173 (0.199)</td>
</tr>
<tr>
<td></td>
<td>IFNγ</td>
<td>-0.108 (0.429)</td>
</tr>
<tr>
<td>TLR3</td>
<td>IL-1β</td>
<td>-0.041 (0.753)</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>0.056 (0.672)</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>-0.152 (0.245)</td>
</tr>
<tr>
<td></td>
<td>IL-12</td>
<td>0.148 (0.316)</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
<td>-0.053 (0.686)</td>
</tr>
<tr>
<td></td>
<td>IFNγ</td>
<td>-0.090 (0.496)</td>
</tr>
<tr>
<td>TLR4</td>
<td>IL-1β</td>
<td>-0.386 (0.126)</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>-0.133 (0.610)</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>-0.278 (0.279)</td>
</tr>
<tr>
<td></td>
<td>IL-12</td>
<td>-0.036 (0.892)</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
<td>-0.036 (0.892)</td>
</tr>
<tr>
<td></td>
<td>IFNγ</td>
<td>-0.036 (0.892)</td>
</tr>
<tr>
<td>TLR9</td>
<td>IL-1β</td>
<td>0.265 (0.075)</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>0.139 (0.356)</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>-0.036 (0.812)</td>
</tr>
<tr>
<td></td>
<td>IL-12</td>
<td>0.047 (0.755)</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
<td>0.216 (0.150)</td>
</tr>
<tr>
<td></td>
<td>IFNγ</td>
<td>0.021 (0.892)</td>
</tr>
</tbody>
</table>

TNFα and IL-12 in particular showed consistent positive correlation across ligands, and scatter plots of TNFα (TLR2, TLR3, TLR4 and TLR9), IL-12 (TLR2, TLR3, TLR9) and IL-10 (TLR3 and TLR9) are presented in Figure 4.4.
Figure 4.4. Scatter plots of cytokine production to Toll-like receptor (TLR) ligands, in relation to 25(OH)D3 levels at 6 months of age. * = p < 0.05.
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 (252). 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 4.3 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).

| Table 4.3, 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   | 95% CI         | p   |
| **a) 25(OH)D3 in cord blood**                    |      |                |     |
| Wheeze - 6 months (n=163)                        | 0.97 | 0.78-1.19      | 0.74|
| - 12 months (n=164)                              | 1.00 | 0.78-1.27      | 0.98|
| - 30 months (n=139)                              | 0.95 | 0.77-1.18      | 0.66|
| Eczema - 6 months (n=163)                        | **0.82** | **0.70-0.96**  | **0.011**|
| - 12 months (n=162)                              | **0.84** | **0.72-0.99**  | **0.034**|
| - 30 months (n=139)                              | 0.86 | 0.72-1.04      | 0.12|
| **b) 25(OH)D3 in peripheral blood at 6 months** |      |                |     |
| Wheeze - 6 months (n=112)                        | 0.95 | 0.76-1.19      | 0.66|
| - 12 months (n=108)                              | 0.90 | 0.68-1.18      | 0.43|
| - 30 months (n=96)                               | 0.83 | 0.67-1.02      | 0.073|
| Eczema - 6 months (n=112)                        | 1.03 | 0.89-1.19      | 0.71|
| - 12 months (n=109)                              | 1.03 | 0.89-1.20      | 0.68|
| - 30 months (n=96)                               | 1.07 | 0.92-1.24      | 0.39|

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 (256). Our findings here demonstrate that 25(OH)D3 was related in a consistent manner to both immune responses and the clinical outcomes. Briefly, IFNγ, 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 4.5). 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 4.5). Infants with a history of reported wheeze by 6 months of age had higher IL-5 production to OVA (p = 0.027) and IFNγ responses to HDM (p = 0.050), although this relationship did not persist at later follow-ups (Figure 4.5).
Figure 4.5. Tukey 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. * = p < 0.05; ** = p ≤ 0.01.
Discussion

We have previously described associations between immune function (256) and CB 25(OH)D3 status (252) 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 (48).

We did not observe differences in the risk of early childhood wheezing based on 25(OH)D3 level, in contrast to other studies (47, 48). 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. (47). 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 (206).

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 (105, 249, 264) that is not as selectively ‘Th2 skewed’ as first thought based on early animal models (265, 266).

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 (96, 249). This suggests that the immunological events underlying the allergic phenotype are initiated very early in development, even before birth (reviewed in (267)). 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 TNFα, IL-6 and IL-1β production, and a parallel increase in Th1 responses (249). In contrast, allergic children show attenuation of both their innate microbial responses and IFNγ Th1 adaptive responses (249). 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 IFNγ 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 (268, 269). Unlike in adults, CB 25(OH)D3 levels are strongly correlated with CB 1,25(OH)2D3 levels (270). Exposure of immune cells to higher levels of 1,25(OH)2D3 may have contributed to the ‘programming’ of the adaptive immune system, inducing Treg (134) and suppressing the expression of T cell costimulatory molecules on DC (271).

It is also known that antigen presenting cells are capable of generating 1,25(OH)2D to act as an autocrine and paracrine signal (163). Jeffrey et al. (163) have demonstrated that Treg in the presence of DC will respond equally when cultured with either 1,25(OH)2D or 25(OH)D. This indicates that DC 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 (245) 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 (272). 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 (245, 273). A recent paper by Kanda et al. (245) 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 (71) or 1600 IU cholecalciferol (D3) for 60 days (274), although a third found no significant improvement after 21 days of supplementation with 4000 IU D3 (93). One antenatal vitamin D supplementation RCT investigating prevention of respiratory and atopic conditions in the offspring was identified (37). 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 (37). 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) (275).

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. It also would have been informative to have measurements of 1,25(OH)2D3 at both time points. If the correlation between 25(OH)D3 and 1,25(OH)2D3 changed from birth to 6 months of age this may help explain the different associations observed between 25(OH)D3 at these time points and immune function.

In addition to the variation in biologically active vitamin D, it is becoming increasingly apparent that ultraviolet radiation (UVR), which stimulates endogenous vitamin D synthesis, has immunomodulatory effects independent of vitamin D production (276). As UVR is the predominant source of vitamin D in the Australian population (277) 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.
Chapter 5
VITAL Trial Design and Methods
Rationale for the **Vitamin D in Allergy (VITAL)** trial

The pattern of infant allergic disease has changed dramatically in last 15-20 years, suggesting new and changing environmental risk factors affecting very young infants (205). While the prevalence of asthma and allergic rhinitis remain high, there has been a substantial increase in both eczema and food allergy, in what has been described as the ‘second wave’ of the allergy epidemic (205). This has called for more detailed investigation of the factors most likely to be affecting the developing immune system. Among these, rising rates of vitamin D insufficiency has become a key candidate, based on both population studies (48, 64, 65), and mechanistic studies (148, 163) which provide clear biological pathways of influence. However, intervention trials examining the effect of vitamin D supplementation in allergy prevention are lacking.

Internationally, research groups are already conducting trials of antenatal vitamin D supplementation for allergic disease prevention (Vitamin D Antenatal Asthma Reduction Trial [NCT00920621], and the Vitamin D Supplementation During Pregnancy for Prevention of Asthma in Childhood [NCT00856947]). However, there remains a gap in the research with regard to infant vitamin D supplementation. Thus, the VITAL trial was designed to determine the effect of direct infant oral vitamin D supplementation in the early postnatal period on the developing immune phenotype.

As a pilot study the VITAL trial was powered to investigate immunological, rather than clinical outcomes. This approach requires a smaller sample size, and as immunological differences between allergic and non-allergic individuals are detectable pre-symptomatically the duration of follow-up is shortened. The 6 month time frame was justified by the fact that allergic disease, particularly eczema, frequently presents prior
to 6 months of age (172), indicating that interventions to reduce the risk of allergic disease should be targeted early. While clinical follow up to investigate the development of allergen sensitization, eczema, food allergy, asthma and allergic rhinitis at 12 and 30 months of age is planned, this thesis focuses only on the infant immunophenotype outcomes at 6 months of age.

In addition to the effect of infant vitamin D supplementation, this study has also quantitatively measured infant UV exposure using personal UV dosimetry during the first 6 months of the post-natal period. UV radiation is the predominant source of vitamin D in humans and interestingly it has also been shown to independently modulate immune function (276). The use of UV dosimeters is unique to this study investigating early life vitamin D exposure and immune function.

**Aims**

The primary aims of the VITAL trial were to determine whether vitamin D supplementation from birth to 6 months of age in infants with a family history of allergic disease will improve vitamin D status and influence immune development.

The specific primary aims were to:

1. Describe the vitamin D status (circulating 25(OH)D3 levels) of the participating infants and the effect of supplementation on 25(OH)D3 levels.
2. Determine the effect of oral vitamin D supplementation on infant immune development, as determined by phenotypical analysis (proportions of immune cell subsets, analysed by flow cytometry) immediately following cessation of supplementation at 6 months of age.
3. Describe the relationship between circulating 25(OH)D3 levels and immunophenotype, irrespective of intervention group.

The secondary aims of the VITAL trial relate to the influence of UV exposure on vitamin D status and immunological outcomes. These secondary aims were as follows:

1. Describe the contribution of UV exposure, as measured by personal UV dosimetry, to circulating plasma 25(OH)D3 concentration.
2. Determine whether UV exposure in the first 6 months of life influences immune development, as determined by phenotypical analysis (proportions of immune cell subsets, analysed by flow cytometry).

**Hypotheses**

The hypotheses were that:

1. The vitamin D supplementation will result in higher circulating plasma 25(OH)D3 concentrations in the intervention group, in comparison to the control (placebo) group.
2. Flow cytometry will reveal that increased vitamin D status influences the proportion and activation status of T regulatory and dendritic cell subsets to a profile representative of that observed in non-allergic individuals.
3. UV radiation exposure will be positively correlated with vitamin D status in both the intervention and control groups.
4. UV radiation exposure will be independently associated with immunological outcomes.
Design

The VITAL trial was a double blinded randomized controlled trial in which infants (n = 120) at high risk of allergic disease due to family history were supplemented with cholecalciferol (vitamin D3) or placebo from birth to six months of age to assess the effect of early vitamin D supplementation on infant immune function.

Participant recruitment

Pregnant women were initially approached through routine antenatal visits to obstetricians, antenatal clinics or education sessions in the metropolitan area of Perth, Western Australia. Women that were eligible (inclusion and exclusion criteria listed in Table 5.1), then consented to participation in an antenatal screening visit between 36 and 40 weeks gestation. Study entry was confined to infants with a family history of allergic disease, defined as a first degree relative (mother, father or full sibling) with a history of asthma, eczema or hayfever, in order to limit cohort heterogeneity and increase the likelihood of detecting differences in immune and allergic outcomes. To reduce the risk of vitamin D deficiency or toxicity in the participating infants during the intervention trial an inclusion reference range for maternal 25(OH)D status in late pregnancy of 50-100 nmol/L was applied as a proxy for infant status at birth.

Table 5.1. Inclusion and exclusion criteria for the VITAL trial

<table>
<thead>
<tr>
<th>Inclusion criteria:</th>
<th>Exclusion criteria:</th>
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</thead>
<tbody>
<tr>
<td>* Family history of allergic disease</td>
<td>* Maternal smoking during pregnancy</td>
</tr>
<tr>
<td>* Singleton pregnancy</td>
<td>* Maternal autoimmune disease</td>
</tr>
<tr>
<td>* Term delivery (&gt;37 weeks gestation)</td>
<td>* Maternal 25(OH)D &lt;50 nmol/L or &gt;100 nmol/L at 36-40 weeks gestation</td>
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</table>
Supplementation group allocation

Infants in the VITAL trial were randomized to receive either vitamin D3 or placebo. Randomization was conducted by the Princess Margaret Hospital for Children Clinical Trials Pharmacy (Pharmacy) and stratified according to maternal allergy (positive or negative for a history of asthma, hayfever or eczema), and participant sex. Pharmacy created a randomization plan from an online source (http://www.randomization.com) for each of the four stratification groups. The initial 40 subjects in each stratification group were randomized into 10 blocks. At each dispensing Pharmacy chose the correct stratification group and dispensed according to the randomization plan. Pharmacy staff had no contact with participants and all research staff were blinded to the allocations.

Intervention

Randomization and enrolment occurred within 28 days after birth, and supplementation was ceased at six months of age.

Intervention group

Infants in the intervention group received one drop (0.03 mL) of a vitamin D supplement per day (Ddrops Company, Woodbridge, ON, Canada), providing 400 IU vitamin D3 dissolved in vegetable oil (purified components of coconut and palm oil). This dose is in line with international recommendations for exclusively and partially breastfed (<1000 mL/day of vitamin D-fortified infant formula) infants (90, 278).
Control group

The control product was identical to the active product with the exception of containing no vitamin D. Infants in the control group received one drop (0.03 mL) of vegetable oil (purified components of coconut and palm oil) per day (Ddrops Company, Woodbridge, ON, Canada).

Both products were packaged identically and had no flavoring, coloring or other quality that would enable discernment between active and control. Following the recommendations for infant vitamin D supplementation (90, 278) caregivers were advised to cease administering the drops if the infants intake of infant formula reached 1000 mL/day.

UV dosimetry

To obtain objective, quantitative data on UV exposure participants in both groups were issued a UV dosimeter (VioSpor blue line Type II, Biosense, Bornheim, Germany) at enrolment, which was subsequently replaced at the 3 month of age appointment and collected at the end of the intervention period.

The dosimeters contain a film of immobilized, UV-sensitive spores, with a sensitivity profile similar to that of human skin. The central part of the film is exposed to the UV source (in this case, sun light), while the outer, unexposed part is used for calibration. The spores are incubated in a bacterial growth medium to stimulate germination and protein production. Proteins are stained for densitometric quantification, with the quantity of protein produced corresponding to the amount of UV-induced DNA damage.
sustained by the spores. From this information total UV exposure (expressed in J/m²) can be calculated for the period of use.

So that the dosimeters were exposed to a similar amount of UV as the infant, parents were instructed to attach the dosimeter either to the infants clothing at shoulder level, or to the inside of the pram or carrier near the infants head. The choice of positioning was based on the assumption that the infants face would be bare, while other parts of the body are regularly covered by clothing or blankets. Research staff noted that some dosimeters were being used incorrectly, potentially resulting in inaccurate data, and these results were excluded from analysis.

Compliance with study product and UV dosimeter use

Compliance with study product and UV dosimeter use were assessed by multiple methods. Firstly, mothers of the participants were provided a diary card on which to record the days that the study product was administered and UV dosimeter used. Secondly, research staff conducted monthly phone calls between clinic appointments to monitor compliance and provide advice or encouragement where compliance was waning. As an additional measure of compliance with the study product use, the bottles were weighed by pharmacy before enrolment and again after completion of the supplementation period.
Questionnaire, clinical and biological data collection

Antenatal questionnaires

At the antenatal visit, questionnaire data was collected on factors that may affect vitamin D status of the mother or the risk of allergy development in the offspring. This included questions on allergy history in first degree relatives of the potential participant, parental ages, occupation and educational status, gravidity, pregnancy complications or illnesses, use of medications, nutritional supplements, number and age of siblings, pet ownership, ethnicity, Fitzpatrick Skin Type, sun exposure habits and anthropometry. The antenatal questionnaire appears as Appendix 3.

Infant assessments

Birth questionnaires

At the enrolment visit, conducted between 1 and 28 days of age, data was collected on factors that may be relevant to infant immune function, allergic disease outcomes, and/or vitamin D status. Questions, which appear as Appendix 4, included mode of delivery, infant sex, birth weight, length and head circumference, and if the infant had received any infant formula to date.

Infant questionnaire data collected by monthly phone calls

Phone interviews were conducted at month 1, 2, 4 and 5 to ascertain compliance with the study product, UV dosimeter use, daily intake (if any) of infant formula and use of dietary supplements. This questionnaire appears as Appendix 5.
Infant questionnaire data collected at the 3 and 6 month visits

Data collected by questionnaire at the 3 and 6 month appointments included diagnosis of eczema or food allergy by a medical doctor, parent reported eczema (dry, red, itchy skin) or wheezing symptoms in the infants, use of medications, vaccination records, intake of infant formula, introduction of solids, use of nutritional supplements, sun exposure habits, and anthropometry. The infant questionnaires used at the 3 and 6 month appointments make up Appendix 6 and 7, respectively.

Allergic outcomes and definitions

Although not a primary outcome of this phase of the trial due to sample size, clinical allergic disease was documented for VITAL participants at the 3 and 6 month of age visits.

1) Eczema: Defined by typical skin lesions on examination (259) or with a doctor diagnosis of eczema. Children with any signs of eczema were assessed by research staff using the Scoring Atopic Dermatitis (SCORAD) index (239) to assess the extent and severity of lesions “on the day”.

2) Wheeze: Questions on wheeze were asked at 6 months of age and were based on those used in The International Study of Asthma and Allergies in Childhood. Parents were asked if their child had ever had wheeze or whistling in the chest, the number of episodes, and additional factors relating to severity, medical management and concurrent respiratory tract infections.
Sample collection

Blood collection

Blood was collected from the umbilical cord at birth by venipuncture of the umbilical vessels into heparinised RPMI 1640 (Life Technologies, Grand Island, NY, USA) for processing of CBMC, and serum clot tubes for collection of serum (Vacuette, Greiner Bio-One GmbH, Kremsmünster, Austria). Samples were transported at room temperature and processed within 12 hours of collection.

Infant blood samples were collected at the 3 and 6 month of age appointments where practically possible and parents consented. Peripheral blood was collected by venipuncture into lithium heparin tubes (Vacuette, Greiner Bio-One GmbH, Kremsmünster, Austria) and processed immediately after the clinic visit for plasma and mononuclear cells.

Mononuclear cells from CB and 6 month peripheral blood samples were separated by means of density gradient centrifugation (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway). Purified MC were cryopreserved in 7.5% dimethyl sulfoxide.

To ensure participants did not develop vitamin D deficiency or toxicity during the trial, plasma collected at 3 months of age was analysed for 25(OH)D, calcium, phosphate and alkaline phosphatase. Test results were sent to a paediatric endocrinologist who informed caregivers and the participants’ nominated general practitioner if any parameters were out of the expected normal range. The researchers involved in data collection and analysis remained blinded to these test results throughout the trial.
Laboratory methods

25(OH)D analysis

Due to the requirement to have 25(OH)D levels assessed by a clinically accredited pathology laboratory in a timely manner during the trial, maternal and infant samples were first analysed for total 25(OH)D levels by a competitive chemiluminescent immunoassay, automated on the Abbott Architect i2000 (Abbott Laboratories, Abbott Park, Illinois, operated by PathWest Laboratory Medicine, Western Australia). Internal quality control data indicates the imprecision of the assay (coefficient of variation) as follows: 11.4% at 22 nmol/L; 5.2% at 48 nmol/L; 4.5% at 68 nmol/L; and 4.0% at 90 nmol/L.

While the Abbott Architect i2000 is accredited by the National Association of Testing Authorities for measurement of 25(OH)D, issues of accuracy have around this form of assay have been raised (279). Thus, stored CB serum and peripheral blood plasma from the 6 month of age appointment were later batch analyzed using a more accurate assay. For analysis of these samples a liquid chromatography tandem mass spectrometry (LC/MS/MS) assay was used, certified by the Centres for Disease Control Vitamin D Standardization Certification Program. The assay, which is described extensively elsewhere (280), is a serum based approach where the vitamin D metabolites are extracted using liquid-liquid extraction, followed by 2-dimensional LC/MS/MS analysis using an Agilent 6460 LC-QQQ mass spectrometer. The imprecision (coefficient of variation) of the assay is consistently <5% at 22.9 through 182 nmol/L, and can report down to 2 nmol/L (280).

Flow cytometry

Detailed methods of flow cytometric analysis are included in Chapter 6.
Sample size and power calculations

At present as there is no direct data available on the effects of infant vitamin D supplementation on immune parameters at this age, so the sample size calculations were an estimate based on 1) previous studies that have examined differences in immune function between allergic and non-allergic infants of the same age and 2) difference in immune function with dietary interventions.

Members of my research group have previously detected differences in infant immune function based on allergic outcomes using group sizes of between n=15 (96) and n=40 (249). In previous studies a sample size of 40-50 per group has been sufficient to detect differences in infant immune function (cytokine protein production in cell culture experiments) associated with dietary changes (35, 256, 281). Based on preliminary data on immune responses (cell culture) at this age and using sample size calculators for non-normal data (282), we would require group sizes of n=25 (for IL-13, effect size 0.27) and n=35 (for IL-1b, effect size 0.305) to detect differences with 80% power at α=0.05 (and 34 to 47 per group respectively for 90% power). Collectively these observations indicated that it was highly likely that we would achieve our aims with a sample size of 100 (50 in each group). To allow for 20% loss to follow-up or withdrawals a total of 120 infants would be recruited into the trial.

This original sample size estimate was made on the assumption of conducting cell culture experiments. However, due to a longer than expected approval and recruitment process, there was insufficient time to complete the intervention and analysis within my period of candidature. Subsequently, it was decided I would utilize multicolor flow cytometry as a means to achieve a statistically significant analysis with a smaller sample
size. Previous studies have identified significant differences in Treg in pediatric asthma (125) and eczema (128) using sample sizes of n=41 and n=60, respectively, and changes in the circulating Treg population with vitamin D supplementation have been reported in a sample of n=46 adults (283). Likewise, DC populations have been studied in cord (n=18) and peripheral blood of atopic and non-atopic children (n=53) (140), and adult asthma (n=59) (158). Based on these observations it was estimated the n=42 matched CB and 6 month of age samples available at the time would be sufficient to proceed with analysis.

**Ethics approval**

The trial was approved by the Princess Margaret Hospital for Children Ethics Committee and Research Governance Office (1959/EP), Joondalup Health Campus Human Research Ethics Committee (1224), Sir Charles Gairdner Hospital Human Research Ethics Committee (2012-070), St John of God Health Care Ethics Committee (561), and the South Metropolitan Area Health Service Human Research Ethics Committee. The trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12606000281594). All participants provided written, informed consent.
Chapter 6

Results of the VITAL Randomized Controlled Trial
**Introduction**

Vitamin D (25\([\text{OH}]\)D3) levels at birth and 6 month of age are associated with peripheral blood mononuclear cell (PBMC) cytokine production to innate and adaptive stimuli *in vitro*, specifically, lower Th2 cytokine responses to allergens, and stronger innate responses to TLR ligands (Chapter 4). The cytokine production patterns associated with higher 25(OH)D3 levels are reflective of those seen in the natural immune maturation of non-allergic children (lower Th2 and stronger innate responses in comparison to allergic counterparts) (249). Although Chapter 4 presents biologically plausible results that are conducive with previous research the observational study design leaves open the possibility for confounding. Therefore, in order to further investigate these observational findings the VITAL randomized controlled trial was designed (described in Chapter 5). The aim of the VITAL trial was to determine whether infant oral vitamin D supplementation and UV exposure over the first 6 months of life modifies the developing immune system. To complement the functional data of Chapter 4 this study investigates phenotypical variables through multicolour flow cytometry.

Research by other groups suggests that regulatory T cells (Treg) and dendritic cells (DC) differ in number and function by allergy status (122, 140, 143), and are also modulated by 25(OH)D3 (134, 162, 163, 284). Thus, in this study attention was turned to these cell populations in attempt to identify differences in the immunophenotype based on vitamin D status. The function of Treg is of particular interest in the pathogenesis of allergic disease as these cells represent a major mechanism in the maintenance of immune tolerance. The important role of these cells can be seen in experiments where transfer of Treg confers allergen tolerance to the recipient (285,
and induction of Treg protects against airway hyperresponsiveness to allergens (287). Aside from their role in immune tolerance Treg are frequently suggested to be key players in the epidemiological link between vitamin D status and immune disorders.

A positive correlation between serum 25(OH)D levels and Treg number has been reported observationally (131), and multiple in vivo studies find that the administration of 1,25(OH)₂D₃ results in Treg expansion (288, 289). This may be explained by the fact that 1,25(OH)₂D acts directly on T cells to promote FoxP3 expression and enhance suppressive activity (134). Due to the significant role of Treg in the development and maintenance of immune tolerance and their direct responsiveness to vitamin D, Treg were the primary focus of the flow cytometric analysis conducted for this study. However, Treg do not operate in isolation. Maintenance of Treg homeostasis and the induction of Treg proliferation is in part controlled by DC, which are independently modulated by vitamin D and associated with allergic disease.

Several non-overlapping subsets of DC are found in circulation, specifically plasmacytoid DC (pDC), and multiple myeloid, or conventional DC (cDC) subsets. DC are relevant to allergy for a number of reasons. This includes not only their role in antigen presentation and T cell priming, but also the development of tolerance (157). Interestingly, the proportions and function of DC has been associated with the availability of vitamin D (162, 163, 284), suggesting a link between vitamin D, DC and allergic disease.
Under *in vitro* experimental conditions the provision of 25(OH)D3 and 1,25(OH)D3 inhibits antigen presentation (expression of MHC II and costimulatory molecules), chemotaxis (expression of CCR7) and the production of inflammatory cytokines by monocyte-derived DC (162, 284). Subsequently, vitamin D-treated DC have a reduced capacity to prime naive T cells into Th phenotypes, instead inducing T cells with suppressive activity (162, 163). In addition to the effects of vitamin D, exposure to ultraviolet radiation (UV), which is the predominant source of vitamin D in humans (1), has been shown to inhibit dermal DC maturation, migration and capacity to stimulate T cells (290, 291). The immunomodulating effect of vitamin D and UV on DC, and the downstream effects on T cells, rendered DC analysis a logical inclusion in the present study.

**Materials and methods**

**Study participants**

All samples were obtained from VITAL trial participants. A detailed description of the trial, including blood collection and processing, and UV dosimetry is given in Chapter 5.

**Measurement of 25(OH)D3**

Two assays for measuring 25(OH)D were used in this trial, as described in Chapter 5. One, a liquid chromatography tandem mass spectroscopy (LC/MS/MS) assay which reports 25(OH)D3 independently of 25(OH)D2 and C3-epi-25(OH)D3. In C3-epimerized vitamin D metabolites the hydroxyl group at carbon 3 of the A-ring is converted from the alpha to beta position (226). Although capable of undergoing C-1α
hydroxylation to 3-epi-$1\alpha,25$(OH)$_2$D3, this metabolite has significantly reduced calcaemic and non-calcaemic effects in comparison to $1\alpha,25$(OH)$_2$D3 (226). *In vitro* studies indicate some biological activity (292), however its function *in vivo* remains unclear and thus it will not be addressed in this thesis. C3-epi-25(OH)D3 may account for ~20% of 25(OH)D3 in infants, and thus the inability to separately measure metabolites could result in misclassification of vitamin D status (226).

The second assay, a chemiluminescent immunoassay (CIA) reports total 25(OH)D (sum of 25[OH]D3 and 25[OH]D2). The 25(OH)D measure given by the CIA is not altered by the presence of C3-epi-25(OH)D3 (226). As the majority of samples tested by LC/MS/MS had undetectable levels of 25(OH)D2 (39/42 CB and 37/40 6 month of age samples) it can be safely assumed that most of the 25(OH)D measured by these assays is 25(OH)D3. Here the terminology “25(OH)D3” is used when referring to results from both assays.

*Flow cytometry*

Mononuclear cells from CB and 6 month peripheral blood were labelled using two novel panels of fluorochrome conjugated monoclonal antibodies (mAb); one for the identification of T cells (13 colour) and one for DC (15 colour). Where MC number was limited preference was given to the T cell panel. Data acquisition was performed on an LSRFortessa (BD Biosciences) and analysed using FlowJo software (TreeStar, CA, USA). The fluorescence minus one (FMO) (293) method was used to obtain a precise definition for cells with high background fluorescence.
Regulatory T cell flow cytometry panel

For quantification of T cells, 2x10^6 MC were first stained for viability with Fixable Viability Stain (FVS660-APC) (BD Biosciences) for 15 minutes at room temperature in phosphate buffered saline [PBS]) then washed in buffer (PBS + 1% fetal calf serum). Nonspecific binding of Abs to Fc receptors was controlled by pre-incubating MC with human Ig (50 μg/ml; BD Biosciences) for 10 minutes at room temperature. After washing in buffer MC were labelled for 30 minutes on ice with optimized concentrations of anti-CD3-FITC (UCHT1), anti-CD4-(APC-H7) (RPA-T4), anti-CD25-(PE-Cy7) (M-A251), anti-CD27-BV786 (L128), anti-CD45RA-BV650 (HI100), anti-CD69-BV605 (FN50), anti-CD127-BV421 (HIL-7R-M21), anti-CCR7-(CD197)-(PE-CF594) (150503), anti-HLA-DR-BV510 (G46-6) (all from BD Biosciences), anti-TCR-Vα24-(PerCP-Cy5.5) (C 15) and anti-TCR-Vβ11 Biotin (C 21) (Beckman Coulter), made up to 100μl in buffer. MC were washed in buffer following the 30 minute incubation before secondary labelling of Vβ11 with streptavidin conjugated with AF700 (BD Biosciences) for 20 minutes on ice. Once all surface labelling was completed intracellular staining was performed using the Transcription Factor Buffer Set (BD Biosciences) for permeabilization and fixation according to the manufacturer’s instructions, and FoxP3 stained using anti-FoxP3-PE (259D/C7).

Phenotypic analysis of regulatory T cells

Conventionally the “natural” Treg population has been defined based on the expression of CD4, CD25 and FoxP3, and low levels of CD127 (CD4⁺CD25⁺ FoxP3⁻ CD127⁻) (117, 294). However, it has become apparent that there is heterogeneity within the natural Treg population, based on the examination of activation and memory status (120, 295, 296), and that FoxP3 and suppressive functionality is also exhibited by cells not fitting this conventional definition (117, 294, 297, 298). The latter include
CD25⁺FoxP3⁺CD127⁺ populations (298), and FoxP3-expressing CD4⁺CD25⁻ T cells which can upregulate CD25 upon disruption of immune homeostasis to resemble the conventional CD25⁺FoxP3⁺ Treg phenotype (299). CD25-FoXP3⁺ cells have thus been referred to as a Treg “reservoir” (299). As vitamin D has been associated with the number and function of circulating conventional Treg (131, 133, 164), and the induction of FoxP3-expressing Treg from CD25⁻ T cells in vitro (163, 300), I have investigated CD25⁺FoxP3⁺CD127⁻ Treg (henceforth referred to as nTreg), in addition to CD25⁺FoxP3⁺CD127⁺ (CD127⁺ nTreg) and CD25⁺FoxP3⁺ (CD25CD127⁻ Treg and CD25CD127⁺ Treg) populations. Representative gating plots are presented in Figure 6.1.

To further examine phenotypical differences between the study groups markers of cell activation and memory were investigated. Here we define naïve, central memory and effector memory as CD45RA⁺CCR7⁺, CD45RA⁻CCR7⁺ and CD45RA⁻CCR7⁻, respectively (301, 302). As can be seen in Figure 6.1 the distribution of the CD127⁺ nTreg population was naïve, effector memory and effector (CD45RA⁺CCR7⁻), without a significant central memory subset. Within each subset the proportion of cells expressing CD69 and HLA-DR (MHC II), and the geometric mean fluorescence intensity (MFI) of these markers, is reported. MFI is quantitative value, providing an indication of the amount of protein expressed by a cell, in comparison to binary “positive” or “negative” categorisation.
Figure 6.1. Representative FACS plots to identify FoxP3 expressing populations in peripheral and cord blood mononuclear cells. Cells were first gated on viability (FSC vs viability dye) (A) then live cells gated on co-expression of CD3 and CD4 to identify T cells (B). CD25 vs FoxP3 was then used to gate conventional CD25^+ FoxP3^+ Treg, and FoxP3-expressing CD25^- populations (C). Plots D – F and G – I represent gating of the CD25^+ and CD25^- populations, respectively, by CD127 expression, and subsequent classification into activation/memory subsets based on CCR7 and CD45RA expression.
Dendritic cell flow cytometry panel

Analysis of DC first involved labelling MC (2x10^6) for viability (BioLegend Zombie NIR; 15 minutes at room temperature in PBS), after which MC were washed in buffer before blocking of high affinity receptors (as per T cell protocol). MC were then incubated for 30 minutes on ice with optimized concentrations of anti-CD11c-(PE-Cy7) (B-ly6), anti-CD14-(PE-Cy5.5) (MφP9), anti-CD16-AF700 (3G8), anti-CD19-BV605 (SJ25C1), anti-CD40-BV421 (5C3), anti-CD56-(PE-Cy5) (B159), anti-CD86-BV650 (2331[FUN-1]), anti-CD123-BV786 (7G3), anti-CCR7-(CD197)-(PE-CF594) (150503), anti-HLA-DR-BV510 (G46-6) and anti-CD141-(BCDA-3)-BV711 (1A4) from BD Biosciences; anti-CD1c-(BDCA-1)-PE (AD5-8E7), anti-CD303-(BDCA-2)-FITC (AC144) from Miltenyi Biotec; and anti-FceR1-APC (AER-37) from eBioscience.

Phenotypic analysis of dendritic cells

Several non-overlapping subsets of DC are found in circulation, specifically CD123^+CD303^+ plasmacytoid DC (pDC), and CD11c^+ myeloid, or conventional DC (cDC), which are further categorized as CD1c^+ , CD141^+ , or CD16^+ (154). This study has adopted the conventional classifications of circulating pDC and cDC subsets (151, 154). Here pDC are defined as lineage (CD14, CD19, CD56) negative (Lin')HLA-DR^+CD123^+CD303/BDCA-2^+. cDC are defined as Lin'HLA-DR^+CD123^dimCD11c^+, and then split into CD1c/BDCA-1^+ or CD141/BDCA-3^+ fractions. CD11c^+ cells that were neither CD1c^+ nor CD141^+ were classified as either CD16^+ or CD16^-. Representative gating plots are presented in Figure 6.2.
Figure 6.2. Representative FACS plots to identify DC populations in peripheral and cord blood mononuclear cells. Cells were first gated on viability (FSC vs viability dye) (A) then selected based on absence of lineage markers (CD14, CD19 and CD56) and positive expression of HLA-DR (B - C). pDC were then gated by the expression of CD123 and CD303(D), and cDC gated as CD11c⁺ (E). Plots F – H represent the four distinct populations of cDC; CD1c⁺ (F), CD141⁺ (G), CD16⁺ and CD16⁻ (H).
In the context of allergic disease the various DC populations appear to have differing roles in the induction or maintenance of allergic inflammation. Functionally, pDC are recognised to be Th1 polarizing on account of their rapid and substantial production of Type 1 interferon in response to viruses, yet are also able to induce regulatory T cells (Treg) and immune tolerance (155). Comparisons of circulating human DC populations’ reveals that pDC have a lower propensity for allergen presentation, induction of T cell proliferation and Th2-polarization than CD1c+ or monocyte-derived DC (153). Human CD1c+ DC are noted to be good activators of naïve T cells with the capacity to induce Th1 or Th2 polarization depending on the context (303). In the presence of TLR ligands (specifically TLR4 and TLR7/8) CD1c+ DC induce Th1 polarization; however, when unstimulated or cultured with the house dust mite antigen Der P1 CD1c+ DC induce a Th2 dominant phenotype (303). Additionally, the epithelial cytokine thymic stromal lymphopoietin (TSLP), which is strongly associated with eczema and asthma, activates CD1c+ DC and enhances their capacity to drive Th2 responses (304). This tendency towards inducing an allergic-like Th2-dominant T cell population in response to allergen and TSLP is in agreement with reports of greater proportions of circulating and respiratory tract CD1c+ DC in allergic individuals (304, 305). The contribution of cDC to allergic inflammation may not be restricted to the CD1c+ DC subset, however, with evidence that CD141+ DC may also play a role.

CD141+ DC display great plasticity in their function, being able secrete large amounts of IL-12p70 in response to TLR3 ligation, but also possessing strong Th2-polarizing capacity (306). It is this ability to induce IL-4- and IL-13-producing T cells that may contribute to allergic inflammation (306). Circulating CD141+ DC are found at higher frequencies in allergic individuals in comparison to controls and CD141 expression in blood leukocytes is elevated during acute episodes of asthma (159, 307). The evidence
for CD16$^+$ and CD16$^-$ DC in allergy is less clear. CD16$^+$ DC have been referred to as “inflammatory DC” on account of their production of large amounts of TNF$\alpha$, and thus upon activation may promote vasodilation, necrosis and fibrosis (308). However, CD16$^+$ DC appear to have a lower capacity for T cell activation than CD1c$^+$ or CD141$^+$ DC (309).

What is apparent from the published literature is that DC maturation and function is highly dependent on the particular antigens and immunological factors encountered. Subsequently, expression patterns on DC subsets can be used to make inferences about their function. Exposure of DC to maturation stimuli, including allergens, results in the upregulation of co-stimulatory molecules (CD40 and CD86) and chemokine receptors (CCR7), facilitating migration from the periphery into lymph nodes, and the subsequent activation and differentiation of naive T cells (310). The circulating DC subsets also display varying levels of immunoglobulin receptors, for example the high affinity IgE receptor Fc$\varepsilon$R1 (305, 311, 312). This receptor is found on pDC and CD1c$^+$ DC, and to a lesser extent CD141$^+$ DC (305, 312). Fc$\varepsilon$R1 expression has been reported to be greater in atopic individuals and correlate with serum IgE levels (311, 313), although its association with allergic disease in children is controversial (312). Therefore, in addition to the proportion and ratios of DC subsets, we have examined the expression of CD40, CD86, CCR7 and Fc$\varepsilon$R1 in relation to supplementation, 25(OH)D3 levels and UV exposure.
Statistical analysis

Analyses were performed according to the intention-to-treat principle. Normally distributed data was analysed using two-tailed, unpaired Student's t tests assuming equal variance. Means of three or more groups were compared using one-way ANOVA with Tukey post-hoc test. Correlations were evaluated with the Pearson’s correlation and linear regression analysis. Where data was not normally distributed Mann-Whitney U tests were used to compare between groups, Wilcoxon rank test for paired analyses, Kruskal-Wallis one-way ANOVA where there were three or more groups, and continuous data analysed by Spearman correlations. To test the independent significance of UV exposure multivariate linear regression analysis was conducted and residuals checked for normality. Pearson’s Chi-square test was used to test associations among categorical variables.

Assay agreement was assessed using Bland-Altman plots, reporting bias and 95% limits of agreement between methods. Agreement in classification of results as deficient (<50 nmol/L), insufficient (50 – 74.9 nmol/L) or sufficient (≥75 nmol/L) (9) between assays was assessed using Cohen's Kappa (κ), where a κ <0.4 corresponds to poor agreement, 0.4 – 0.75 fair to good, and >0.75 is excellent. A P value <0.05 was considered significant. Statistical analysis was performed using SPSS version 20 for Windows (SPSS Inc, Chicago, IL). Figures were generated using FlowJo software and GraphPad Prism v 6.05 (GraphPad Software, La Jolla, CA).
Results

Population characteristics

This study included n = 42 participants from the VITAL trial for whom both CB (serum and CBMC) and peripheral blood at 6 months of age (plasma and PBMC) was obtained. These study subjects were representative of the main trial cohort, and a comparison of their characteristics relative to the remainder of the cohort is given in Table 6.1. As described for the main trial cohort in Chapter 5, all participants had all been randomized to receive vitamin D supplementation (20/42) (47.6%) or placebo (22/42) (52.4%) from the perinatal period. There was no difference in season of birth between vitamin D supplemented and placebo groups, analysed by the traditional four season method (summer, autumn, winter, spring, p = 0.290), or by the half of the year with the highest (summer and autumn) or lowest (winter and spring) average UV index (p = 0.434).

<table>
<thead>
<tr>
<th>Table 6.1 VITAL trial population characteristics</th>
</tr>
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<tbody>
<tr>
<td>Study subset (n = 42)</td>
</tr>
<tr>
<td>Remainder of cohort (n = 60)</td>
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<tr>
<td>Maternal age, mean years (SD)</td>
</tr>
<tr>
<td>33.8 (4.5)</td>
</tr>
<tr>
<td>32.4 (5.8)</td>
</tr>
<tr>
<td>Maternal atopy, n (%) atopic</td>
</tr>
<tr>
<td>30 (71.4)</td>
</tr>
<tr>
<td>44 (73.3)</td>
</tr>
<tr>
<td>Ethnicity, n (%) white</td>
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<tr>
<td>38 (90.4)</td>
</tr>
<tr>
<td>49 (81.7)</td>
</tr>
<tr>
<td>Infant sex, n (%) male</td>
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<tr>
<td>21 (50)</td>
</tr>
<tr>
<td>31 (51.7)</td>
</tr>
<tr>
<td>Gestation at birth, mean weeks (SD)</td>
</tr>
<tr>
<td>39.2 (0.9)</td>
</tr>
<tr>
<td>39.3 (1.2)</td>
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<tr>
<td>Birth weight, g (SD)</td>
</tr>
<tr>
<td>3438 (312)</td>
</tr>
<tr>
<td>3430 (405)</td>
</tr>
<tr>
<td>Mode of delivery, n (%) vaginal</td>
</tr>
<tr>
<td>27 (64.3)</td>
</tr>
<tr>
<td>38 (63.3)</td>
</tr>
</tbody>
</table>

Sample data distribution

Due to limitations in both blood collection and blood volume in this young age group not all participants had complete sets of 25(OH)D3 measurements for CIA and LC/MS/MS assays, or flow cytometry panels. In addition, UV dosimeters were only
issued to a subset of the study population. The distribution of data is outlined in Table 6.2.

<table>
<thead>
<tr>
<th></th>
<th>CIA</th>
<th>LC/MS/MS</th>
<th>Both assays</th>
<th>UV dosimetry</th>
<th>FACS: T cell panel</th>
<th>FACS: DC panel</th>
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<tr>
<td>CB</td>
<td>41</td>
<td>42</td>
<td>41</td>
<td>31 (0 – 3 months)</td>
<td>42</td>
<td>41</td>
</tr>
<tr>
<td>6 months</td>
<td>42</td>
<td>40</td>
<td>40</td>
<td>22 (3 – 6 months)</td>
<td>42</td>
<td>36</td>
</tr>
<tr>
<td>Matched</td>
<td>41</td>
<td>40</td>
<td>39</td>
<td>21</td>
<td>42</td>
<td>36</td>
</tr>
</tbody>
</table>

**25(OH)D3 levels at birth and 6 months of age: a comparison of assays**

Mean (SD) 25(OH)D3 levels at birth (CB) were 67.8 (26.0) nmol/L by LC/MS/MS and 70.8 (24.0) nmol/L when measured by CIA. The two assays had a correlation coefficient of 0.461 (p = 0.002), with the CIA displaying a positive bias of 2.7 nmol/L (95% limit of agreement -48.6, 53.9) (Figure 6.3). 7/42 (16.7%) CB samples registered 25(OH)D3 <50 nmol/L when measured by LC/MS/MS, with 8/41 (19.5%) falling in the deficient range when measured by CIA. Insufficiency was more common, with 24/42 (57.1%) samples in the 50 – 74.9 nmol/L range by LC/MS/MS, and 18/41 (43.9%) when measured by CIA. The agreement between assays in classifying samples as deficient was fair to good (κ 0.429), but was poor when classifying between the three categories of vitamin D status (deficient, insufficient, sufficient) (κ 0.216).

At 6 months of age there was significant discrepancy between the two assays, although the measures were strongly correlated (0.774, p <0.0001). Mean (SD) 25(OH)D3 levels reported by LC/MS/MS were 98.8 (26.5) nmol/L, and 89.3 (35.2) nmol/L by CIA; a bias of -8.8 nmol/L (95% limit of agreement -52.3, 34.7) for the CIA (Figure 6.3). The LC/MS/MS assay detected only 1/40 (2.5%) participant with deficient 25(OH)D3.
levels, and 5/40 (12.5%) with insufficiency, whereas the CIA reported 4/42 (9.5%) and 12/42 (28.6%) participants with vitamin D deficiency and insufficiency, respectively. This represents fair to good agreement when classifying samples as deficient (κ 0.481), slipping to poor when classifying between the three cut-off groups (κ 0.378).

**Figure 6.3** Bland-Altman plots of CIA vs LC/MS/MS. Dotted lines indicate 95% limits of agreement.

**Effect of vitamin D supplementation on circulating 25(OH)D3 levels**

Baseline (CB) 25(OH)D3 levels were not significantly different between the vitamin D supplemented and placebo groups (mean [SD] by LC/MS/MS 67.2 [17.7] nmol/L and 68.4 [32.1] nmol/L, respectively, p = 0.880, and by CIA 77.5 [24.9] nmol/L and 65.1 [22.2] nmol/L, respectively, p = 0.099). By 6 months of age circulating 25(OH)D3 levels were higher in the vitamin D supplemented group infants compared to placebo group infants when analysed by LC/MS/MS (p = 0.047) (**Figure 6.4**). However there was no difference between the groups when analysed by the CIA methodology (p = 0.118) (**Figure 6.4**).
Figure 6.4. Comparison of 25(OH)D3 levels at 6 months of age between intervention groups, as determined by LC/MS/MS (left) and CIA (right). Bars represent mean and SD. * = p <0.05.

It is worth noting that of the 19/22 participants in the placebo group who had blood drawn at 3 months of age, 5/19 (26.3%) had a plasma 25(OH)D3 <50 nmol/L. As an ethical requirement of the study these infants were referred to their family physician for treatment, and 4/5 deficient participants were treated with cholecalciferol (3/4 with 400 IU, 1/4 with 1000 IU). All five recorded levels of 25(OH)D3 >50 nmol/L at 6 months of age by LC/MS/MS, although one was still classified as deficient by CIA at this time point (26 nmol/L).

Within the vitamin D supplemented group 2/15 (13.3%) participants with successful blood draw at 3 months of age had a 25(OH)D3 level <50 nmol/L, one of whom was subsequently treated with 400 IU D3 (equivalent dose as provided by the study
product). This participant was borderline sufficient at 6 months of age (50 nmol/L by CIA, insufficient plasma collected to run matched LC/MS/MS analysis) whilst the untreated participant achieved a sufficient status by 6 months of age.

**UV radiation exposure and its influence on 25(OH)D3 levels**

The median (IQR) average daily UV exposure as measured by personal dosimetry was 16.0 (11.5) J/m\(^2\), from 0 – 3 months of age, and 17.5 (11.8) J/m\(^2\) from 3 – 6 months of age. In a Wilcoxon rank test average daily UV exposure was not significantly different between the ages of 0 – 3 months and 3 – 6 months (p = 0.322), indicating that sun exposure behaviours did not change substantially over the first 6 months of life. Average daily UV exposure from 0 – 3 months of age was significantly greater in the vitamin D supplemented group, in comparison to placebo group (p = 0.012); likely a chance result secondary to uneven seasonal distribution of dosimeters (p <0.001) rather than a true indication of a between-group disparity. Thus, this finding would not be expected to alter the interpretation of between-group analyses, or the extrapolation of UV-related findings.

Average daily UV exposure from 0 – 3 months of age was positively correlated with 25(OH)D3 levels at 6 months of age (R\(^2\) = 0.137, p = 0.052 for LC/MS/MS, and R\(^2\) = 0.183, p = 0.018 for CIA) (Figure 6.5). This association between low levels of average daily UV exposure and 25(OH)D3 levels is consistent with previous findings that 25(OH)D3 levels are increased with low dose UV, and that synthesis depends on total UV dose but not dose rate (314). Although average daily UV exposure in infants was similar for the 3 – 6 months of age period this variable was not significantly correlated
with 25(OH)D3 levels at 6 months of age (p = 0.955 for LC/MS/MS and p = 0.388 for CIA), possibly due to the smaller sample size.

Figure 6.5. Correlation between average daily UV exposure from 0 – 3 months of age and circulating 25(OH)D3 levels at 6 months of age, as determined by LC/MS/MS and CIA. * = p < 0.05.

As the UV index in Perth varies significantly by season, resulting in seasonal variation in 25(OH)D3 levels (described in Chapter 2), the differences in 25(OH)D3 levels were examined by season of blood draw (independent of group allocation). Results from both assays indicate significantly higher 25(OH)D3 levels during summer (December through February), in comparison to spring (September through November) (mean difference [SEM] for LC/MS/MS 27.4 [10.0] nmol/L, p = 0.046, and for CIA 39.4 [13.3] nmol/L, p = 0.026).
Treg analysis

Percentage and characteristics of Treg populations at birth and 6 months of age

The percentage of CD4$^+$ T cells (as a percent of live cells), and FoxP3$^+$ T cell populations (as a percent of CD4$^+$ T cells) was analysed in CBMC and PBMC. The median (IQR) percent of CD4$^+$ T cells increased marginally from birth to 6 months of age (37.3 [15.6] and 39.9 [13.9], respectively, p = 0.045), as has recently been reported by others (315). In agreement with the findings of Collier et al (315) there was a significant increase in the percentage of CD25$^+$FoxP3$^+$ cells from birth to 6 months, which was driven predominantly by an increase in nTreg (Figure 6.6). Conversely, the percentage of CD25$^-$FoxP3$^+$ cells decreased to 6 months of age, with a differential change in the CD127$^-$ and CD127$^+$ subsets (increasing and decreasing, respectively).

![Cell population and time point](image)

**Figure 6.6.** Change in the percentage of FoxP3$^+$ T cell populations from birth (grey boxes) to 6 months of age (orange boxes) (whiskers represent 5th and 95th percentile). A) CD25$^+$FoxP3$^+$ cells; B) nTreg; C) CD127$^+$ nTreg; D) CD25$^+$FoxP3$^+$ cells; E) CD25$^-$CD127$^-$ Treg; F) CD25$^+$CD127$^+$ Treg. ** = p < 0.001, *** = p < 0.0001.
Despite reports of inverse correlations between CD127 and FoxP3 expression (294) the MFI of FoxP3 was significantly higher in the CB CD127\(^+\) nTreg population, in comparison to CB nTreg (2122 [742] and 1890 [536], respectively, \(p = 0.034\)). By 6 months of age, however, the FoxP3 MFI of nTreg had increased so that it was not statistically different from the CD127\(^+\) nTreg population (2176 [444] and 2170 [682], respectively, \(p = 0.847\)). The MFI of FoxP3 in the CD25\(^-\) subsets was comparable to that of the CD25\(^+\) subsets, and did not vary significantly between birth and 6 months of age (CB CD25 CD127\(^-\): 2107 [586]; CB CD25 CD127\(^+\): 2136 [596]; 6 month CD25 CD127\(^-\): 2343 [588]; and 6 month CD25 CD127\(^+\): 1919 [519]).

In terms of memory status of the FoxP3\(^+\) subsets there were significant changes in the percentages of naïve, central memory and effector memory subsets from birth to 6 months in the CD25\(^-\)FoxP3\(^+\) populations. Specifically, in both the CD127\(^-\) and CD127\(^+\) subsets the proportion of naïve cells decreased while there was an increase in central memory cells (Figure 6.7). The CD127\(^+\) subset also saw an increase in the percentage of effector memory cells (Figure 6.7). This may indicate a shift towards a greater “reservoir” of inducible conventional Treg, as it is reported that only the CD45RB\(^{\text{low}}\) (largely equivalent to CD45RA\(^{\text{low}}\)) upregulate CD25 and exert suppressive function (299). These trends were not observed in the CD25\(^+\) subsets (data not shown).
Figure 6.7. Change in CD25\(^{\text{FoxP3}^+}\) memory subsets from birth to 6 months of age. * = \(p < 0.05\), ** = \(p < 0.001\), *** = \(p < 0.0001\).

As sexual dimorphism is noted in pediatric allergy (316) cell populations were analysed by sex. There was no significant sex effect for the percentages of CD4\(^+\) T cells, FoxP3\(^+\)
T cell populations or their respective memory subsets at birth or 6 months of age (data not shown).

*Baseline differences between intervention groups*

Prior to comparing differences in immune phenotype between intervention groups at 6 months of age pre-existing differences between groups at baseline (CB) that may influence subsequent findings were examined. Whilst the majority of parameters were not significantly different between groups, there was a trend for lower HLA-DR expression in infants randomized to the vitamin D supplement group. Specifically, in the nTreg effector memory subset HLA-DR MFI was lower in comparison to the placebo group ($p = 0.034$), with similar associations for the percentage of HLA-DR$^+$ cells and HLA-DR MFI in the CD25$^-$CD127$^-$ population ($p = 0.052$ and $p = 0.027$, respectively) and effector memory subset ($p = 0.009$ and $p = 0.022$, respectively). These baseline differences were accounted for by calculating the change in magnitude of expression from birth to 6 months of age, with results described below.

*Difference in Treg populations between vitamin D supplement and placebo groups*

Vitamin D supplementation in adults has previously been reported to increase the frequency of circulating nTreg (164). In contrast to these findings there was no difference in the frequency of nTreg ($p = 0.921$), CD127$^+$ nTreg ($p = 0.900$), CD25$^-$CD127$^+$ ($p = 0.348$) nor CD25$^+$CD127$^+$ ($p = 0.835$) subsets between vitamin D and placebo groups in the present study. There were, however, differences in the expression of activation markers and co-stimulatory molecules between the groups.
The vitamin D supplemented group had a significantly higher MFI of HLA-DR in the total nTreg population (median MFI 2437 [1208] in the vitamin D group compared with 1942 [776] in placebo group, p = 0.041). The supplemented group also experienced a greater change from baseline in the MFI of HLA-DR in the nTreg effector memory subset (p = 0.004). A greater expression of HLA-DR on Treg is characteristic of a more effectively suppressive population (120).

Within the CD127+ nTreg effector memory cell subset vitamin D supplementation was associated with a higher MFI for CD45RA (p = 0.021) and CCR7 (p = 0.047). The vitamin D supplemented group had a significantly greater change in HLA-DR MFI in the CD25 CD127− population (p = 0.010) and CD25 CD127− effector memory subset (p = 0.025), in addition to a higher percentage of HLA-DR+ CD25− CD127− effector memory cells (p = 0.027).

In an exploratory analysis breaking from the intention-to-treat principle, the n = 4 participants from the placebo group that received vitamin D supplementation on medical advice were excluded from analysis in order to determine if supplementation of these infants had negatively affected the strength of the results. Contrary to this hypothesis, exclusion of the infants that received supplementation post-three months of age resulted in weaker statistical differences between the groups, although all remained significant with the exception of the MFI of CCR7 in the CD127+ nTreg effector memory subset (data not shown). Thus, although these infants had achieved a 25(OH)D3 level >50 nmol/L by 6 months of age, this was not sufficient to compensate for the deficient levels experienced earlier in infancy.
Differences in Treg populations by 25(OH)D levels

Multiple associations were observed between 25(OH)D3 level and Treg parameters. CB 25(OH)D3 levels were first examined in relation to CBMC and PBMC, due to the significant associations between CB 25(OH)D3 and immune function at 6 months of age detected in Chapter 4. In the present study, however, associations between CB 25(OH)D3 level and Treg parameters were weak and did not display consistent patterns.

CB 25(OH)D3, as measured by CIA, was positively correlated with the MFI of HLA-DR of CB nTreg cells (rho = 0.313, p = 0.046). This correlation, however, was not observed when using the LC/MS/MS data (rho = 0.151, p = 0.339). A number of significant correlations were observed within the CD127+ nTreg subsets, although results varied between assays. Specifically, CB 25(OH)D3 as determined by LC/MS/MS was inversely correlated with the percentage of CD127+ nTreg effector cells (rho = -0.341, p = 0.029), whereas the CIA measure was not (rho = -0.050, p = 0.755). CIA 25(OH)D3 was highly significantly correlated with the MFI of CD45RA in the CD127+ nTreg naïve population (rho = -0.432, p = 0.005), in contrast to the results given by LC/MS/MS (rho = -0.178, p = 0.266). Given the lack of association between CB 25(OH)D3 and any other CB Treg parameter these may be chance findings secondary to the large number of tests conducted.

When analysing the relationship between CB 25(OH)D3 level and Treg parameters at 6 months of age there was a weak inverse correlation between CB 25(OH)D3 and the MFI of CD45RA in the CD127+ nTreg effector population (rho = -0.326, p = 0.038 for LC/MS/MS, and rho = -0.308, p = 0.05 for CIA). No further significant correlations were detected between CB 25(OH)D3 levels and immune phenotype at 6 months of age,
however, more consistent findings were observed when 25(OH)D3 levels at 6 months of age were investigated.

In agreement with the finding of a higher MFI of HLA-DR in nTreg population with vitamin D supplementation, there were significant positive correlations between this parameter and both LC/MS/MS and CIA measures of 25(OH)D3 at 6 months of age (rho = 0.454, p = 0.003 and rho = 0.512, p = 0.001, respectively). 25(OH)D3 at 6 months of age was inversely correlated with the percentage of CD69+ nTreg (Table 6.3), representative of an inactive naïve Treg phenotype (317). This was also reflected in a lower percentage of CD69+ cells in the nTreg naïve subset (rho = -0.329, p = 0.041 for LC/MS/MS and rho = -0.417, p = 0.007 for CIA).

A similar pattern was observed for the CD127+ nTreg population, with a positive correlation between 25(OH)D3 measured by CIA and the MFI of HLA-DR (rho = 0.342, p = 0.027). Both LC/MS/MS and CIA measures of 25(OH)D3 were inversely correlated with the percentage of CD69+ CD127+ nTreg, in addition to the MFI of CD69 (Table 6.3). Again, this trend was observed in the naïve subset where the percentage of CD69+ cells and the MFI was inversely correlated with 25(OH)D3 measured by LC/MS/MS (rho = -0.331, p = 0.04, and rho = -0.384, p = 0.016, respectively), and CIA (rho = -0.407, p = 0.008, and rho = -0.357, p = 0.022, respectively). Furthermore, the percentage of CD69+ effector cells was inversely correlated with 25(OH)D3 (rho = -0.372, p = 0.02 for LC/MS/MS and rho = -0.331, p = 0.034 for CIA).
A consistent pattern between 25(OH)D3 level at 6 months of age and CD69 expression emerged again in the CD25FoxP3+ population. The percentage of CD69+ cells in both the CD127− and CD127+ subsets was inversely correlated with both 25(OH)D3 measures, with significant correlations also noted for the MFI of CD69 in the CD127+ subset (Table 6.3).

<p>| Table 6.3. Correlation between plasma 25(OH)D3 at 6 months of age and CD69 expression in Treg subsets |
|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>LC/MS/MS</th>
<th>CIA</th>
<th>Spearman’s rank correlation coefficient</th>
<th>Spearman’s rank correlation coefficient</th>
<th>P</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>nTreg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>- % CD69+</td>
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<td>-0.370</td>
<td>0.016</td>
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<td>- CD69 MFI</td>
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<td>-0.274</td>
<td>0.079</td>
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</tr>
<tr>
<td>CD127+ nTreg</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>- % CD69+</td>
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<tr>
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<tr>
<td>CD25 CD127 Treg</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- % CD69+</td>
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<td>-0.418</td>
<td>0.007</td>
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<td>- CD69 MFI</td>
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<td>-0.254</td>
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<tr>
<td>CD25 CD127+ Treg</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>- % CD69+</td>
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<td>-0.406</td>
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</tr>
<tr>
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<td>-0.367</td>
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</table>

Differences in Treg populations by UV exposure

Average daily UV exposure from 0 – 3 months of age, but not 3 – 6 months of age was significantly correlated with multiple parameters within the populations of interest. In the nTreg population, average daily UV exposure from 0 – 3 months of age was positively correlated with the MFI of CD45RA in the central and effector memory populations (rho = 0.414, p = 0.023, and rho = 0.436, p = 0.016, respectively). This was also reflected in seasonal analysis, where the MFI of CD45RA in the central memory population was lowest in spring (p = 0.039) when 25(OH)D3 levels are at their lowest.
The majority of significant correlations with UV exposure from 0 – 3 months of age were observed in the subsets of the CD127+ nTreg population (Table 6.4). A higher UV exposure was associated with a reduced proportion of effector memory cells, and a concurrent increase in effector and naïve subgroups. Accordingly, there were strong positive correlations between UV and the MFI of CCR7 and CD45RA in the effector memory subgroup. The percentage of HLA-DR+ cells in the effector memory and effector subgroups was also noted to correlate positively with UV exposure. The influence of UV exposure on these subsets was further supported when analysed by season of blood collection. The percentage of CD127+ nTreg effector memory cells was highest in spring (p = 0.040), whilst the percentage of naïve cells was greatest in summer (p = 0.027).

<table>
<thead>
<tr>
<th>Table 6.4. Correlations between average daily UV exposure from 0 – 3 months of age, and subsets of the CD127+ nTreg population</th>
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</thead>
<tbody>
<tr>
<td><strong>Effector Memory (%)</strong></td>
</tr>
<tr>
<td>- CD45RA MFI</td>
</tr>
<tr>
<td>- CCR7 MFI</td>
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<tr>
<td>- CD69+ (%)</td>
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<tr>
<td>- CD69 MFI</td>
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<td>- HLA-DR+ (%)</td>
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<tr>
<td>- HLA-DR MFI</td>
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<tr>
<td><strong>Effector (%)</strong></td>
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<tr>
<td>- CD45RA MFI</td>
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<td>- CCR7 MFI</td>
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<tr>
<td>- CD69+ (%)</td>
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<td>- CD69 MFI</td>
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<tr>
<td>- HLA-DR+ (%)</td>
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<tr>
<td>- HLA-DR MFI</td>
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<tr>
<td><strong>Naive (%)</strong></td>
</tr>
<tr>
<td>- CD45RA MFI</td>
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<tr>
<td>- CCR7 MFI</td>
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<tr>
<td>- CD69+ (%)</td>
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<tr>
<td>- CD69 MFI</td>
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<tr>
<td>- HLA-DR+ (%)</td>
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<tr>
<td>- HLA-DR MFI</td>
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</tbody>
</table>
The CD25\textsuperscript{+}FoxP3\textsuperscript{+} populations also displayed significant associations with UV, as measured by personal dosimetry and season. In the CD25\textsuperscript{+}CD127\textsuperscript{−} subset average daily UV exposure from 0 – 3 months of age correlated with the MFI of CD45RA in the central and effector memory subsets (\(\rho = 0.494, p = 0.006\), and \(\rho = 0.374, p = 0.046\), respectively). Although not correlated with personal dosimetry data, the proportion of naïve CD25\textsuperscript{+}CD127\textsuperscript{−} was greatest in summer (\(p = 0.032\)). As was observed for the CD25\textsuperscript{+}FoxP3\textsuperscript{+} populations, the MFI of CD45RA in the CD25\textsuperscript{−}CD127\textsuperscript{+} effector memory subset was positively correlated with average daily UV exposure from 0 – 3 months of age (\(\rho = 0.388, p = 0.038\)), and was lowest in spring (\(p = 0.024\)).

To test the hypothesis that UV exposure was independently associated with the aforementioned statistically significant correlations, multiple regression analysis was used with 25(OH)D\textsubscript{3} as determined by both assays included as independent variables. Several parameters within the CD127\textsuperscript{+} nTreg population remained significantly correlated with average daily UV exposure from 0 – 3 months of age independently of plasma 25(OH)D\textsubscript{3} levels. Specifically, UV exposure was inversely correlated with the proportion effector memory cells (\(\beta -0.295 [95\% \ CI -0.50, -0.09], p = 0.007\)), while being positively correlated with the MFI of CCR7 (\(\beta 2.93 [95\% \ CI 0.95, 4.91], p = 0.005\)). Within the effector subset the MFI of CCR7 (\(\beta 3.68 [95\% \ CI 1.71, 5.64], p = 0.001\)) and the proportion of HLA-DR\textsuperscript{+} effector cells (\(\beta 0.578 [95\% \ CI 0.1, 1.06], p = 0.020\)) were positively correlated with UV exposure.
**DC analysis**

**Percentage of DC populations at birth and 6 months of age**

The percentage of pDC, CD1c\(^+\), CD141\(^+\), CD16\(^+\) and CD16\(^-\) cDC, as a percent of live cells, was analysed and compared at birth and 6 months of age. The percentage of pDC, CD1c\(^+\) and CD141\(^+\) cDC were stable from birth to 6 months (Figure 6.8). The percentage of CD16\(^+\) cDC increased from birth to 6 months of age (Figures 6.9 and 6.10), in conjunction with an increase in the MFI of CD11c (median [IQR] at birth 2265 [785] and at 6 months of age 3542 [1213], p <0.0001). The increase in the percentage of CD16\(^+\) cDC was accompanied by a decrease in CD16\(^-\) cDC.

![Figure 6.8](image)

**Figure 6.8.** Change in the percentage of DC populations from birth (grey boxes) to 6 months of age (orange boxes) (whiskers represent 5\(^{th}\) and 95\(^{th}\) percentile). A) pDC; B) CD1c\(^+\) cDC; C) CD141\(^+\) cDC. ns = not significant.
Figure 6.9. Change in the percentage of DC populations from birth (grey boxes) to 6 months of age (orange boxes) (whiskers represent 5th and 95th percentile). A) CD16+ cDC; B) CD16− cDC. *** = p <0.0001, * = p <0.05.

Figure 6.10. Representative gating plot presenting the CD16− and CD16+ cDC populations in CB (left) and at 6 months of age (right). From birth to 6 months of age there is a significant increase in CD16+ cells that express greater amounts of CD11c.
Definitions of DC vary substantially between studies which can make direct comparisons of populations challenging. The absence of a significant change in the percentage of pDC from birth to 6 months of age is supported by the findings of Koumbi et al (318); however the current study did not witness an increase in the percentage of cDC (analogous to the combined CD1c+, CD141+ and CD16– cDC populations in the current study) reported by these authors. The ratios of DC populations observed in the current study also differ from those previously reported in CB or infant samples. The CD11c+ cDC:pDC ratio of 3.9 (3.6) (median [IQR]) is significantly higher than has been reported by others, and more closely resembles the ratio observed in adult blood (319).

**Baseline differences**

Percentages of the DC populations and MFI of cell surface markers under investigation were compared at baseline (CB) between the vitamin D supplemented and placebo groups. No significant differences were detected between the groups (data not shown).

**Difference in DC populations between vitamin D supplement and placebo groups**

DC populations as a percentage of live cells, the ratios of DC populations, their activation markers and co-stimulatory molecules were first compared between the vitamin D supplemented and placebo groups. The only statistically significant difference between the intervention groups was a lower percentage of CD11c+CD16– CCR7+ cDC in the vitamin D supplemented group (p = 0.023). While not statistically significant a similar association was observed for the percentage of CD16+CCR7+ cDC, being lower in the supplemented group (p = 0.061).
As per the Treg analysis, participants from the placebo group that were supplemented post-three months of age were excluded to see if this impacted on the results. Analogous to the prior analyses exclusion of these participants lessened the strength of the association between vitamin D supplementation and a reduced percentage of CCR7$^+$ CD16$^-$ (p = 0.031) and CD16$^+$ (p = 0.070) cDC. This finding further supports the notion that deficiency earlier in infancy has impacts on the immunophenotype that are not rapidly amenable to correction of the deficiency.

**Differences in DC populations by 25(OH)D3 levels**

First CB 25(OH)D3 levels were correlated with CB DC parameters. CB 25(OH)D3 showed little association with the percentages of pDC or cDC populations, nor cell surface protein expression. Exceptions to this were a possible inverse correlation with the percentage of pDC when compared to 25(OH)D3 measured by LC/MS/MS (rho = -0.392, p = 0.011, rho = -0.169, p = 0.297 for CIA) and a positive correlation with the percentage of CD16$^+$CD86$^+$ cDC (rho = 0.321, p = 0.041 for LC/MS/MS, and rho = -0.298, p = 0.062 for CIA).

Following this the associations between CB 25(OH)D3 levels and DC parameters at 6 months of age were investigated. In this set of analyses CB 25(OH)D3 by LC/MS/MS was positively correlated with the percentage of CCR7$^+$ pDC (rho = 0.298, p = 0.078) and the MFI of CCR7 (rho = 0.355, p = 0.046), however this result was not observed when using the CIA 25(OH)D3 measurement (rho = 0.234, p = 0.176 for the percentage CCR7$^+$, and rho = 0.150, p = 0.391 for the MFI). Both the LC/MS/MS and CIA assays were associated with a positive trend for the percentage of FceR1$^+$ pDC (rho = 0.326, p = 0.052, and rho = 0.355, p = 0.036, respectively) and FceR1$^+$ CD16$^+$ cDC (rho = 0.386, p = 0.024, and rho = 0.354, p = 0.043, respectively) at 6 months of age. Lastly, CB
25(OH)D3 measured by LC/MS/MS was inversely correlated with the percentage of CD40\(^+\) CD141\(^+\)cDC (rho = -0.369, p = 0.029, and rho = -0.194, p = 0.257 for CIA). However, at 6 months of age some of these associations were reversed.

At 6 months of age 25(OH)D3 levels by CIA were inversely correlated with the percentage of pDC and CD16\(^+\) cDC expressing CCR7, with a non-significant trend for the CD16\(^-\) cDC (rho = -0.351, p = 0.036, rho = -0.395, p = 0.021 and rho = -0.305, p = 0.079, respectively). The MFI of CCR7 in the pDC and CD16\(^+\) cDC subsets also appeared to inversely correlate with 25(OH)D3 by CIA (rho = -0.352, p = 0.035 and rho = -0.321, p = 0.064, respectively). Although the trend for a lower proportion of CCR7\(^+\) cells was consistent across DC subsets, and in line findings from the intervention group comparisons, the correlations were non-significant for the LC/MS/MS measure (rho = -0.232, p = 0.173, rho = -0.255, p = 0.145, and rho = -0.076, p = 0.670 for pDC, CD16\(^+\) cDC and CD16\(^-\) cDC, respectively). Aside from the associations between 25(OH)D3 and CCR7 no further significant correlations emerged. Interestingly, the associations between CB 25(OH)D3, co-stimulatory molecules or FceR1 were not observed for 25(OH)D3 levels at 6 months of age.

**Differences in DC populations by UV exposure**

As was the case with the T cell analysis, only average daily UV exposure from 0 – 3 months of age was significantly associated with DC parameters. Proportions of most DC subsets, as a percentage of live cells or as ratios to other subsets, did not vary with UV exposure, although there was a positive correlation with the percentage of CD16\(^-\) cells (rho = 0.438, p = 0.029). However, while there was not a strong association between UV exposure and the proportion of cell types there was indication that DC
maturation and capacity to promote allergic inflammation is inhibited with increasing UV exposure.

Most notably, the percentage of pDC and cDC expressing CCR7 was consistently inversely correlated with UV exposure from 0 – 3 months of age (Figure 6.11). CCR7 is upregulated as DC mature, indicating that fewer DC are being activated and trafficking to lymph nodes, whereupon they would activate T cells. In addition, the percentage of pDC and CD1c+ cDC positive for CD86, a dominant co-stimulatory molecule in the activation CD4+ T cell and development of Th2 responses (320, 321), was inversely correlated with average daily UV exposure from 0 – 3 months of age (rho = -0.645, p = 0.0005 and rho = -0.443, p = 0.018, respectively). While CD86 appears to be suppressed by UV the expression of co-stimulatory molecule CD40, which is involved in the development and maintenance of Th1 responses (321), was positively correlated with UV exposure in CD1c+ (rho = 0.376, p = 0.049) and CD16– (rho = 0.417, p = 0.034) subsets, with significant correlations with CD40 MFI detected in the CD141+ (rho = 0.488, p = 0.008) and CD16– (rho = 0.420, p = 0.033) subsets.
Figure 6.11. Correlation between average daily UV exposure from 0 - 3 months of age and CCR7 expression on dendritic cell subsets.
In multivariate linear regression, testing UV exposure and plasma 25(OH)D3, the percentage of CCR7$^+$ pDC, CD1c$^+$, CD141$^+$, CD16$^+$ and CD16$^-$ remained significantly correlated with UV exposure from 0 – 3 months of age ($\beta$ [95% CI] -0.191 [-0.32, -0.07], $p = 0.004$; $\beta$ -0.223 [-0.44, -0.01], $p = 0.039$; $\beta$ -0.390 [-0.66, -0.12], $p = 0.007$; $\beta$ -0.205 [-0.36, -0.04], $p = 0.014$; and $\beta$ -0.208 [-0.33, -0.08], $p = 0.002$, respectively). An inverse correlation remained between UV exposure and the percentage of CD86$^+$ pDC and CD1c$^+$ cDC, although the latter did not retain statistical significance ($\beta$ -0.707 [-1.15, -0.26], $p = 0.003$; and $\beta$ -0.478 [-0.96, 0.005], $p = 0.052$, respectively). In this model, however, the positive correlations between UV exposure and CD40 expression were not maintained (data not shown).

**Discussion**

The VITAL trial is the first randomized, double-blinded, placebo controlled trial to investigate the effects of vitamin D supplementation over the first 6 months of life on the developing immune phenotype. Furthermore, it is the first trial to utilize personal UV dosimetry in infants for the purposes of investigating the immunomodulatory effects of UV exposure. The novel multicolour flow cytometry panels reveal consistent patterns in Treg and DC surface protein expression associated with vitamin D supplementation, plasma 25(OH)D3 levels and UV exposure. The expression patterns observed with higher vitamin D status and UV exposure are consistent with a tolerogenic immune phenotype with lower propensity for Th2 allergic inflammation. Results from this trial provide crucial immunological support for observational associations between vitamin D status in early life and the development of allergic disease.
In the current trial both vitamin D supplementation and plasma 25(OH)D3 level at 6 months of age were positively associated with the MFI of HLA-DR in nTreg, while 25(OH)D3 level also correlated with HLA-DR MFI in the CD127+ nTreg population. As Baecher-Allen et al (120) elegantly demonstrated, HLA-DR expression on CD4+CD25+ Treg is associated with a distinct, terminally differentiated Treg population, with earlier and stronger suppressive function than HLA-DR- Treg. Interestingly, treatment with glucocorticoids – drugs known to supress Th2 responses – has been shown to increase the proportion of differentiated HLA-DR+ Treg (322), supporting a role for these cells in the suppression of allergic inflammation.

The consistent inverse correlations between 25(OH)D3 level at 6 months of age and the proportion of FoxP3+CD69+ cells may be highly relevant to the reduced risk of allergic disease observed with increasing 25(OH)D3 levels, although there is conflicting data. A recent publication reports that CD69 expression on murine Treg is necessary for suppression of T cell proliferation and the maintenance of tolerance to allergens (296). The CD69+ Treg cells show higher expression of cytokine genes including IL-2 and IL-10, and appear to exert their suppressive effects via mechanisms dependent on secretion of TGF-β (296). This differs from the contact-dependent suppression of classical Treg (323) and HLA-DR+ Treg (120). Yet in contrast to the findings of Cortes et al (296) there is evidence from human and murine studies that CD69 expression on Treg is inversely associated with allergic disease.

One study finds that the frequency of circulating CD4+CD25+CD69+ cells was significantly higher in allergic children (mean age 7.8 years) in comparison to non-allergic controls (123), and this is supported by data from mouse models of allergic
airway disease. Faustino et al (317) have shown that the frequency of CD4⁺CD25⁺FoxP3⁺CD69⁺ cells increases substantially in the lung tissue of allergic, compared to tolerant mice, following OVA challenge. The authors note that while the lung Treg of allergic mice were able to effectively inhibit T cell proliferation they lacked the capacity to suppress Th2 cytokine (IL-5 and IL-13) production upon anti-CD3 or OVA stimulation (317). Lastly, and of most relevance to the current study, McGlade et al (323) have demonstrated that UV-irradiation of mice modulates of the number of CD4⁺CD25⁺CD69⁺ cells. Rather than inducing an IL-10- or TGF-β-producing Treg phenotype, UV-induced suppression of allergic airway disease is associated with fewer CD4⁺CD25⁺CD69⁺ cells (323). While a direct correlation between personal UV dosimetry data and CD69 expression was not observed in the present study, the fact that 25(OH)D3 levels in the study population are largely dependent on UV exposure is conducive with these findings. Further evidence for the maintenance of an inactivated Treg pool by UV exposure was seen in the correlations between average daily UV exposure and CD45RA and CCR7 expression.

In subsets of both the nTreg and CD127⁺ nTreg populations there were positive correlations between average daily UV exposure and the MFI of CD45RA, with further positive correlations for the MFI of CCR7 within the CD127⁺ cells. This phenotype corresponds to a previously described suppressive “natural naïve Treg” population, which are precursors of antigen-experienced natural CD45RO⁺/RA⁻ Treg (324). As per HLA-DR⁺ and classical Treg cells, these natural naïve Treg exert their suppressive function by cell-cell contact mechanisms and do not produce detectable levels of IL-10 or TGF-β (324). Thus, the data presented here suggest that vitamin D and UV both play a role in maintaining a suppressive Treg pool characterized by a greater expression of HLA-DR and CD45RA, and fewer CD69⁺ cells. The fact that these subsets work via
cell-cell contact may explain the absence of strong positive correlations between 25(OH)D3 level and IL-10 production in PBMC cell culture experiments, while supporting the inverse association between 25(OH)D3 levels and clinical outcomes presented in Chapter 4. This pattern of a more naïve, non-inflammatory immune profile associated with vitamin D and UV exposure also emerged in the DC analysis where consistent trends were observed across the DC subsets.

In the steady state, immature DC expressing low amounts of co-stimulatory molecules migrate to lymph nodes where they induce anergic, apoptopic or regulatory T cells (325). In the presence of microbial products or inflammatory mediators DC undergo maturation, upregulating CCR7 and co-stimulatory molecules, facilitating trafficking to secondary lymphoid organs and activation of naïve T cells (325). The most strikingly consistent observation to emerge from the analysis of DC subsets in the present study was the lower percentage of CCR7+ DC associated with vitamin D supplementation, higher plasma 25(OH)D3 at 6 months of age, and average daily UV exposure. At the same time, there was a lower percentage of pDC and CD1c+ cDC expressing the co-stimulatory molecule CD86, yet an increase in CD40 on CD1c+, CD16- and CD141+ subsets. Some studies find that CD86, but not CD40, is required for Th2 activation (321, 326, 327) whereas CD40 is associated with Th1 responses (321); a potentially important finding given the hereditary risk of Th2-associated allergic disease in this population. However, this is not a universally held view as depending on the context CD40 can also contribute to Th2 responses (328). Thus, in the absence of functional data in the present study the significance of this observation is speculative.
The results obtained here are highly analogous to previous research on the effects of vitamin D and UV radiation on DC. *In vitro* studies have demonstrated that 25(OH)D3 and 1,25(OH)2D3 suppresses CD86 and CCR7 expression by monocyte-derived DC and cDC (271, 329). In contrast to the current findings, however, Penna et al (329) report that vitamin D did not suppress the expression of these markers in pDC. The inverse correlation between 25(OH)D3 level and CCR7+ pDC in the present study may therefore be a reflection of the local environment and cytokine milieu. The role of vitamin D in modulation of DC function is further supported by an *in vivo* mouse study, where nutritional vitamin D deficiency was associated with a higher MFI of CD86 on CD11c+ splenocytes, and increased CD86 expression in lung CD11c+ cells (pDC were not investigated) (330). Again it is interesting to note that one mechanism by which glucocorticoids exert their anti-inflammatory effects is by inhibiting DC maturation, including inhibiting the upregulation of CCR7 and CD86 (331). Therefore these results indicate that exposure of DC to vitamin D *in vivo* contributes to the development of a DC population with tolerogenic properties and lower Th2-stimulating capacity. There is some indication in the current study and that of others (290, 291) that UV may play a role in these findings independently of vitamin D production.

In *in vitro* experiments involving human skin two groups report that emigrating DC express less CD86+ and CCR7+ following UVA (290) or low dose UVB irradiation (291), and that the irradiated DC have a reduced capacity to stimulate T cells (291). While the results of Richters et al (291) do not exclude vitamin D as the mediating factor, the use of non-vitamin D producing UVA wavelengths by Furio et al (290) support an independent effect of UV radiation. As of yet researchers have not identified a non-vitamin D mechanism that adequately explains these observations; frequently cited UV-induced immunomodulating molecules including PGE2, *cis*-urocanic acid or
reactive oxygen species having either no effect or upregulating DC maturation markers (332-334). However, a potential mechanism that remains underexplored is the effect of UV radiation on the regulation of immune-related genes (335).

Solar-simulated UV irradiation alters the regulation of numerous genes, downregulating immune function pathways including complement (335). Several DC subsets including pDC and CD1c⁺ cDC have receptors for multiple components of the complement pathway (336), and Liu et al (337) have demonstrated that the complement molecule C1q enhances the chemotaxis of mature DC by upregulating CCR7. Therefore, gene regulation by UV represents an alternative, non-vitamin D mechanism for immunomodulation, and an area for future research.

An unexpected finding in this study is that CB 25(OH)D3 level showed little association with Treg and DC markers in CBMC and PBMC samples, or in some cases correlations that were opposite to those observed when using 6 month of age 25(OH)D3 levels. Furthermore, the significant correlations between CB 25(OH)D3 and immune cell markers at 6 months of age initially appear unsupportive of our earlier functional study where CB 25(OH)D3 was associated with reduced Th2 responses (Chapter 4). This was most prominent in the DC analysis where there was a positive trend between CB 25(OH)D and CCR7 expression on pDC, in addition to the percentage of FcεR1⁺ pDC and CD16⁻ cDC at 6 months of age.

FcεR1 expression on DC increases the efficiency of allergen presentation, contributing to Th2 allergic inflammation, and higher levels of FcεR1 have been detected on DC of
allergic, in comparison to non-allergic, adults (305, 313). In a study of children aged 12 months – 15 years, however, Vasudev et al (312) find that FcεR1 expression on pDC and cDC was not associated with sensitization status, and in the case of pDC did not correlate with serum IgE levels. The expression of FcεR1 on cDC was correlated with serum IgE, although interestingly when IgE levels were <60 kU/L FcεR1 expression was greater on cDC of non-sensitized children (312). The complex relationship between IgE and FcεR1 on DC in children may explain why the increased FcεR1 expression with higher CB 25(OH)D3 status did not translate to increased Th2 responses or sensitization in our previous studies (Chapters 3 and 4). Cell culture experiments and clinical follow up of the current study population will be required in order to confirm this.

A second unexpected outcome was that, although effective at reducing the proportion of participants with vitamin D deficiency, the supplement product was only able to marginally raise the mean 25(OH)D3 level in comparison the placebo group. Supplementation had less of an effect than seasonal UV variation on circulating 25(OH)D levels at 6 months of age. This most likely explains the modest statistical associations observed for supplementation in comparison to other measures of vitamin D exposure, and in comparison to results of other studies. For example, whereas Prietl et al (164) find that vitamin D supplementation is efficacious in raising the percentage of nTreg in adults (164), here there was no association between supplementation and the percentage of nTreg, nor any other Treg subset. An important difference between the two studies is the high dose protocol employed by Prietl et al (164). These researchers used a dose approximately 12 times greater than in the current trial (140,000 IU/month), and subsequently were able to produce a significant change in serum 25(OH)D levels
(~85 nmol/L difference between supplemented and placebo groups after 12 weeks). It is possible that the magnitude of change in vitamin D status was responsible for the change in Treg percentage by providing high local levels of 25(OH)D and simultaneously increasing circulating levels of active 1,25(OH)₂D (164). The subtle phenotypical changes observed in the present, as opposed to a more dramatic changes in the proportion or frequency of cell populations, may in fact be preferable in terms of limiting the risk of intended and negative consequences resulting from significant changes to immune homeostasis.

A finding that requires further exploration is that UV exposure from 0 – 3 months of age, but not 3 – 6 months of age, correlated with 25(OH)D level at 6 months of age, in addition to multiple immune parameters. The absence of association between the recorded UV data from 3 – 6 months of age and 25(OH)D₃ levels in particular is puzzling due to our previous findings that average UV index over the 3 months preceding blood draw correlates significantly with 25(OH)D₃ levels (Chapter 2). One possible explanation for this observation is that the small data set with 3 – 6 month UV exposure prohibited the detection of a statistical signal. As the quantity of UV exposure did not appear to differ between age groups this explanation is plausible, and will be confirmed by the future examination of our remaining data. A second possible explanation is that infant skin fundamentally changes over the first months of life, such that it is more sensitive to UV in early infancy. It is well documented that infant skin is thinner than that of adults, and that infants under 12 months of age have less melanin than children aged 16 – 24 months (reviewed in (338)). Theoretically this would permit greater penetrance of UV radiation; however the consequences of this in terms of vitamin D production or the development immune system are unknown.
Finally, the results from this study further highlight the recognized discrepancies between vitamin D assays. Here a CDC accredited LC/MS/MS assay has been utilized as the gold standard. The CIA results correlated strongly with the LC/MS/MS, however the CIA had fair-to-poor performance in terms of categorising participants by their vitamin D status. By presenting the correlations for immune parameters with both assays it is apparent that somewhat differing results could be obtained from the same study based solely on the choice of assay. Correlations with the CIA measures needn’t be distrusted entirely, but should be considered in the context of the pattern of results and biological plausibility.

In summary, this study presents novel findings that vitamin D supplementation and UV exposure in infancy have important consequences for the developing immune phenotype in a population at hereditary risk of allergic disease. The findings offer insight into possible immunological mechanisms behind the reported inverse associations between vitamin D status and allergic disease. This includes the presence of an effectively suppressive Treg pool and a reduced propensity for Th2-priming by DC with vitamin D supplementation, higher 25(OH)D3 levels and a greater exposure to UV. While it was beyond the scope of this study to investigate the potential mechanisms there was indication that UV exposure may be associated with changes in immune phenotype independently of vitamin D levels. However, given the strong relationship between UV exposure and vitamin D this will require investigation by future studies. Based on these findings clinical follow up and replication studies are justified to determine if these immunological changes translate into disease prevention.
Chapter 7

General Discussion
The work of this thesis is framed around the key question of whether vitamin D status in early infancy is an important determinant of the infant immune phenotype and the risk of developing symptomatic allergic disease. To address this question, this thesis examined observational data relating to vitamin D status at birth and 6 months of age, and conducted the first randomized controlled trial designed to investigate the effect of infant vitamin D supplementation on the developing immune phenotype. The key findings of this thesis are discussed in the context of the existing literature, followed by study limitations and conclusions and future directions.

**Summary of findings and discussion in the context of existing literature**

The primary aims of this thesis were to: 1) describe the 25(OH)D status of pregnant women in Perth, Western Australia, in order to quantify the proportion of this population with suboptimal 25(OH)D levels, and identify intrinsic and extrinsic factors associated with 25(OH)D status; 2) investigate the relationship between 25(OH)D3 status at birth (cord blood) and in infancy (6 months of age) in relation to risk of clinical allergic disease in infancy and childhood; and 3) identify differences in infant immune phenotype between participants stratified by vitamin D supplementation, 25(OH)D3 status and UV exposure.

*Chapter 2 - 25(OH)D status of pregnant women in Perth, Western Australia*

This cross-sectional study identified that a significant proportion of Perth women have suboptimal 25(OH)D levels in late gestation (36 – 40 weeks), despite the predominantly white Caucasian population and sun-rich climate. Overall, 15.1% (25/165) of women were vitamin D deficient (<50 nmol/L), with a further 34.5% (57/165) in the insufficient range of 50 – 75 nmol/L. While UV radiation was a major determining factor in
25(OH)D status, vitamin D intake via antenatal vitamin supplements was also positively correlated with 25(OH)D levels, and use of such supplements was associated with a reduced risk of vitamin D deficiency. This study extends the existing scientific knowledge by examining the vitamin D status of a low risk population in a contemporaneous setting, and being the first Australian study to describe the contribution of supplementation to 25(OH)D levels.

Due to the high prevalence of vitamin D insufficiency in this study it is important to consider the possibility that suboptimal vitamin D levels in pregnancy are a contributing factor to the high rates of allergic disease in Australian children. Whether vitamin D status at birth (strongly correlated with maternal levels) translates to the risk of allergic disease in infancy was then explored in Chapter 3. Furthermore, based on the evidence that vitamin D supplementation significantly reduced the risk of vitamin D deficiency in a free-living, low risk adult population, it is logical to ask whether the infant population in Perth may also benefit from routine supplementation. To address this question infant 25(OH)D3 levels and the effect of low dose supplementation was investigated in Chapter 6.

Chapters 3 and 4 - Risk of allergic disease in relation to 25(OH)D3 status at birth and in infancy

The studies presented in Chapters 3 and 4 examined the relationship between 25(OH)D3 status at birth (CB) and the risk of allergic disease in infancy, finding a significantly reduced risk of eczema at 6 and 12 months of age with higher CB 25(OH)D3 levels. In both of these studies sensitization and wheeze were also examined, but neither was associated with CB 25(OH)D3 levels. Since the initial study (Chapter
3) was published in 2012 (252) the findings have been replicated by two other groups (48, 50).

Both of these later studies reported an inverse association between CB 25(OH)D3 and the risk of eczema at 1 year of age, with the strength of the associations mirroring the results of this thesis. The 12% reduction in risk per 10 nmol/L reported by Palmer et al (50), and 16% reduction per 12.5 nmol/L reported by Baiz et al (48) is highly comparable to the 13% - 16% reduction in odds in Chapters 3 and 4, respectively. In agreement with the results of this thesis Palmer et al also found no association between CB 25(OH)D3 and sensitization, however do report a significant inverse association for the cumulative incidence of atopic eczema (that is, eczema plus a positive skin prick test) by 3 years, and the presence of IgE food allergy at 1 year of age. While the studies presented in this thesis did not detect an association between CB 25(OH)D3 and IgE food allergy this may be related to the relative infrequency of food allergy in comparison to eczema, and thus a low probability of detecting relationships in a small sample size.

In contrast to these reported inverse associations between CB 25(OH)D3 and allergic outcomes, a recent study in a German birth cohort study (LINA) has reported that CB 25(OH)D3 is not associated with the risk of eczema at age 1 and 2 years, but is positively associated with the risk of food allergy, specifically in the second year of life (44). There are, however, a number of issues with the design and analysis of the study that may have affected the findings.

Firstly, the adequacy of the sample size for detection of food allergy as a clinical outcome may be questioned. As would be expected in a cohort of mixed hereditary risk,
the incidence of food allergy was fairly low; 8/272 (2.9%) in the second year of life (44). In analyses involving only a small number of events a random clustering into one quartile or another could easily skew the results. Secondly, food allergy was determined by parental report. This is a less objective and definitive measure than the gold standard of oral food challenge, or a history of immediate reaction with positive sIgE or skin prick test results (45). Aside from issues in the sample size and diagnosis, there was also considerable disparity between the 25(OH)D3 levels recorded in the LINA cohort in comparison to the other studies, and variation in the approach to statistical analysis.

The median CB 25(OH)D3 level in the LINA cohort (27.3 nmol/L) is substantially lower than that reported by Palmer et al (mean 55.9 nmol/L), Baiz et al (median 44.5 nmol/L) or in Chapters 3 and 4 (mean 58.4 nmol/L and 57.9 nmol/L, respectively). As such, the proportion of infants in the LINA cohort with what would be considered adequate or “optimal” levels is substantially lower; a factor that becomes apparent in subsequent categorical analyses. Rather than analyzing 25(OH)D3 as a continuous variable as per the four other studies, Weisse et al analysed their data by quartiles. While this can be a legitimate method of statistical analysis it is noteworthy that the quartiles contained highly variable ranges of 25(OH)D3. The first 3 quartiles combined spanned 3.7 – 43.4 nmol/L, a range of 39.7 nmol/L, whilst the top quartile had a range of 56.6 nmol/L (43.4 – 100.1 nmol/L). Given the discordance between the quartile values and the more commonly applied reference ranges (i.e. <50 nmol/L, 50 – 75 nmol/L and >75 nmol/L (9)), and the fact that the top quartile overlaps the three categories of status, one may query whether a different analytical approach might have yielded different results.
With regard to the analysis of infant (6 months of age) 25(OH)D3 in relation to allergic disease, no significant associations with eczema, wheeze or sensitization were detected (Chapter 4). There are two possible explanations for this lack of association. Firstly, the smaller sample size analysed at 6 months of age (n = 112) lacked power for clinical outcomes; or secondly, that 25(OH)D3 levels beyond the perinatal period are not relevant to the development of allergic disease. The latter explanation would, however, seem to contradict the findings of other studies on the relationship between infant and childhood vitamin D status and allergic disease (64, 86).

Allen et al (64), for example, have reported that infants with 25(OH)D3 levels ≤50 nmol/L at 1 year of age are more likely to have challenge-proven egg, peanut or multiple food allergies than infants with higher 25(OH)D3 levels. These researchers had a sample size approximately five times greater (n = 577) than was available for analysis in Chapter 4, thus providing greater statistical power to detect clinical outcomes. Furthermore, Liu et al (86) found that children who have persistent low 25(OH)D levels in early life (from birth and throughout infancy), in comparison to children with low levels at a single time point, are at increased risk of food sensitization. Therefore, the absence of statistically significant associations between 25(OH)D3 at 6 months of age and clinical outcomes in Chapter 4 should not be interpreted as an absence of clinical relevance of vitamin D beyond the perinatal period.

In summary, work conducted for this thesis provided the first evidence that 25(OH)D3 status at birth is inversely associated with the risk of eczema at 6 and 12 months of age (Chapters 3 and 4); a finding that has subsequently been replicated by other researchers (48, 50). In support of these clinical findings this thesis also provides evidence that
vitamin D status is associated with functional and phenotypical immune parameters in infancy, compatible with a reduced risk of allergic disease.

*Chapters 4 and 6 - Vitamin D supplementation, 25(OH)D3 status, UV exposure and infant immunophenotype*

In two novel studies this thesis identifies consistent and significant associations between higher infant vitamin D status and the development of a non-allergic immunophenotype. Firstly, in an observational study CB 25(OH)D3 levels were inversely correlated with allergen-induced IL-5, IL-13 and IFNγ production by peripheral blood mononuclear cells (PBMC) at 6 months of age; cytokines that were concurrently associated with the risk of eczema and wheeze in infancy (*Chapter 4*). Secondly, flow cytometric analysis of PBMC from infants in the VITAL RCT revealed multiple and consistent patterns in the immunophenotype of Treg and DC dependent on intervention group, plasma 25(OH)D levels and UV exposure (*Chapter 6*).

These results support experimental data in the literature implicating both vitamin D and UV exposure as modulators of the immune phenotype. The immunomodulating properties of the active vitamin D metabolite, 1,25(OH)2D, have long been recognized, but being the minor, homeostatically controlled, metabolite had limited explanatory power for observations linking vitamin D status with immunological disorders. Later investigations, however, identified the enzymatic machinery necessary for vitamin D activation in DC (148), and have demonstrated that the presence of 25(OH)D3 is sufficient to facilitate the suppression of inflammatory T cell cytokines, and induction of FoxP3+ regulatory T cells via 1,25(OH)2D3 paracrine signaling (163). *In vitro* studies wherein DC are cultured with 25(OH)D3 or 1,25(OH)2D3 provide results analogous to
those presented in Chapter 6, including the inhibition of CD86 (148, 284) and CCR7 (329) expression. In addition to the correlations between 25(OH)D3 levels and immune parameters, the analysis in Chapter 6 also provides evidence suggesting that UV exposure in infancy may be independently associated with certain aspects of the developing immune phenotype.

Figure 7.1. Summary and proposed mechanism of the immunomodulatory effects of vitamin D observed in the VITAL trial. Circulating 25(OH)D3 is taken up by DC wherein it is hydroxylated to 1,25(OH)2D3 by CYP27B1. 1,25(OH)2D3 activates the vitamin D receptor (VDR) which binds to hormone response elements on DNA, resulting in decreased transcription of CD86 and CCR7. 1,25(OH)2D3 produced by DC also acts locally on Treg, altering cell surface marker expression. UV radiation may also contribute to the altered immune phenotypes observed in this study through non-vitamin mechanisms, although the particular UV-induced factors are yet to be confirmed.
UV irradiation has previously been associated with the suppression of allergic airway disease in murine studies – a finding proposed to be related to fewer CD4\(^+\)CD25\(^+\)CD69\(^+\) cells (323), or the production of soluble factors such as prostaglandin E2 (PGE2) by bone marrow CD11b\(^+\) DC (203). While a lower proportion of CD4\(^+\)CD25\(^+\)CD69\(^+\) cells with UV exposure is analogous to the findings of Chapter 6, UV-induced production of PGE2 is not compatible with the results observed here, for reasons discussed in Chapter 6. However, the consistent and significant inverse correlation between UV exposure and DC maturation/activation markers requires explanation.

As discussed in Chapter 6, in vitro experiments have found similar changes in DC surface marker expression through the addition of 25(OH)D3 or 1,25(OH)\(_2\)D3 (271, 329), leaving open the possibility that UV-induced vitamin D production was responsible for the results of the current study. The one caveat is the finding by Furio et al that in vitro UVA irradiation of dermal sheets obtained from human skin reduced the expression of CCR7 and co-stimulatory molecules on emigrating dermal DC (290). As UVA does not induce vitamin D synthesis these findings imply a non-vitamin D mechanism, although the authors did not explore this further. However, as mentioned by the authors, epidermal thickness influences the extent of UVA penetration to the dermal DC (290), and subsequently the thinner epidermis in young infants (338) may be a contributing factor to the correlations between low dose UV exposure from 0 – 3 months of age and DC phenotype. Although the issue of sample size remains to be addressed (discussed in Chapter 6) the findings of this thesis may represent the first evidence that in the first few months of infancy (0 – 3 months of age) even low dose UV exposure has immunomodulating effects. The nature of the relationship between UV exposure, age and immunomodulation will require further investigation, with specific recommendations given in section 7.3.
Study limitations

While attempts have been made to be scientifically rigorous throughout the studies conducted for this thesis, there are a number of factors which may potentially influence the significance and interpretation of the data. These considerations are described below in relation to each of the key areas of investigation.

Chapter 2 - 25(OH)D status of pregnant women in Perth, Western Australia

Measurement of 25(OH)D

25(OH)D levels presented in Chapter 2 were determined by chemiluminescent immunoassay, results of which vary from the gold standard liquid chromatography tandem mass spectroscopy (LC/MS/MS) approach (as demonstrated in Chapter 6). As a result participants may have been misclassified by their vitamin D status. Despite the recognised limitations of this assay it was an ethical requirement that the study use a National Association of Testing Authorities, Australia (NATA) accredited assay, and obtain the results in a timely manner to report to the participants treating physician. Although more accurate, the LC/MS/MS assay utilized in Chapter 6 is not NATA accredited, and samples are batch-analyzed rather than being run as collected. The use of different assays also introduces variability between chapters of this thesis, although analyses within each chapter are internally consistent.
**Chapters 3 and 4 - Risk of allergic disease in relation to 25(OH)D3 status at birth and in infancy**

**Observational study design**

The observational study design of Chapters 3 and 4 prohibits the determination of causality. Observational studies are open to confounding; a significant confounder in the case of vitamin D being UV exposure. In both studies UV radiation was a significant contributing factor to vitamin D status, as evidenced by the seasonal variation in 25(OH)D3 levels. As discussed throughout this thesis UV may potentially impact immunological and clinical findings, independent of vitamin D. While season was controlled for in statistical analyses the results cannot be interpreted as a cause-effect relationship. To address this limitation the VITAL trial (Chapter 5 and 6) issued UV dosimeters to a subset of participants; analyzing personal UV exposure data in relation to plasma 25(OH)D3 levels and immune parameters.

**Chapters 4 and 6 - Vitamin D supplementation, 25(OH)D3 status, UV exposure and infant immune phenotype**

**Incomplete data sets**

Chapters 4 and 6, relating to immune function and variation in Treg and DC populations, are limited by incomplete data sets. The major contributing factor to missing cell culture or flow cytometry data is the difficulty associated with blood collection from infants, resulting in low PBMC counts and the necessity to prioritize experiments. Additionally, while Chapter 6 revealed novel and interesting associations between immune cell markers and UV exposure, dosimetry data is limited to a subset of the infants under investigation. Personal dosimetry was employed in approximately half
of infants in the VITAL trial as this was an exploratory, secondary outcome, with significant costs associated. Not all of the dosimetry data collected was able to be used in this thesis as the other participants did not meet the criteria for inclusion into the Chapter 6 analysis (25[OH]D3 measures and mononuclear cell samples at both birth and 6 months of age). There are, however, plans to analyze the remaining samples with matching UV dosimetry data and PBMC at a later date.

**Homogeneity of the study population**

This limitation applies predominantly to the VITAL trial (Chapter 6), and manifests in a few ways. Firstly, 90% of the infants in the study are of white Caucasian ethnicity. This may be of relevance not solely in terms of 25(OH)D3 synthesis and status, but also in relation to recent reports that children born to Asian migrant parents are at greatest risk of developing allergic conditions (339, 340). However, given the small sample size of the trial, limiting genetic and lifestyle variables may actually be a benefit in finding statistically significant results.

The second limitation in terms of homogeneity of the VITAL trial population comes as a result of the antenatal screening process and responsibility to exclude participants based on vitamin D deficiency in late pregnancy. Although ethically justified, by excluding mothers with 25(OH)D3 levels <50 nmol/L we have potentially excluded the participants that may have most benefitted from supplementation, and theoretically restricted the study to vitamin D-replete infants.
This same criticism applies to testing 25(OH)D3 levels at 3 months of age, with the subsequent cessation of the trial product where vitamin D deficiency is detected. The requirement to do so resulted in approximately 20% (4/19) of participants in the placebo group commencing a vitamin D supplement following the 3 month of age appointment, and may have contributed to the slightly lower prevalence of vitamin D deficiency at 6 months of age in the VITAL trial (1/40, 2.5%, determined by LC/MS/MS) compared to participants analysed for Chapter 4 (9/112, 8%, determined by LC/MS/MS). However, the presence of a wide range of 25(OH)D3 levels at 6 months of age (minimum and maximum levels 25.8 and 154.6 nmol/L by LC/MS/MS, and 26.0 and 182.0 nmol/L by CIA), and the fact that statistically significant differences in immune parameters were detected, suggest that this restriction did not fundamentally effect the study.

It is acknowledged, however, that the prevalence of vitamin D deficiency in the populations investigated in this thesis were much lower than has been reported in other mother/infant populations (208, 209). Subsequently the results presented here, particularly in relation to the effects of vitamin D supplementation, may not be applicable to populations with lower UV exposure and higher rates of vitamin D deficiency.

Low dose of cholecalciferol used in VITAL trial

At 6 months of age the difference in plasma 25(OH)D3 levels between the vitamin D supplemented and placebo groups was only marginally statistically significant. Most likely, the subtle change in 25(OH)D3 level produced by the 400 IU supplement, on the background of UV-induced endogenous production, was difficult to detect in the small
sample size. The future analysis of the entire VITAL trial population would be expected to correct this issue by providing greater statistical power.

Sample size

As described in Chapter 5, candidature time limitations meant that analysis of VITAL trial samples had to proceed prior to the completion of recruitment and follow-up. As a result the sample size was smaller than initially planned. While the sample size presented is within the range of other studies utilizing flow cytometry (see sample size estimation in Chapter 5), being the first trial of its kind there was no firm basis from which to predict an effect size for VITAL. While the consistency of findings between intervention group, plasma 25(OH)D levels and UV exposure does give confidence in the results, certain analyses would be strengthened by the addition of more data points. This applies in particular to comparisons between supplement and placebo groups, where the small sample size and modest effect on 25(OH)D3 levels likely limited the ability to detect significant differences.

Conclusions and future directions

The work presented for this thesis contributes novel and important findings for the role of vitamin D in the developing infant immune system and the risk of allergic disease. It has identified prevalent suboptimal 25(OH)D levels in pregnant women and infants residing in Perth, Western Australia, and produced the first evidence that a low vitamin D status at birth is associated with an increased risk for eczema in infancy. The latter finding was supported by functional immune data linking 25(OH)D3 status at birth and 6 months of age to adaptive and innate responses. Together, the findings from these observational studies suggest that suboptimal vitamin D levels from birth to 6 months of
age may be a contributing factor to the high prevalence of allergic disease in our community.

Perhaps most significantly, in the first RCT specifically designed to investigate the effects of vitamin D on aspects of the developing infant immunophenotype, favourable and consistent differences in Treg and DC populations were detected in relation to vitamin D supplementation, higher 25(OH)D3 levels and greater UV exposure in the first 6 months of life. Thus, results of the VITAL trial provide evidence for a cause-effect relationship between vitamin D status and immunophenotype; indicating that the risk of allergic disease may be attenuated by vitamin D supplementation in early infancy. At this time the clinical implications of the VITAL trial are an extrapolation based on previously reported data; the efficacy of pre-symptomatic intervention (i.e. commencing in the early postnatal period, or even prenatally) in the prevention of allergic disease remaining to be demonstrated. Randomized, double-blind, placebo controlled trials with well documented objective clinical outcomes will be require to avoid further ambiguity in answering the question of efficacy. A thorough review of the existing literature and results of this thesis suggest that such trials should be designed to address the following issues:

i) Critical windows of opportunity - prenatal versus postnatal interventions;

ii) Duration of intervention – prenatal only; commencing pre- and continuing post-natally; first 6 months of infancy versus longer or ongoing interventions;

iii) Optimal circulating 25(OH)D levels and supplementation doses – identification of a target range for 25(OH)D for immunological outcomes; determination of appropriate dose to achieve target 25(OH)D range based on variables such as baseline level and capacity for endogenous production (i.e. skin type and geographical location).
The VITAL trial results also advise the future direction of laboratory studies, particularly as it relates to phenotypical analysis. Vitamin D supplementation and status was not associated with dramatic shifts in major cell populations, but rather more subtle changes in activation and memory markers. Therefore, detailed phenotypical analyses are required in order to avoid committing type II errors. While this thesis focussed on circulating Treg and DC populations there remains a large scope for the analysis of other immune cell subsets that are subject to regulation by vitamin D; monocytes, macrophages and B cells (341) to name but a few. The examination of other cell subsets will help to build a holistic picture of the role of vitamin D in immunological outcomes. In addition to vitamin D, VITAL trial results also raise questions related to the immunomodulating effects of UV exposure in infancy.

Results from this observational component of the trial (personal UV dosimetry) indicate a potential role for UV exposure in the developing immunophenotype. Although the vitamin D-independent effects of UV on immunomodulation have been demonstrated previously (201, 290) the mechanisms by which low dose UV exposure might cause the observed phenotypical differences in Treg and DC populations have not been clarified. Extension and replication of these preliminary results by the examination of as-yet unanalysed VITAL data, and use of personal dosimetry in similar trials, will help to confirm these findings. However, elucidation of the precise mechanisms is likely to be highly complex.

The UV radiation reaching the Earth’s surface is comprised of varying percentages of UVA and UVB radiation, depending on cloud cover and the solar zenith angle (342).
Both UVA and UVB are immunosuppressive but differ in their skin penetrance and time courses of action, in addition to having interactive effects (343). Accordingly, experiments must consider the not just the individual UV components, but their interactive effects in models physiologically relevant to infants (UV dose, skin thickness and pigmentation). In terms of the cellular mechanisms future studies must look beyond the known UV-induced soluble factors (e.g. PGE2, cis-urocanic acid) as such molecules are not conducive with the findings presented here (332, 333). Instead, the effects of UV exposure on gene regulation may be more useful in explaining the findings of this thesis.

There is evidence that solar-simulated UV irradiation downregulates genes related to immune function, including the complement system (335). While vitamin D is also recognised to regulate genes related to immune function (344) experimental designs that block the vitamin D receptor, inhibit the function of vitamin D hydroxylase enzymes or control UV light would allow direct comparisons of the effects of UV and vitamin D on gene regulation. The presence of significant correlations between UV exposure in the first, but not the second, three months of life (Chapter 6) may be indicative of a greater sensitivity to UV at this younger age. Hence, the natural maturation of the skin over infancy should be considered a potential confounding factor. Due to the long term deleterious effects of UV exposure in childhood (345) a better understanding of the risk-benefit relationship of UV exposure in infancy is required before practical recommendations can be devised.

This study has identified associations between vitamin D status in early infancy, the development of a tolerogenic immune profile, and the risk of allergic disease. Various
aspects of vitamin D status were explored including 25(OH)D3 levels at birth and 6 months of age, vitamin D supplementation and UV exposure. The novel findings presented herein provide evidence that the risk of allergic disease may be attenuated through the improvement of vitamin D status in early life; either by supplementation or exposure to UV radiation. This research paves the way for future, conclusive studies, and makes important contributions towards the search for preventative strategies targeting conditions of increasing importance in the 21st century.
References


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Appendix 1

Published article: Cord blood 25-hydroxyvitamin D3 and allergic disease during infancy

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abstract

OBJECTIVE: There has been growing interest in vitamin D insufficiency as a predisposing factor for allergy development based on immunoregulatory properties and epidemiological studies. The aim of this study was to investigate the association between vitamin D exposure in utero and allergic outcomes in the first year of life.

METHODS: Cord blood (CB) vitamin D was measured in 231 high-risk infants from an Australian prospective birth cohort. CB 25-hydroxyvitamin D₃ (25(OH)D₃) concentration was analyzed in relation to maternal vitamin D intake and the development of infant eczema, allergen sensitization, and immunoglobulin E-mediated food allergy.

RESULTS: Maternal intake of supplemental vitamin D was significantly correlated with CB 25(OH)D₃ concentration (r = 0.244, P = 0.003), whereas dietary vitamin D did not influence CB levels. There was significant seasonal variation in CB 25(OH)D₃ concentration suggesting that sunlight exposure was an important determinant. Lower CB vitamin D status was observed in infants that developed eczema (P = 0.018), and eczema was significantly more likely in those with concentrations <50 nmol/L in comparison with those with concentrations ≥75 nmol/L (odds ratio 2.66; 95% confidence interval 1.24–5.72; P = 0.012). This association remained significant after adjustment for multiple confounding factors. The associations between CB 25(OH)D₃ concentration and allergen sensitization, immunoglobulin E-mediated food allergy, and eczema severity (SCORing Atopic Dermatitis) were not significant.

CONCLUSIONS: Reduced vitamin D status in pregnancy may be a risk factor for the development of eczema in the first year of life, reinforcing the need to explore the role of vitamin D exposure during development for disease prevention. Pediatrics 2012;130:e1128–e1135

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KEY WORDS
vitamin D, eczema, cord blood, pregnancy, season of birth, maternal diet, supplementation, food allergy, sensitization, SCORAD, infant, DOHaD

ABBREVIATIONS
25(OH)D₃—25-hydroxyvitamin D₃
CB—cord blood
CI—confidence interval
IgE—immunoglobulin E
OR—odds ratio
SCORAD—SCORing Atopic Dermatitis

Mr Jones contributed to the design of the study, analysis of data, drafting and revising the article, and approval of final version; Dr Palmer contributed to the study design and provided intellectual input in revising the article and approving the final version; Dr Zhang assisted with statistical analysis, revising the article, and approving the final version, and Dr Prescott contributed to the conception and design of the study and acquisition of data, supervised the overall project, and contributed intellectual input in the drafting and revision of the article and approval of the final version.

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Allergic diseases are now the most common chronic disorders of childhood, with a pressing need to define the causal pathways and better prevention strategies. In particular, the rates of food allergy and eczema have continued to increase dramatically in children as part of what appears to be a “second wave” of the allergy epidemic. Progressively earlier presentations with disease clearly implicate early environmental influences such as exposures in pregnancy. Although this is likely to be multifactorial, there has been growing speculation that vitamin D insufficiency in pregnancy may have adverse consequences for early immune development of the fetus.

Previous studies provide a persuasive basis for the hypothesis that vitamin D may protect against allergic disease. Reduced maternal dietary vitamin D intake in pregnancy has been reported as a risk factor for respiratory conditions such as wheezing, asthma, and allergic rhinitis; however, these studies were largely based on questionnaire-derived data rather than biological measures. Other studies have examined “season of birth” as a surrogate marker of vitamin D status through sunlight exposure and found significantly higher rates of food allergy in children born in autumn and winter (compared with spring and summer), providing indirect evidence that seasonal variations in sunlight exposure may make an important contribution to early disease risk. In older children, there is also evidence of lower serum 25-hydroxyvitamin D levels associated with allergen sensitization, eczema severity, and asthma.

Here, we have examined cord blood (CB) serum 25(OH)D levels as an indicator of fetal exposure to vitamin D in relation to infant eczema outcomes. Our prospective cohort study with well-defined allergic outcomes, documented maternal dietary and supplement intakes in pregnancy, and CB serum samples provided an ideal opportunity to examine this question in addition to other early allergic outcomes.

METHODS

Study Design and Subjects

The mother-infant pairs included in this study were derived from a larger (n = 669) prospective birth cohort recruited between 2002 and 2009 for the investigation of dietary exposures in relation to infant allergy outcomes. Only nonsmoking mothers with healthy, uncomplicated term pregnancies were recruited. The population used for this study was selected on the basis of (1) =2 frozen CB serum samples in storage (317/669), (2) allergic outcomes assessed at 12 months of age (259/317), and (3) at least 1 parent with a history of allergic disease (eczema, asthma, or hay fever) (231/259). The latter criterion was included because infants with a family history of atopy have a greater risk of developing allergic disease (50%–80%) than those with no family history (20%), providing greater statistical power, and results from this population are the most relevant and transferable to allergy prevention strategies. The cohort was recruited in Perth, Western Australia, as approved by the Princess Margaret Hospital Ethics Committee. Mothers provided written, informed consent.

Assessment of Antenatal Vitamin D Intake

In the last trimester of pregnancy, mothers completed a validated semi-quantitative food frequency questionnaire developed and analyzed by the Commonwealth Scientific and Industrial Research Organization in Adelaide, Australia. This recorded the frequency of consumption of 212 individual foods, mixed foods, and beverages from which vitamin D intake in IUs could be calculated. Energy intake cutoffs were applied to identify unreliable records. In addition to food and drink items, details of all dietary supplements taken in the last trimester of pregnancy (including doses, brands, and frequency of consumption) were collected. Reported supplement intake was converted into daily vitamin D intake (IU) by using composition information provided by the manufacturers.

Collection and Analysis of Umbilical CB Samples for 25(OH)D Concentration

At delivery, blood was collected from the umbilical cord or placental vein, and an aliquot (7–9 mL) transferred to a serum clot activator tube, which was kept out of light and processed within 8 hours. Aliquots of serum were stored at −80°C and transported in dry ice for analysis by RMIT Drug Discovery Technologies (Melbourne, Australia) by using liquid chromatography-tandem mass spectrometry (Applied Biosystems 4000 Q Trap and Agilent LC-MS/MS). The lower limit of quantification was 4.69 ng/mL for 25(OH)D, and the intra-assay precision had a coefficient of variation of <5%.

For categorical analysis by CB 25(OH)D concentrations we used cutoffs of <50 nmol/L, 50 to 74.99 nmol/L, and ≥75 nmol/L, as described in the literature.

Although similar cutoffs have been used by others in relation to CB concentrations, there is evidence to suggest that neonatal 25(OH)D concentrations are generally lower than CB concentrations. Therefore we used these values as categorical descriptors of vitamin D status rather than diagnostic criteria.
Assessment of Allergic Status

The primary outcome measures in the infants at 12 months of age were eczema and allergen sensitization. Infants were defined as having eczema if they had a doctor’s diagnosis of eczema, or evidence of typical skin lesions. The extent and severity of the eczema was determined by the standardized SCORing Atopic Dermatitis (SCORAD) severity index, measured on the day and as mother-reported worst ever episode. An objective SCORAD of <15 was classified as mild, 15 to 40 as moderate, and >40 as severe. Allergen sensitization was assessed by use of the skin prick test by using common allergen extracts (whole egg, cow’s milk, peanut, house dust mite, Dermatophagoides pteronyssinus, cat, rye grass pollen, mold mix; Hollister-Stier Laboratories, Spokane, WA). A wheal diameter of ≥3 mm was considered positive. The secondary outcome was immunoglobulin E (IgE)-mediated food allergy, which was defined as a history of immediate symptoms (typically within 60 minutes) after contact with and/or ingestion of food and a positive skin prick test to the implicated food. Information on respiratory symptoms (recurrent wheeze) and physician-diagnosed asthma were also collected, but these were not analyzed because of the limitations in diagnosis at this age.

Statistical Analysis

In this high-risk population, we estimated that >40% of infants would have vitamin D deficiency and ~40% infants at age 1 who would have either food allergy, eczema, or atopy (defined by skin prick test). Based on these assumptions, a sample size of 230 was estimated to detect an odds ratio (OR) of 2.5 with a power >0.8 in the vitamin D deficiency group, at a significance of 0.05. The distribution of CB 25(OH)D₃ concentration represented approximate normality after adjusting an outlying value. Because all results between the natural and adjusted data set were unaffected, parametric tests were performed by using adjusted data. Objective SCORAD data and maternal vitamin D intake from diet or supplements were not normally distributed. Means were compared by using the Mann-Whitney U test. Differences in CB 25(OH)D₃ concentration by month of birth were analyzed by 1-way analysis of variance and Bonferroni post hoc test. Logistic regression was used to estimate the risk of allergic outcomes by CB 25(OH)D₃ status while adjusting for confounders. We included recognized confounders of vitamin D status and allergic disease in our analyses, specifically season of birth, pets in the home, infant gender, maternal age, maternal education, and ethnicity. All statistics were performed by using SPSS software (version 19 for IBM, SPSS Inc, Chicago, IL).

RESULTS

Population Characteristics

The characteristics of the study population (n = 231) are shown in Table 1. The maternal population was predominately of white ethnicity and tertiary educated. A history of maternal allergic disease was reported for 86.1% of the infants, and 51.9% of the infants had both parents with a history of allergic disease.

CB 25(OH)D₃ Concentrations

The mean (SD) CB 25(OH)D₃ concentration was 58.4 (24.1) nmol/L, with a range of 9.18 to 246.34 nmol/L. The distribution of CB 25(OH)D₃ concentrations are displayed in Fig 1.

Season of Birth Effect

Concentrations of CB 25(OH)D₃ varied significantly by month of birth, with the Australian summer/autumn months of January, February, and March representing significantly higher levels than the winter/spring months of August, September, and October (Fig 2). Summer births showed a significantly greater percentage of CB 25(OH)D₃ concentrations ≥75 nmol/L in comparison with spring births (43.9% and 12.1%, respectively) in addition to a smaller proportion with concentrations <50 nmol/L (12.2% and 65.5%, respectively, P < .001) (Fig 3).

Maternal Characteristics

Although the seasons of birth were not significantly different for Asian and white participants, CB 25(OH)D₃ concentrations were significantly higher for those of white (59.39 [23.77] nmol/L, n = 184) in comparison with Asian ethnicity (37.01 [18.36] nmol/L, n = 9) (P = .008).

There was no relationship between CB 25(OH)D₃ concentrations and maternal age controlling for season of birth, nor was maternal education a significant predictor of vitamin D status.

Maternal Vitamin D Intake From Diet and Supplements in Pregnancy

The reported intakes of vitamin D from background dietary sources suggest that 85.1% of women in the main cohort had dietary vitamin D intakes that were less than the recommended dietary intake during pregnancy of 200 IU/day. Antenatal supplement use was reported by 212/231 (91.8%) mothers, but information on brand, type, and frequency was only provided by 146/231 (63.2%). Vitamin D intake from diet and supplements is presented in Table 1.

The Relationship Between Maternal Intake and CB 25(OH)D₃ Concentrations

Maternal intake of vitamin D from supplements was significantly correlated with CB vitamin D status (r = 0.244, P = .003), whereas the relationship between
CB levels and background dietary intake was not significant ($P = -0.105, P = .173$).

**Association Between CB 25(OH)D$_3$ and Allergic Outcomes in Infants**

**Eczema**

Consistent with other similar populations at high risk of allergic disease, eczema was the most common expression of the allergic phenotype, affecting 34% of the infants in this study$^{22}$ (Table 2). CB 25(OH)D$_3$ concentrations were significantly ($P = .018$) lower in infants who had developed eczema by 12 months of age (Fig 4). The risk of eczema declined significantly as CB 25(OH)D$_3$ increased, with a 10 nmol/L rise in CB 25(OH)D$_3$ reducing risk by 13.3% (OR 0.87, 95% confidence interval [CI] 0.77–0.98; $P = .020$). The association remained significant after adjustment for multiple confounding factors (Table 3). We found a significant negative dose-response trend across categories of vitamin D status (OR 0.63, 95% CI 0.44–0.90, $P = .013$) and an OR 2.66 for infants with CB 25(OH)D$_3$ $<50$ nmol/L compared with the reference group of $\geq$75 nmol/L (95% CI 1.24–5.72; $P = .012$) (Fig 5). Mean CB 25(OH)D$_3$ concentration was not significantly different between IgE- and non–IgE-associated eczema.

Objective SCORAD measures were conducted on 65/78 infants with eczema (severity categories described in Table 2). The median (interquartile range) SCORAD score on the day of assessment and worst ever were 7.8 (14) and 18.1 (13), respectively. We found no correlation between CB 25(OH)D$_3$ concentration and SCORAD on the day of examination ($r = 0.018$, $P = .9$) or worst ever ($r = 0.092$, $P = .467$). There was no difference in SCORAD score between IgE- or non–IgE-associated eczema.

**Allergen Sensitization**

Skin prick tests were conducted on 217/231 infants. Of these 21.2% were found to be sensitized to at least 1 allergen (see Table 2). CB 25(OH)D$_3$ concentration was not significantly associated with an increased risk for allergen sensitization (OR 1.0, 95% CI 0.9–1.01; $P = .642$).

**IgE-Mediated Food Allergy**

IgE-mediated food was present in 24/231 infants. Egg was the most common allergy affecting 6.5% of infants in this study, followed by milk and peanut allergy affecting 1.7% and 1.3%, respectively. Four of the 231 infants (1.7%) displayed allergy to $\geq$1 food. The risk of developing IgE-mediated food allergy was not related to CB 25(OH)D$_3$ (OR 1.00, 95% CI 0.99–1.02; $P = .584$).

**Recurrent Wheeze**

There was no significant difference in mean CB 25(OH)D$_3$ concentration between infants with or without recurrent wheeze (56.94 [24.44] nmol/L and 58.6 [24.1] nmol/L, respectively).

---

**TABLE 1** Characteristics of Selected Population Compared With Whole Cohort

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Study Population, $n$ (%)</th>
<th>Total Cohort, $n$ (%)</th>
<th>$P$ Value for Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>184 (79.7)</td>
<td>321 (73.5)</td>
<td>.674</td>
</tr>
<tr>
<td>Asian</td>
<td>9 (3.9)</td>
<td>16 (3.7)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>6 (2.6)</td>
<td>16 (3.7)</td>
<td></td>
</tr>
<tr>
<td>Not reported</td>
<td>32 (13.9)</td>
<td>84 (19.2)</td>
<td></td>
</tr>
<tr>
<td>Age, y ± SD</td>
<td>33.4 (4.5)</td>
<td>32.6 (4.6)</td>
<td>.042</td>
</tr>
<tr>
<td>Bachelor degree or higher</td>
<td>88 (59.0)</td>
<td>154 (55.4)</td>
<td>.468</td>
</tr>
<tr>
<td>Average daily supplemental</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vitamin D intake, IU ± SD</td>
<td>200 (248)</td>
<td>176 (228)</td>
<td>.315</td>
</tr>
<tr>
<td>Average daily dietary vitamin D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intake, IU ± SD</td>
<td>125 (88)</td>
<td>119 (88)</td>
<td>.500</td>
</tr>
<tr>
<td>Pets in the home</td>
<td>130 (57.3)</td>
<td>243 (61.4)</td>
<td>.316</td>
</tr>
<tr>
<td><strong>Neonatal</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender, female</td>
<td>112 (48.5)</td>
<td>201 (53.2)</td>
<td>.261</td>
</tr>
<tr>
<td>Birth weight, g ± SD</td>
<td>3455.8 (420.3)</td>
<td>3468.4 (458.9)</td>
<td>.986</td>
</tr>
<tr>
<td>Gestational age, wk ± SD</td>
<td>38.1 (1.1)</td>
<td>39.3 (1.2)</td>
<td>.190</td>
</tr>
<tr>
<td>Gravidity, median (IQR)</td>
<td>2.3 (1.5)</td>
<td>2.3 (1.3)</td>
<td>.442</td>
</tr>
</tbody>
</table>

IQR, interquartile range.

---

**FIGURE 1**

Distribution of CB 25(OH)D$_3$ concentrations ($n = 231$). Shading represents the 3 status categories referred to in our analysis: light bars $<50$ nmol/L ($n = 88$), medium bars 50–74.9 nmol/L ($n = 87$), and dark bars $\geq$75 nmol/L ($n = 56$).
nor was risk of this outcome related to CB 25(OH)D3 concentration (OR 1.00, 95% CI 0.98–1.01; P = .731).

**Association Between Maternal Vitamin D Intake and Allergic Outcomes in Infants**

Maternal vitamin D intake from supplements was not different for infants with or without eczema (P = .571), allergen sensitization (P = .563), or IgE-mediated food allergy (P = .341). Supplemental intake (analyzed in increments of 50 IU) displayed no association with the risk of eczema (OR 1.02, 95% CI 0.95–1.11; P = .517), allergen sensitization (OR 0.98, 95% CI 0.90–1.07; P = .698), and IgE-mediated food allergy (OR 1.08, 95% CI 0.97–1.19; P = .169).

**DISCUSSION**

There is an increasing body of evidence linking vitamin D status and immune function, raising important questions about the relationship between fetal vitamin D status and the rising predisposition for allergic disease in young infants. This is the first study to report that reduced CB 25(OH)D3 levels, as an indicator of vitamin D status in utero, are associated with an increased risk of eczema in the first 12 months of life. Interestingly, although 25(OH)D3 concentrations were significantly lower in infants with eczema, there was no association between vitamin D status and allergen sensitization or presence of IgE-mediated food allergy in this cohort. In addition, we found that only 24.2% of participants had adequate vitamin D concentrations (≥75 nmol/L) despite the sunny and temperate climate experienced in Perth, Australia (although we recognize that CB 25(OH)D3 concentrations are generally lower than neonatal concentrations\(^\text{17}\)). We did find marked seasonal variation in CB vitamin D status; summer births displayed significantly greater concentrations.

Consistent with our findings, Miyake et al\(^\text{23}\) found an association between lower maternal vitamin D consumption in pregnancy and increased risk of eczema in infants. These observations are also in keeping with a series of pregnancy studies that found that lower vitamin D intakes were associated with increased risk of other potential (respiratory-based) indicators of an allergic phenotype including recurrent wheeze,\(^\text{5,24,25}\) subsequent asthma, and allergic rhinitis.\(^\text{6}\) In addition, several studies using indirect measures of nondietary vitamin D such as season of birth (surrogate for sunlight exposure) found that birth in
winter months was associated with higher rates of subsequent eczema and food allergy. Although these studies support a protective role for improving status, they cannot exclude confounding effects of other seasonal factors such as variations in humidity and viral infections. The confirmed seasonal variations in vitamin D levels observed in our study provide support for the hypothesis that vitamin D is independently associated with eczema. Contrary to our results, Gale et al. found that maternal serum 25(OH)D₃ concentrations >75 nmol/L were associated with an increased risk of visible eczema on examination at 9 months of age. The method of diagnosis may be key here, because the risk of eczema was not significant when assessed by using the modified UK Working Party’s diagnostic criteria for atopic dermatitis.

There are relatively few other studies that use CB 25(OH)D₃ to assess neonatal vitamin D in relation to allergic and immune outcomes. A recent longitudinal study conducted in New Zealand reported a protective association between CB 25(OH)D₃ and the risk of wheezing and respiratory infection at 15 months of age, but consistent with our findings, no effect on sensitization. Also supporting our findings that CB 25(OH)D₃ was not associated with IgE-mediated outcomes is a study by Liu et al. that reports no overall association between vitamin D status and food-specific IgE levels (however, the authors did find that risk was increased for particular genotypes). The protective effects of vitamin D on eczema in our study, together with protective effects on wheezing in other studies, appear to be independent of IgE-related features, raising questions about the potential mechanisms.

The role of vitamin D in both skin barrier function and local antimicrobial defense could contribute to protective effects at mucosal and cutaneous surfaces. In the skin, the CYP27B1 enzyme (possessed by keratinocytes and monocytes) is required to hydroxylate 25(OH)D₃ to the active 1,25(OH)₂D₃. This active form facilitates the production of the antimicrobial peptide cathelicidin. Notably, in subjects with atopic dermatitis, 25(OH)D₃ levels are positively correlated with serum cathelicidin and its production in both keratinocytes and neutrophils. It is possible that insufficient vitamin D levels contribute to the impaired barrier function characteristic in eczema, because diminished CYP27B1 and reduced production of 1,25(OH)₂D₃ result in hyperproliferation of the basal layers and disrupted barrier integrity, coupled with impaired antimicrobial activity. Cathelicidin levels in lesional skin of established eczema increase significantly in response to oral 25(OH)D₃ supplementation, supporting a role of vitamin D in promoting antimicrobial functions and barrier integrity. As in the study by Kanda et al., we did not find that serum vitamin D levels predicted the severity of disease (SCORAD).

A limitation of this study is that the high-risk nature of the population may not be reflective of other populations. In the absence of serial blood collections throughout pregnancy and infancy, it is

### TABLE 2 Allergy Characteristics of the 231 Infants at 1 Year of Age

<table>
<thead>
<tr>
<th>Infant Characteristics</th>
<th>n (%)</th>
<th>Unless Otherwise Stated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any allergic disease</td>
<td>100 (43.3)</td>
<td></td>
</tr>
<tr>
<td>Eczema</td>
<td>78 (34.1)</td>
<td></td>
</tr>
<tr>
<td>Objective SCORAD at 12 mo</td>
<td>7.6 (14)*</td>
<td></td>
</tr>
<tr>
<td>Mild (&lt;15)</td>
<td>51 (78.5)</td>
<td></td>
</tr>
<tr>
<td>Moderate (15–40)</td>
<td>13 (20)</td>
<td></td>
</tr>
<tr>
<td>Severe (&gt;40)</td>
<td>1 (1.5)</td>
<td></td>
</tr>
<tr>
<td>Objective SCORAD at worst in preceding 12 mo</td>
<td>18.1 (13)*</td>
<td></td>
</tr>
<tr>
<td>Mild (&lt;15)</td>
<td>20 (30.8)</td>
<td></td>
</tr>
<tr>
<td>Moderate (15–40)</td>
<td>42 (64.6)</td>
<td></td>
</tr>
<tr>
<td>Severe (&gt;40)</td>
<td>3 (4.6)</td>
<td></td>
</tr>
<tr>
<td>IgE-mediated food allergy</td>
<td>24 (10.4)</td>
<td></td>
</tr>
<tr>
<td>Sensitized to ≥1 allergen (SPT+)</td>
<td>46 (21.2)</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>36 (16.7)</td>
<td></td>
</tr>
<tr>
<td>Peanut</td>
<td>15 (6.9)</td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td>4 (1.8)</td>
<td></td>
</tr>
<tr>
<td>HDM</td>
<td>7 (3.2)</td>
<td></td>
</tr>
<tr>
<td>Cat</td>
<td>2 (0.9)</td>
<td></td>
</tr>
<tr>
<td>Rye grass pollen</td>
<td>2 (0.9)</td>
<td></td>
</tr>
<tr>
<td>Mold mix</td>
<td>1 (0.5)</td>
<td></td>
</tr>
</tbody>
</table>

SPT, skin prick test.

* Median (interquartile range).

### FIGURE 4

Mean CB 25(OH)D₃ concentrations for infants that were positive or negative for allergic outcomes by 12 months of age.
also not possible to determine if variations in vitamin D levels at different stages of development differentially influence eczema risk. Likewise, although infant vitamin D supplementation and food fortification is not standard practice in Australia, we have not accounted for variations in infant vitamin D intake. Although we acknowledge that CB 25(OH)D₃ concentrations reflect recent vitamin D status, mainly in the last trimester, this biological measure remains a more accurate indicator of vitamin D status than dietary intake or other surrogate measures.

**CONCLUSIONS**

The findings of this study provide new evidence that reduced fetal exposure to vitamin D increases the risk of eczema in infants by 12 months of age. This adds to the growing body of evidence that vitamin D status is important for many aspects of health and that interventions to improve vitamin D status in pregnancy may be an important part of preventive strategies. This will be more definitively assessed through randomized controlled trials to assess the effects of maternal and/or infant vitamin D supplementation on immune development and clinical outcomes in childhood.

**ACKNOWLEDGMENTS**

Acknowledgments are extended to the Commonwealth Scientific and Industrial Research Organization Food and Nutritional Sciences for the food frequency questionnaire data entry and analysis, and to RMIT Drug Discovery Technologies laboratories for the CB 25(OH)D₃ analysis.

**REFERENCES**

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Appendix 2

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

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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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Clinical & Experimental Allergy

Summary

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 recognized, 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 (q = −0.57; P = 0.0002) and IL-5 (q = −0.29; P = 0.009) responses, with a similar trend for IL-5 (q = −0.59; P = 0.0001) 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.

Keywords 25(OH)D3, allergens, allergic disease, cord blood, cytokines, eczema, Toll-like receptor, Vitamin D

Submitted 16 July 2014; revised 27 October 2014; accepted 31 October 2014

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 Ref. 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, 1α,25-dihydroxyvitamin D (1,25(OH)2D), which can be synthesized 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 Ref. 16) and altered Th cytokine responses in vitro [17, 18]. However, understanding of the relationship between 25(OH)D status and allergic disease has been limited by the small number of studies with both immunological and clinical outcomes, or of a longitudinal design. This study aims to address these issues by investigating the association between 25(OH)D 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 (SPT)] were randomly assigned to receive 650 mg of fish oil (Ocean Nutrition, Canada Ltd, Dartmouth, Nova Scotia, Canada) 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)D analysis and matched to cell culture and clinical follow-up data. Participant numbers are presented in Fig. 1. Plasma for 25(OH)D 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 (CB) was collected from the umbilical artery or vein at delivery into a serum clot activator tube and processed within 8 h of collection. Peripheral blood from 6-month-old infants was collected by venipuncture 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 sulphoxide) at a concentration of no more than 1 × 10^6.

Assessment of 25(OH)D3

Plasma and serum samples were analysed using isotope-dilution liquid chromatography–tandem mass spectrometry (RDDT, Melbourne, Vic., Australia) 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 months 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 months of age 25(OH)D3 measures is indicated below.

Assessment of innate immune function

Peripheral blood mononuclear cells were cultured at 1 × 10^6 cells/mL either alone (control) or with optimized...
concentrations of Toll-like receptor (TLR) ligands for
(a) TLR3 (polyinosinic : polycytidylic acid, 50 µg/mL, paired with CB 25(OH)D3 n = 60, paired with 6 month 25(OH)D3 n = 73), (b) TLR2 (lipoteichoic acid, 20 µg/mL, paired with CB 25(OH)D3 n = 57, paired with 6 month 25(OH)D3 n = 68), (c) TLR9 (CpG oligonucleotide, 3 µg/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% foetal 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 µg/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, WA, 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 the 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 whether 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 SPT at 12 and 30 months of age using common food- and aero-allergen extracts (milk, peanut, HDM, cat, grass, mould; Hollister-Stier Laboratories, Spokane, WA, USA) 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 nonparametric 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, College Station, TX, USA). Figures were generated using BIOVENN [28] (Fig. 1), and PRISM v 6 (GraphPad Software Inc., San Diego, CA, USA) (Figs 2 through 5).

Results

Study Population

Cord blood 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 this 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 Fig. 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 categorized into quartiles of 25(OH)D3 based on season of collection.

Fig. 4. Scatter plots of cytokine production to Toll-like receptor (TLR) ligands, in relation to 25(OH)D3 levels at 6 months of age. Significance *P < 0.05.
(season-standardized quartiles) for both the birth and 6 months of age 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 ($r = -0.57$; $P = 0.0002$) and IL-5 ($r = -0.59$, $P = 0.0001$) responses at 6 months, with similar negative correlations between CB 25(OH)D3 levels and IL-5 ($r = -0.29$; $P = 0.009$) and IL-13 ($r = -0.21$; $P = 0.061$) Th2 responses to OVA (Fig. 3). Accordingly, when analysed according to category of 25(OH)D3 status, HDM IL-5 and IL-13 were significantly higher in infants with CB 25(OH)D3 $< 50$ nmol/L compared to those with levels $\geq 75$ 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 $\geq 75$ nmol/L ($P = 0.032$).

Infants who had higher 25(OH)D3 levels at birth also had lower production of the inflammatory Th1 cytokines INF-$\gamma$ ($r = -0.43$; $P = 0.008$) (Fig. 3) and TNF-$\alpha$ ($r = -0.41$; $P = 0.012$) 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 [INF-$\gamma$ $r = -0.111$, $P = 0.320$ (Fig. 3) and TNF-$\alpha$ $r = -0.130$, $P = 0.243$] There was also an inverse correlation for IL-10 to HDM ($r = -0.35$; $P = 0.034$), but not OVA ($r = -0.07$; $P = 0.531$).

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 $r = -0.080$, $P = 0.620$ and IL-13 $r = 0.059$, $P = 0.712$; OVA IL-5 $r = 0.022$, $P = 0.833$ and IL-13 $r = 0.188$, $P = 0.071$). 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-$\alpha$ ($r = 0.23$; $P = 0.027$) and IL-17A ($r = 0.24$; $P = 0.023$) to OVA. There were similar trends for TNF-$\alpha$ responses to HDM, but this was not statistically significant ($P = 0.067$).

There were no relationships between immune responses to milk allergen BLG 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 response to allergens.
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 with 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). TNFα and IL-12 in particular showed consistent positive correlation across ligands, and scatter plots of TNFα (TLR2, TLR3, TLR4 and TLR9), IL-12 (TLR2, TLR3, TLR9) and IL-10 (TLR3 and TLR9) are presented in Fig. 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 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-

### Table 2. Correlations between 25(OH)D3 and cytokine production to Toll-like receptor (TLR ligands)

<table>
<thead>
<tr>
<th>TLR2</th>
<th>Spearman’s rank correlation coefficient ((r))</th>
<th>CB 25(OH)D3</th>
<th>6 month 25(OH)D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>0.058 (0.668)</td>
<td>0.00 (1.00)</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>0.047 (0.727)</td>
<td>0.141 (0.251)</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>−0.144 (0.286)</td>
<td>0.203 (0.097)</td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td>0.005 (0.975)</td>
<td>0.288 (0.031)</td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>−0.173 (0.199)</td>
<td>0.285 (0.018)</td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>−0.108 (0.429)</td>
<td>0.145 (0.237)</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TLR3</th>
<th>Spearman’s rank correlation coefficient ((r))</th>
<th>CB 25(OH)D3</th>
<th>6 month 25(OH)D3</th>
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<tr>
<td>IL-1β</td>
<td>−0.041 (0.753)</td>
<td>0.109 (0.357)</td>
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</tr>
<tr>
<td>IL-6</td>
<td>0.056 (0.672)</td>
<td>0.234 (0.046)</td>
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</tr>
<tr>
<td>IL-10</td>
<td>−0.152 (0.245)</td>
<td>0.299 (0.010)</td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td>0.148 (0.316)</td>
<td>0.265 (0.045)</td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>−0.053 (0.686)</td>
<td>0.191 (0.105)</td>
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</tr>
<tr>
<td>IFNγ</td>
<td>−0.090 (0.496)</td>
<td>0.142 (0.230)</td>
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</tbody>
</table>

<table>
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<tr>
<th>TLR4</th>
<th>Spearman’s rank correlation coefficient ((r))</th>
<th>CB 25(OH)D3</th>
<th>6 month 25(OH)D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>−0.386 (0.126)</td>
<td>0.230 (0.344)</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>−0.133 (0.610)</td>
<td>−0.368 (0.121)</td>
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<tr>
<td>IL-10</td>
<td>−0.278 (0.279)</td>
<td>0.236 (0.331)</td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>−0.036 (0.892)</td>
<td>0.494 (0.032)</td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>−0.036 (0.892)</td>
<td>0.184 (0.450)</td>
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<table>
<thead>
<tr>
<th>TLR9</th>
<th>Spearman’s rank correlation coefficient ((r))</th>
<th>CB 25(OH)D3</th>
<th>6 month 25(OH)D3</th>
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<tbody>
<tr>
<td>IL-1β</td>
<td>0.265 (0.075)</td>
<td>0.007 (0.959)</td>
<td></td>
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<tr>
<td>IL-6</td>
<td>0.139 (0.356)</td>
<td>0.209 (0.123)</td>
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</tr>
<tr>
<td>IL-10</td>
<td>−0.036 (0.812)</td>
<td>0.204 (0.132)</td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td>0.047 (0.755)</td>
<td>0.212 (0.086)</td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>0.216 (0.150)</td>
<td>0.291 (0.029)</td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>0.021 (0.892)</td>
<td>0.056 (0.680)</td>
<td></td>
</tr>
</tbody>
</table>

CB, cord blood. 
P<0.05 considered significant.

| 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 |
|-----------------|------------------|-----------------|
| CB 25(OH)D3: cord blood |
| Wheeler – 6 months (\(n = 163\)) | 0.97 (0.74) | 0.78–1.19 |
| 2 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 |
| CB 25(OH)D3: peripheral blood at 6 months |
| Wheeler – 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 |

\(P<0.05\) considered significant.
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 months 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).

Fig. 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 *\( P < 0.05 \); **\( P \leq 0.01 \).
**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, IFNγ, 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) (Fig. 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 (Fig. 5). Infants with a history of reported wheeze by 6 months of age had higher IL-5 production to OVA (P = 0.027) and IFNγ responses to HDM (P = 0.050), although this relationship did not persist at later follow-ups (Fig. 5).

**Discussion**

We have previously described the 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 Ref. 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 TNFα, IL-6 and IL-1β production, and a parallel increase in Th1 responses [4]. In contrast, allergic children show attenuation of both their innate microbial responses and IFNγ Th1 adaptive responses [4]. In this study, 25(OH)D3 levels at 6 months were associated with enhanced innate responses, although this was not associated with any parallel effect on IFNγ 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 APC [34, 35]. It is also known that APC 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 recognized 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 utilizes 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 a 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.

Acknowledgements

We wish to acknowledge and sincerely thank the mothers and infants who took part in the study. We are particularly grateful to obstetricians and midwives at Osborne Park Hospital, St John of God Hospital Subiaco, Mercy Hospital and King Edward Memorial Hospital in Western Australia, as well as the Childhood Allergy and Immunology Research (CAIR) group. We also extend acknowledgments to RDDT Laboratories, Melbourne, Victoria, for 25(OH)D3 analysis.

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Conflict of interest

The authors declare no conflict of interest.
References


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Appendix 3

Antenatal Questionnaire
VITAL Study   CONFIDENTIAL   CRF File Number V1T___ ID _______  

VITAL Screening Number: _______  

Date of Screening Appointment: ___/___/____  

Section A: Antenatal Questionnaire  

A1 PERSONAL DETAILS  

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<td></td>
</tr>
<tr>
<td>LAST NAME</td>
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<td></td>
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<tr>
<td>DOB</td>
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<td><em><strong>/</strong></em>/_____</td>
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<td>dd</td>
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</tr>
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</tr>
<tr>
<td>DESCRIPTION OF USUAL OCCUPATION</td>
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<td></td>
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<tr>
<td>--------</td>
<td>--------</td>
<td></td>
</tr>
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<td><strong>EDUCATION</strong>&lt;br&gt;Completed secondary school?</td>
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<tr>
<td>□ Yes □ No □ Unknown</td>
<td>□ Yes □ No □ Unknown</td>
<td></td>
</tr>
<tr>
<td><strong>EDUCATION</strong>&lt;br&gt;Any further study? (Cross highest level attained only)</td>
<td><strong>EDUCATION</strong>&lt;br&gt;Any further study? (Cross highest level attained only)</td>
<td></td>
</tr>
<tr>
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<td>□ No further study □ Cert/Dip □ Degree □ Higher Degree □ Unknown</td>
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<tr>
<td><strong>ETHNICITY</strong></td>
<td><strong>ETHNICITY</strong></td>
<td></td>
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<td>□ White European</td>
<td>□ White European</td>
<td></td>
</tr>
<tr>
<td>□ Black African</td>
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<td>□ North African / Middle Eastern / Mediterranean</td>
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<td></td>
</tr>
<tr>
<td>□ Aboriginal / Torres Strait Islander</td>
<td>□ Aboriginal / Torres Strait Islander</td>
<td></td>
</tr>
<tr>
<td>□ East Asian (China, Japan, Korea)</td>
<td>□ East Asian (China, Japan, Korea)</td>
<td></td>
</tr>
<tr>
<td>□ South / South East Asian</td>
<td>□ South / South East Asian</td>
<td></td>
</tr>
<tr>
<td>□ Maori / Pacific Islander</td>
<td>□ Maori / Pacific Islander</td>
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</tr>
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<td>□ Other, specify:_______________</td>
<td>□ Other, specify:_______________</td>
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<th>A2 OTHER CONTACTS</th>
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<tr>
<td><strong>A 2.2 Family or friend contact details</strong></td>
</tr>
<tr>
<td>CONTACT #1</td>
</tr>
<tr>
<td>LAST NAME</td>
</tr>
<tr>
<td>RELATIONSHIP TO INFANT</td>
</tr>
<tr>
<td>ADDRESS</td>
</tr>
<tr>
<td>_______________________________</td>
</tr>
<tr>
<td>_______________________________</td>
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<tr>
<td>State _____ Post Code: __ __ __</td>
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<td>EMAIL ADDRESS</td>
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### TELEPHONE NUMBERS

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<td>Work: _______________________</td>
</tr>
<tr>
<td></td>
<td>Mobile: _____________________</td>
<td>Mobile: _____________________</td>
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### A 2.3 Mothers Physician details

**DOCTOR'S NAME**

**AREA OF CLINICAL PRACTICE**

- ☐ GP
- ☐ Obstetrician
- ☐ Other: ___________________________

**CLINIC NAME and ADDRESS**

________________________________________________________________________

State _____________    Post Code: __ __ __ __

**TELEPHONE NUMBER**

### A3 FAMILY ALLERGY HISTORY

**A 3.1**

<table>
<thead>
<tr>
<th></th>
<th>Hay Fever</th>
<th>Eczema/Dermatitis</th>
<th>Asthma</th>
<th>Food Allergies confirmed by skin prick test or RAST</th>
<th>Other/Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>You</strong></td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
</tr>
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<td>☐ No</td>
<td>☐ No</td>
<td>☐ No</td>
<td>☐ No</td>
</tr>
<tr>
<td><strong>The Father</strong></td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
</tr>
<tr>
<td></td>
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<td>☐ No</td>
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<td>☐ No</td>
</tr>
<tr>
<td><strong>Your Children</strong></td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
</tr>
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<td></td>
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<td>☐ No</td>
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</table>

### A4 PRESENT OBSTETRIC HISTORY

**A 4.1** How many pregnancies of gestation >20 weeks have you had in total, including this one?  

…………………

**A 4.2** Have you had any complications during this pregnancy? ☐ Yes ☐ No (go to A 4.3)

- ☐ Bleeding
- ☐ Gestational Diabetes
- ☐ Hypertension/ Pre-eclampsia

Other complications?

...........................................................................................................................................

............................................................................................................................................
**A 4.3** What is your estimated delivery date? __ __ / __ __ / __ __ __ __

Gestation at screening ........................................

Hospital .........................................................

Obstetrician .....................................................

**A 4.4** Have you been on any prescribed medication during this pregnancy?

□ Yes  □ No (go to A 4.5)

<table>
<thead>
<tr>
<th></th>
<th>During pregnancy?</th>
<th>Last trimester (≥ 27 weeks)</th>
<th>Details (eg. name, duration etc.)</th>
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</thead>
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<tr>
<td>Antibiotics</td>
<td>□ Yes □ No</td>
<td>□ Yes □ No</td>
<td></td>
</tr>
<tr>
<td>Asthma preventers</td>
<td>□ Yes □ No</td>
<td>□ Yes □ No</td>
<td></td>
</tr>
<tr>
<td>Asthma relievers</td>
<td>□ Yes □ No</td>
<td>□ Yes □ No</td>
<td></td>
</tr>
<tr>
<td>Antidepressants</td>
<td>□ Yes □ No</td>
<td>□ Yes □ No</td>
<td></td>
</tr>
<tr>
<td>Other prescription medication</td>
<td>□ Yes □ No</td>
<td>□ Yes □ No</td>
<td></td>
</tr>
</tbody>
</table>

**A 4.5** Have you had any over the counter medication during pregnancy?

□ Yes  □ No (go to A 4.6)

<table>
<thead>
<tr>
<th></th>
<th>During pregnancy?</th>
<th>Last trimester (≥ 27 weeks)</th>
<th>Details (eg. name, duration etc.)</th>
</tr>
</thead>
<tbody>
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<td>Hay fever medication</td>
<td>□ Yes □ No</td>
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</tr>
<tr>
<td>Asthma medication</td>
<td>□ Yes □ No</td>
<td>□ Yes □ No</td>
<td></td>
</tr>
<tr>
<td>Pain relief</td>
<td>□ Yes □ No</td>
<td>□ Yes □ No</td>
<td></td>
</tr>
<tr>
<td>Heartburn or Reflux</td>
<td>□ Yes □ No</td>
<td>□ Yes □ No</td>
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<tr>
<td>Other over counter medication</td>
<td>□ Yes □ No</td>
<td>□ Yes □ No</td>
<td></td>
</tr>
</tbody>
</table>

**A 4.6** Have you taken any dietary supplements or health food preparations from 27 weeks gestation?
(This includes multivitamins, fish oil, probiotics, individual vitamins or minerals and herbal supplements)

□ Yes  □ No (go to A 4.7)
**Supplement Coding**

<table>
<thead>
<tr>
<th>Supplement Code</th>
<th>Manufacturer and Product Name</th>
<th>No. of tablets/wk</th>
<th>Duration of tablets (wks) (27wks – today)</th>
</tr>
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<tbody>
<tr>
<td>MV – Multivitamin</td>
<td>FO – Fish Oil</td>
<td></td>
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</tr>
<tr>
<td>M – Mineral only</td>
<td>H – Herbal supplement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P – Probiotics</td>
<td>V – Vitamin only</td>
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<td></td>
</tr>
<tr>
<td>O – Other</td>
<td>U – Unknown</td>
<td></td>
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</table>

**A 4.7** Have you had any vaccinations during this pregnancy? □ Yes □ No (go to A 4.8)
Details:........................................................................................................................................

**A 4.8** Have you had any infections/conditions during this pregnancy? □ Yes □ No (go to A 5)

<table>
<thead>
<tr>
<th>Episode</th>
<th>Gestation</th>
<th>Type of Infection</th>
</tr>
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<tbody>
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<td>1</td>
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<td>2</td>
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<tr>
<td>5</td>
<td></td>
<td>□ respiratory □ ENT □ gastrointestinal □ urinary □ skin □ other ________</td>
</tr>
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</table>

**A 5 PREVIOUS MEDICAL HISTORY**

**A 5.1** Have you had any medical problems prior to this pregnancy? □ Yes □ No (go to A 6)
........................................................................................................................................
........................................................................................................................................
A6  LIFESTYLE AND ENVIRONMENT

6.1  Have you ever smoked cigarettes?  □ Yes  □ No (go to A 6.2)

6.1.1  Age started smoking?  ……………………………..  

6.1.2  Age quit smoking  ……………………………..  

6.1.3  Average amount cigarettes smoked per day?  ……………………………..  

A 6.2  Does your partner currently smoke?  □ Yes  □ No  

A 6.3  How would you describe your exposure to passive smoking during pregnancy?
□ Heavy  □ Moderate  □ Light  □ Not Exposed  

A 6.4  Where are you most frequently exposed to passive smoking?
□ Home  □ Work  □ Recreation  □ None  

A 6.5  Did you drink alcohol before becoming pregnant?  □ Yes  □ No (go to A 6.6)

6.5.1  What type of drink did you usually have?
□ Beer  □ Fortified Wines  

□ Wine/Champagne  □ Spirits/Liqueurs  

6.5.2  How many standard drinks per week?  ……………………………..  

A 6.6  How many standard drinks have you had per week during your pregnancy?………..  

A 6.7  Have you ever used any other recreational drugs?  □ Yes  □ No (go to A 6.8)

6.7.1  Have you used any recreational drugs during your pregnancy?
□ Yes  □ No (go to A 6.8)  

Details of recreational drug use during pregnancy:  
…………………………………………………………………………………………………………………  
…………………………………………………………………………………………………………………  

A 6.8  How many children in each age group live in your home?

0 – 4 yrs  ……………………………..  

4 – 13 yrs  ……………………………..  

13 – 18 yrs  ……………………………..  

VITAL study  Section A: Antenatal Questionnaire  Version 1, 01/08/12
A 6.9  Do you have any pets?  □ Yes  □ No (go to A 6.10)

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<thead>
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<th>How long (months and years)</th>
<th>Lives? (please circle)</th>
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<td>Rabbit / Guinea Pig</td>
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<td></td>
<td>Inside / outside / both</td>
</tr>
<tr>
<td>Bird</td>
<td>□ Yes  □ No</td>
<td></td>
<td>Inside / outside / both</td>
</tr>
</tbody>
</table>

A 6.10  Is there a free-standing gas heater without a chimney in your home?

□ Yes  □ No
A 7. FITZPATRICK SKIN TYPE

A 7.1 Genetic disposition

A 7.1.1 What is your eye colour?
- Light blue, light gray or light green
- Blue, grey or green
- Hazel or light brown
- Dark brown
- Brownish black

A 7.1.2 What is your natural hair colour?
- Red or light blonde
- Blonde
- Dark blonde or light brown
- Dark brown
- Black

A 7.1.3 What is your natural skin colour (before sun exposure)?
- Ivory white
- Fair or pale
- Fair to beige, with golden undertone
- Olive or light brown
- Dark brown or black

A 7.1.4 How many freckles do you have on unexposed areas of your skin?
- Many
- Several
- A few
- Very few
- None

A 7.2 Reaction to Extended Sun Exposure

A 7.2.1 How does your skin respond to the sun?
- Always burns, blisters and peels
- Often burns, blisters and peels
- Burns moderately
- Burns rarely, if at all
- Never burns

A 7.2.2 Does your skin tan?
- Never - I always burn
- Seldom
- Sometimes
- Often
- Always

A 7.2.3 How deeply do you tan?
- Not at all or very little
- Lightly
- Moderately
- Deeply
- My skin is naturally dark

A 7.2.4 How sensitive is your face to the sun?
- Very sensitive
- Sensitive
- Normal
- Resistant
- Very resistant/Never had a problem
## A8 SUN EXPOSURE HABITS

### A 8.1 General Questions

<table>
<thead>
<tr>
<th>Question</th>
<th>Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1.1 In the past 2 months, when outdoors did you use sunscreen?</td>
<td>☐ Always ☐ Sometimes ☐ Never (Go to A 8.1.3)</td>
</tr>
<tr>
<td>8.1.2 What factor (SPF) sunscreen (if known)?</td>
<td>☐ &lt; SPF 30 ☐ SPF 30 ☐ &gt; SPF 30 ☐ Unknown</td>
</tr>
<tr>
<td>8.1.3 Do you usually wear a hat?</td>
<td>☐ Yes ☐ No</td>
</tr>
</tbody>
</table>

### A 8.2. Sun Exposure Last Weekend

(Please place a cross in appropriate boxes)

1. When did you spend time in the sun last Saturday? (Please estimate for how long)
   - ☐ Before 11.00AM Estimate of time in sun _______ minutes
   - ☐ 11.00AM to 3.00PM Estimate of time in sun _______ minutes
   - ☐ After 3.00PM Estimate of time in sun _______ minutes

2. When did you spend time in the sun last Sunday? (Please estimate for how long)
   - ☐ Before 11.00AM Estimate of time in sun _______ minutes
   - ☐ 11.00AM to 3.00PM Estimate of time in sun _______ minutes
   - ☐ After 3.00PM Estimate of time in sun _______ minutes

3. Where did you spend most of your time over last weekend?
   - ☐ Indoors ☐ Outdoors in shade ☐ Outdoors in direct sunlight

4. Amount of skin exposed
   - ☐ Face and hands ☐ Face, hands and arms ☐ Face, hands, arms and legs

### A 8.3. Sun Exposure Last Week

(Based on a regular weekday)

(Please place a cross in appropriate boxes)

1. When did you spend time in the sun on a regular weekday? (Please estimate for how long)
   - ☐ Before 11.00AM Estimate of time in sun _______ minutes
   - ☐ 11.00AM to 3.00PM Estimate of time in sun _______ minutes
   - ☐ After 3.00PM Estimate of time in sun _______ minutes

2. Where did you spend most of your time last week?
   - ☐ Indoors ☐ Outdoors in shade ☐ Outdoors in direct sunlight

4. Amount of skin exposed
   - ☐ Face and hands ☐ Face, hands and arms ☐ Face, hands, arms and legs
A9 Blood samples

A 9.1 Blood Sample Collected?

□ No, specify reasons *(check one only)*:

□ Unsuccessful □ Refused □ Other specify: __________________________

□ Yes, volume collected __________________________

A 9.2 Cord blood kit given?

□ Yes, number __________________________

□ No, reason __________________________

Pre-pregnancy weight (kg) ....................... 
Pregnancy weight at visit (kg) ....................... 
Height (cm) ..................................
Appendix 4

Infant Questionnaire
SECTION B: INFANT APPOINTMENT (0-4 weeks of age)

B1 Infant information

Date: __ __ / __ __ / __ __

B1.1 Infant’s PMH UMRN __ __ __ __ __ __ __ __

B1.2 Infant’s Medicare Number __ __ __ __ __ __ __ __ __ __

B1.3 Baby’s Name ___________________________ ___________________________

First name(s) Last name

B1.4 Baby’s date of birth __ __ / __ __ / __ __ __ __

B1.5 Baby’s current age __ __ (days)

B1.6 Sex

☑ Male ☐ Female

B1.7 Ethnicity

☐ White European

☐ Black African

☐ North African / Middle Eastern / Mediterranean

☐ Aboriginal / Torres Strait Islander

☐ East Asian

☐ South / South East Asian

☐ Maori / Pacific Islander

☐ Other, specify: ___________________________

B2 Birth details

B2.1 Gestational age at birth __ __ weeks __ __ days

B2.2 Birth Anthropometry

B2.1.1 Weight __ __ __ __ grams

B2.1.2 Recumbent Length __ __ . __ cm

B2.1.3 Head circumference __ __ . __ cm

B2.3 Mode of delivery

☐ Natural vaginal

☐ Assisted vaginal (vacuum, forceps)

☐ Caesarean section
B 2.4 Baby’s Physician Details

<table>
<thead>
<tr>
<th>DOCTOR’S NAME</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AREA OF CLINICAL PRACTICE</td>
<td>☐ GP ☐ Paediatrician ☐ Other: ____________________________</td>
</tr>
<tr>
<td>CLINIC NAME</td>
<td></td>
</tr>
<tr>
<td>CLINIC ADDRESS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>State _____________ Post Code: __ __ __ __</td>
</tr>
<tr>
<td>TELEPHONE NUMBER</td>
<td></td>
</tr>
</tbody>
</table>

B 3 Feeding history

B 3.1 Breastfeeding

B3.1.1 Has your baby ever been fed any breast milk?

☐ No (Go to B3.2) ☐ Yes

B3.1.2 Is your baby currently breastfed?

☐ Yes, number of feeds per 24 hours __ __

☐ No, how old was your baby when you stopped breastfeeding? __ __ (days)

B 3.2 Formula Feeding

B 3.2.1 Has your baby ever been fed any infant formula?

☐ No (Go to B4) ☐ Yes

B 3.2.2 What was your baby’s age when they were first given any infant formula?

__ __ (days)

B 3.2.3 Which infant formula has your baby been fed most since birth?

__________________________________________________________

Full name of formula

B 3.2.4 Is your baby currently fed infant formula?

☐ Yes, number of feeds per 24 hours __ __

☐ No, how old was your baby when you stopped feeding infant formula? __ __ (days)
VITAL Study  CONFIDENTIAL  CRF File Number  V 1 T ___ ID ___

B 4 Study products

- Ddrops provided
- Detector clip provided, number:________

B 5 Education provided on the following:

- Use of study drops (applying single drop to teat/nipple/pacifier)
- Tick chart sheet given and explained
- Instruction on use of detector clip

Appointment completed by:

Name: _________________________________

Signature: ______________________________

Date: __ ___ / __ ___ / __ ___ ___
Appendix 5

Monthly Phone Call Questionnaire
SECTION C: 1 MONTH OF AGE PHONE CALL QUESTIONNAIRE

C 1 Date ___ / ___ / ___ ___ ___

C 2 Date on which drops were commenced? ___ / ___ / ___ ___ ___

C 3 How many days per week of supplementation have been missed since study commencement?

- None *(go to C4)*
- 1 day or less per week
- 2-3 days per week
- 4-5 days per week
- 6 or more days per week
- Stopped use completely on this date: ___ / ___ / ___ ___ ___

**And please specify reason**

- Does not want to give supplement any more
- Health professional advice
- Perceived adverse reaction, please specify_____________________

_________________________________________________________________

________

- Other, please specify__________________________

_________________________________________________________________

________

If the supplement use has been unnecessarily stopped please provide encouragement to recommence use.
C 4 How many days per week of wearing the detector clip have been missed since study commencement?

- None (go to C5)
- 1 day or less per week
- 2-3 days per week
- 4-5 days per week
- 6 or more days per week
- Stopped use completely on this date: __ __ / __ __ / __ __ __ __

And please specify reason

- Does not want to wear detector clip any more
- Other, please specify ________________________________

C 5 Feeding History

C 5.1 Breast feeding

C 5.1.1 Has your baby been fed any breast milk between birth and 1 month of age?  
- No (go to C5.2)  
- Yes

C 5.1.2 Is your baby currently breastfed?

- Yes, number of feeds per 24 hours __ __
- No, how old was your baby when you stopped breastfeeding? __ __ days

C 5.2 Formula feeding

C 5.2.1 Has your baby been fed any infant formula between birth and 1 month of age?

- No (go to C6)  
- Yes

C 5.2.2 What was your baby’s age when formula was first introduced? __ __ (days)

C 5.2.3 What brand of formula has your baby been fed most between birth and 1 month of age? ________________________________

Full name of formula
C 5.2.4 Is your baby currently fed infant formula?
□ Yes, number of feeds per 24 hours __ __
□ No, how old was your baby when you stopped feeding infant formula? ___ (days)

C 5.2.5 How much infant formula (on average) per 24 hours (day+night) is your baby currently having? ___ ___ ___ ml per 24 hours
□ Remind parent to cease drops if baby is consuming ≥ 1000ml formula /day

C 6 Is your baby currently consuming any other fluids? □ No (go to C7)

<table>
<thead>
<tr>
<th></th>
<th>≤ 1 times/week</th>
<th>2-3 times/week</th>
<th>4-5 times/week</th>
<th>≥ 6 times/week</th>
<th>Amount (ml/serve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow’s milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit juice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>_______________</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C 7 Has your baby taken any other vitamin, mineral, fish oil/fatty acid, probiotic or herbal supplement between birth and 1 month of age?
□ No □ Yes, what type and product name:______________
____________________________________________________________________________________

Frequency: □ 1 day or less per week
□ 2-3 days per week
□ 4-5 days per week
□ 6 or more days per week

Phone call completed by: ________________________________

Signature: ________________________________

Date: __ __ / __ __ / __ __ __ __
Appendix 6

3 Month of Age Questionnaire
SECTION E: INFANT 3 MONTH APPOINTMENT

E1 Date of appointment  __ __ / __ __ / __ __ __ __ (dd/mm/yyyy)

E 2 Current Anthropometry

E 2.1 Weight  __  __  __  __  kg
E 2.2 Length  __  __  __  __  cm
E 2.3 Head circumference  __  __  __  __  cm
E 2.4 Abdominal circumference  __  __  __  __  cm

E 3  How many days per week of supplementation have been missed in the past month?

- None (go to E 4)
- 1 day or less per week
- 2-3 days per week
- 4-5 days per week
- 6 or more days per week
- Stopped use completely on this date:  __ __ / __ __ / __ __ __ __

And please specify reason

- Does not want to give supplement any more
- Health professional advice
- Perceived adverse reaction, please specify________________________
  ____________________________________________________________________
- Other, please specify________________________
  ____________________________________________________________________

If the supplement use has been unnecessarily stopped please provide encouragement to recommence use.
E 4  How many days per week of wearing the detector clip have been missed in the past month?

- None *(go to E 5)*
- 1 day or less per week
- 2-3 days per week
- 4-5 days per week
- 6 or more days per week
- Stopped use completely on this date: __ __ / __ __ / __ __ __ __

And please specify reason

- Does not want to wear detector clip any more
- Other, please specify __________________________________________

E 5  Feeding History

E 5.1 Breastfeeding

E 5.1.1 Has your baby been fed any breast milk between 2 and 3 months of age?  □ No *(go to E 5.2)*  □ Yes

E 5.1.2 Is your baby currently breastfed?

- Yes, number of feeds per 24 hours ___ ___
- No, how old was your baby when you stopped breastfeeding?
  ___ ___ months & ___ ___ weeks

E 5.2 Formula feeding

E 5.2.1 Has your baby been fed any infant formula between 2 and 3 months of age?

- No *(go to E 6)*  □ Yes

E 5.2.2 What was your baby’s age when formula was first introduced?

- Before 2 months of age, or ___ months & ___ weeks

E 5.2.3 What brand of formula has your baby been fed most between 2 and 3 months of age?

Full name of formula

E 5.2.4 Is your baby currently fed infant formula?

- Yes, number of feeds per 24 hours ___ ___
- No, how old was your baby when you stopped feeding infant formula?
  ___ ___ months & ___ ___ weeks
E 5.2.5 How much infant formula (on average) per 24 hours (day+night) is your baby currently having? ___ ___ ___ ml per 24 hours

☐ Remind parent to cease drops if baby is consuming > 1000ml formula /24hr

E 6 Is your baby currently consuming any other fluids? ☐ No (go to E 7)

<table>
<thead>
<tr>
<th>Fluid</th>
<th>&lt; 1 times/week</th>
<th>2-3 times/week</th>
<th>4-5 times/week</th>
<th>&gt; 6 times/week</th>
<th>Amount (ml/serve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cow’s milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit juice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E 7 Introduction of Solid Food

E 7.1 Have you introduced solid foods to your baby?

☐ No (Go to E 8) ☐ Yes

E 7.2 How old was your baby when solid foods were first introduced?

___ ___ months & ___ ___ weeks

E 8 Has your baby taken any other vitamin, mineral, fish oil/fatty acid, probiotic or herbal supplement between 2 and 3 months of age?

☐ No ☐ Yes, what type and product name: ____________

Frequency:

☐ 1 day or less per week
☐ 2-3 days per week
☐ 4-5 days per week
☐ 6 or more days per week

E 9 Eczema / atopic dermatitis history

E 9.1 Has your baby shown signs of dry, red, itchy, scaly skin (eczema) between birth and 3 months of age?

☐ No (Go to E 10) ☐ Yes, complete SCORAD

E 9.2 At what age did the symptoms first occur?

___ ___ months & ___ ___ weeks
E 9.3 Has a medical doctor diagnosed your baby as having eczema/atopic dermatitis?

☐ No  ☐ Yes

E 9.4 Is your baby currently on any treatments for eczema/atopic dermatitis?

☐ No  ☐ Yes, please indicate all treatments currently used

☐ Dietary changes, please specify

☐ Oral medications, please specify

☐ Prescription steroid cream, please specify

☐ Over the counter steroid cream, please specify

☐ Moisturiser, please specify

☐ Other, please specify

E 10 General Health

E 10.1.1 Has your baby seen a doctor because he/she was unwell between birth and 3 months of age? (Do not include vaccinations or well baby checkups)

☐ No (Go to E 10.2)  ☐ Yes, specify number of times: __ __

E 10.1.2 Have any of these symptoms been reasons for the consultations (cross all that apply):

☐ Noisy breathing (wheeze or stridor)  ☐ Floppy unresponsive baby

☐ Vomiting  ☐ Swelling of face or body

☐ Loose watery stools  ☐ Hives

☐ Blood stained stools  ☐ Generalised skin rash

☐ Poor sleeping  ☐ Irritability
E 10.2 Has a doctor medically diagnosed your baby with a food allergy between birth and 3 months of age?

- No *(Go to E 10.3)*
- Yes *(please specify all foods)*

- Egg  confirmed by IgE RAST  confirmed by SPT
- Cow’s milk confirmed by IgE RAST confirmed by SPT
- Soy confirmed by IgE RAST confirmed by SPT
- Wheat confirmed by IgE RAST confirmed by SPT
- Other *(please specify)*

- __________  confirmed by IgE RAST  confirmed by SPT
- __________  confirmed by IgE RAST  confirmed by SPT

E 10.3 Has your baby taken any antibiotics between birth and 3 months of age?

- No
- Yes, how many courses? ____ *(please complete the table below)*

<table>
<thead>
<tr>
<th>Course 1</th>
<th>Reason / Type of Infection <em>(cross all that apply below)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>respiratory  ENT  GI  urinary  skin</td>
</tr>
<tr>
<td></td>
<td>other .........................................................</td>
</tr>
<tr>
<td>Course 2</td>
<td>respiratory  ENT  GI  urinary  skin</td>
</tr>
<tr>
<td></td>
<td>other .........................................................</td>
</tr>
<tr>
<td>Course 3</td>
<td>respiratory  ENT  GI  urinary  skin</td>
</tr>
<tr>
<td></td>
<td>other .........................................................</td>
</tr>
<tr>
<td>Course 4</td>
<td>respiratory  ENT  GI  urinary  skin</td>
</tr>
<tr>
<td></td>
<td>other .........................................................</td>
</tr>
</tbody>
</table>
E 10.4 Has your baby taken any over the counter medication between birth and 3 months of age?

- ☐ No
- ☐ Yes, indicate reason and provide details
  - Fever - number of occasions ___
    - Medication
  - Constipation - number of occasions ___
    - Medication
  - Stuffy nose - number of occasions ___
    - Medication
  - Cough/cold - number of occasions ___
    - Medication
  - Other - number of occasions ___
    - Medication

E 10.5 Has your baby had any vaccinations between birth and 3 months of age?

- ☐ No (Go to E 10.6)
- ☐ Yes, tick all that apply
  - Hepatitis B
  - Diphtheria, tetanus and whooping cough (DTPa)
  - *Haemophilus influenzae* type b
  - Polio (IPV)
  - Pneumococcal conjugate (13vPCV)
  - Rotavirus

E 10.6.1 Has your baby been admitted to hospital or attended a hospital emergency department between birth and 3 months of age?

- ☐ No (Go to E 11)
- ☐ Yes, how many times? ___

(please complete the following table)

For each admission to hospital or attendance at a hospital emergency department document the date, name of the hospital, length of stay and reason in the table below.

<table>
<thead>
<tr>
<th>Admission date</th>
<th>Hospital Name</th>
<th>Length of stay in days (any part of day = 1)</th>
<th>Primary diagnosis or reason for hospitalisation</th>
<th>Secondary diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>/</strong>/__</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>/</strong>/__</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
E 10.6.2 Has your baby had a hospital admission that required admission to intensive care between birth and 3 months of age?

☑ No ☐ Yes, Which hospital? ________________________________

Date of admission to the hospital ___ / ___ / _____

Reason for admission? ______________________________________

E 11 Sun exposure

E 11.1 What times of day do you normally take your baby outdoors?

☐ Before 11am  Estimate time in direct sunlight: ______ minutes

Estimate time in shade: ______ minutes

☐ 11am to 3 pm  Estimate time in direct sunlight: ______ minutes

Estimate time in shade: ______ minutes

☐ After 3pm  Estimate time in direct sunlight: ______ minutes

Estimate time in shade: ______ minutes

E 11.2 When outdoors, does your baby wear sun screen?

☐ Always ☐ Sometimes ☐ Never (go to E 11.4)

E 11.3 What factor sunscreen?

☐ < Factor 30    ☐ Factor 30    ☐ > Factor 30  ☐ Unknown

E 11.4 Amount of skin exposed

☐ Face and hands    ☐ Face, hands and arms

☐ Face, hands, arms and legs
E 12  Infant biological samples
   E 12.1 Blood collected?
      ☐ No, specify reasons (check one only):
         ☐ Unsuccessful  ☐ Refused  ☐ Other specify: _______________________
      ☐ Yes, time of collection: ___:___ (use 24 hr clock)  Volume: ___ . ___ ml

E 13 Trial products

      ☐ New detector clip provided, number______________
Detector clip collected  ☐ Yes  ☐ No, reason_____________________

      ☐ Stool sample pot given with instructions on use

      ☐ Diary card photocopied

Information provided on the following
      ☐ Advised caregiver that they will be contacted to cease supplement if blood tests are outside of normal range
E 14 Eczema Assessment (complete at the same time as anthropometrics)

**SCORAD INDEX**

**EUROPEAN TASK FORCE ON ATOPIC DERMATITIS**

---

**A:** EXTENT Please indicate the area involved

**B:** INTENSITY

**C:** SUBJECTIVE SYMPTOMS

PRURITUS + SLEEP LOSS

---

**CRITERIA**

<table>
<thead>
<tr>
<th>Erythema</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oedema/Populat</td>
<td></td>
</tr>
<tr>
<td>Oozing/Crust</td>
<td></td>
</tr>
<tr>
<td>Excoriation</td>
<td></td>
</tr>
<tr>
<td>Lichenification</td>
<td></td>
</tr>
<tr>
<td>Dryness*</td>
<td></td>
</tr>
</tbody>
</table>

---

**MEANS OF CALCULATION**

**PRURITUS**

(0 to 10) ____________________________

---

**SLEEP LOSS**

(0 to 10) 0 ____________________________

---

**Objective SCORAD**

A/5+7B/2 83

**SCORAD**

A/5+7B/2+C 103

---

*Dryness is evaluated on uninvolved areas

---

**Derived from SCORAD INDEX, Dermatology 1993: 186-23-31**

---

Appointment completed by:

Name: ________________________________

Signature: _____________________________

Date: __ __ / __ __ / __ __ __ __
Appendix 7

6 Month of Age Questionnaire
SECTION H: INFANT 6 MONTH APPOINTMENT

H1 Date of appointment  ____ / ____ / ____ ____ (dd/mm/yyyy)

H2 Current Anthropometry

H 2.1 Weight  __ __ . ____ kg
H 2.2 Length  ____ . ____ cm
H 2.3 Head circumference  ____ . ____ cm
H 2.4 Abdominal circumference  ____ . ____ cm

H 3 How many days per week of supplementation have been missed in the past month?

- None (go to H 4)
- 1 day or less per week
- 2-3 days per week
- 4-5 days per week
- 6 or more days per week
- Stopped use completely on this date:  ____ / ____ / ____ ____

And please specify reason

- Does not want to give supplement any more
- Health professional advice
- Perceived adverse reaction, please specify____________________
  ________________________________
  ________________________________
- Other, please specify____________________
  ________________________________
  ________________________________

If the supplement use has been unnecessarily stopped please provide encouragement to recommence use.
H 4  How many days per week of wearing the detector clip have been missed in the past month?

- None *(go to H 5)*
- 1 day or less per week
- 2-3 days per week
- 4-5 days per week
- 6 or more days per week
- Stopped use completely on this date: ___ / ___ / ___ ___ ___

And please specify reason

- Does not want to wear detector clip any more
- Other, please specify __________________________

H 5  Feeding History

H 5.1 Breastfeeding

H 5.1.1 Has your baby been fed any breast milk between 5 and 6 months of age?

- No *(go to H 5.2)*
- Yes

H 5.1.2 Is your baby currently breastfed?

- Yes, number of feeds per 24 hours ___ ___
- No, how old was your baby when you stopped breastfeeding?
  ___ ___ months & ___ ___ weeks

H 5.2 Formula feeding

H 5.2.1 Has your baby been fed any infant formula between 5 and 6 months of age?

- No *(go to H 6)*
- Yes

H 5.2.2 What was your baby’s age when formula was first introduced?

- Before 5 months of age, or ___ months & ___ weeks

H 5.2.3 What brand of formula has your baby been fed most between 5 and 6 months of age?

- Full name of formula

H 5.2.4 Is your baby currently fed infant formula?

- Yes, number of feeds per 24 hours ___ ___
- No, how old was your baby when you stopped feeding infant formula?
  ___ ___ months & ___ ___ weeks
H 5.2.5 How much infant formula (on average) per 24 hours (day+night) is your baby currently having? __ __ __ __ ml per 24 hours

H 6 Is your baby currently consuming any other fluids? □ No (go to H 7)

<table>
<thead>
<tr>
<th>Fluid</th>
<th>≤ 1 times/week</th>
<th>2-3 times/week</th>
<th>4-5 times/week</th>
<th>≥ 6 times/week</th>
<th>Amount (ml/serve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cow’s milk</td>
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<tr>
<td>Fruit juice</td>
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<tr>
<td>Other</td>
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</tr>
</tbody>
</table>

H 7 Introduction of Solid Food

H 7.1 Have you introduced solid foods to your baby?
   □ No (Go to H 8) □ Yes

H 7.2 How old was your baby when solid foods were first introduced?
   __ __ months & __ __ weeks

H 8 Has your baby taken any other vitamin, mineral, fish oil/fatty acid, probiotic or herbal supplement between 5 and 6 months of age?
   □ No □ Yes, what type and product name:________________

   Frequency: □ 1 day or less per week
               □ 2-3 days per week
               □ 4-5 days per week
               □ 6 or more days per week

H 9 Eczema / atopic dermatitis history

H 9.1 Has your baby shown signs of dry, red, itchy, scaly skin (eczema) between 3 and 6 months of age?
   □ No (Go to H 10) □ Yes, complete SCORAD

H 9.2 At what age did the symptoms first occur?
   __ __ months & __ __ weeks
**H 9.3** Has a medical doctor diagnosed your baby as having eczema/atopic dermatitis?

- [ ] No
- [ ] Yes

**H 9.4** Is your baby currently on any treatments for eczema/atopic dermatitis?

- [ ] No
- [ ] Yes, please indicate all treatments currently used
  - [ ] Dietary changes, *please specify*
  - [ ] Oral medications, *please specify*
  - [ ] Prescription steroid cream, *please specify*
  - [ ] Over the counter steroid cream, *please specify*
  - [ ] Moisturiser, *please specify*
  - [ ] Other, *please specify*

---

**H10 Wheezing**

**H 10.1** Has your baby ever had wheeze or whistling in the chest?

- [ ] No (*go to H 11*)
- [ ] Yes, at what age did the wheeze first occur? ___ ___ month

**H 10.2** Since birth, how many different episodes of wheezing has your baby had?

- [ ] 1-3
- [ ] 4-6
- [ ] 7-9
- [ ] 10-12

**H 10.3** With regard to your baby’s wheezing, please indicate all that apply

- [ ] Baby has been short of breath with this wheezing
- [ ] Baby’s sleep has been disturbed with this wheezing
- [ ] Baby’s feeding has been affected with this wheezing
- [ ] Baby has been treated with medication
- [ ] Medication use improved your baby’s symptoms
- [ ] Baby has had wheezing when he/she *did not* have a cold
H 11 General Health

H 11.1.1 Has your baby seen a doctor because he/she was unwell between 3 and 6 months of age? (Do not include vaccinations or well baby checkups)

☐ No (Go to H 11.2) ☐ Yes, specify number of times: __ __

H 11.1.2 Have any of these symptoms been reasons for the consultations (cross all that apply):

☐ Noisy breathing (wheeze or stridor) ☐ Floppy unresponsive baby
☐ Vomiting ☐ Swelling of face or body
☐ Loose watery stools ☐ Hives
☐ Blood stained stools ☐ Generalised skin rash
☐ Poor sleeping ☐ Irritability

H 11.2 Has a doctor medically diagnosed your baby with a food allergy between 3 and 6 months of age?

☐ No (Go to H 11.3) ☐ Yes (please specify all foods)
☐ Egg ☐ confirmed by IgE RAST ☐ confirmed by SPT
☐ Cow’s milk ☐ confirmed by IgE RAST ☐ confirmed by SPT
☐ Soy ☐ confirmed by IgE RAST ☐ confirmed by SPT
☐ Wheat ☐ confirmed by IgE RAST ☐ confirmed by SPT
☐ Other (please specify)

_____________ ☐ confirmed by IgE RAST ☐ confirmed by SPT
_____________ ☐ confirmed by IgE RAST ☐ confirmed by SPT
H 11.3 Has your baby taken any antibiotics between 3 and 6 months of age?
- No
- Yes, how many courses? ___ ___ *(please complete the table below)*

<table>
<thead>
<tr>
<th>Reason / Type of Infection (cross all that apply below)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Course 1</td>
</tr>
<tr>
<td>☐ respiratory ☐ ENT ☐ GI ☐ urinary ☐ skin</td>
</tr>
<tr>
<td>☐ other</td>
</tr>
<tr>
<td>Course 2</td>
</tr>
<tr>
<td>☐ respiratory ☐ ENT ☐ GI ☐ urinary ☐ skin</td>
</tr>
<tr>
<td>☐ other</td>
</tr>
<tr>
<td>Course 3</td>
</tr>
<tr>
<td>☐ respiratory ☐ ENT ☐ GI ☐ urinary ☐ skin</td>
</tr>
<tr>
<td>☐ other</td>
</tr>
<tr>
<td>Course 4</td>
</tr>
<tr>
<td>☐ respiratory ☐ ENT ☐ GI ☐ urinary ☐ skin</td>
</tr>
<tr>
<td>☐ other</td>
</tr>
</tbody>
</table>

H 11.4 Has your baby taken any over the counter medication between 3 and 6 months of age?
- No
- Yes, indicate reason and provide details
- Fever - number of occasions ___ ___
  - Medication__________________________________________
- Constipation - number of occasions ___ ___
  - Medication__________________________________________
- Stuffy nose - number of occasions ___ ___
  - Medication__________________________________________
- Cough/cold - number of occasions ___ ___
  - Medication__________________________________________
- Other - number of occasions ___ ___
  - Medication__________________________________________

H 11.5 Has you baby had any vaccinations between 3 and 6 months of age?
- No *(Go to H 11.6)*
- Yes, tick all that apply
- Hepatitis B
- Diphtheria, tetanus and whooping cough (DTPa)
- *Haemophilus influenzae* type b
- Polio (IPV)
- Pneumococcal conjugate (13vPCV)
- Rotavirus
H 11.6.1 Has your baby been admitted to hospital or attended a hospital emergency department between 3 and 6 months of age?

☐ No (Go to H 12)  ☐ Yes, how many times? ___ ___ (Please complete the following table)

For each admission to hospital or attendance at a hospital emergency department document the date, name of the hospital, length of stay and reason in the table below.

<table>
<thead>
<tr>
<th>Admission date</th>
<th>Hospital Name</th>
<th>Length of stay in days (any part of day = 1)</th>
<th>Primary diagnosis or reason for hospitalisation</th>
<th>Secondary diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>__ / __ / ___</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>__ / __ / ___</td>
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</tr>
</tbody>
</table>

H 11.6.2 Has your baby had a hospital admission that required admission to intensive care between 3 and 6 months of age?

☐ No  ☐ Yes, Which hospital? ________________________________

Date of admission to the hospital  __ ___ / __ ___ / __ ___ __

Reason for admission? ______________________________________

H 12 Sun exposure

H 12.1 What times of day do you normally take your baby outdoors?

☐ Before 11am  Estimate time in direct sunlight: _____ minutes
                Estimate time in shade: _____ minutes

☐ 11am to 3 pm  Estimate time in direct sunlight: _____ minutes
                Estimate time in shade: _____ minutes

☐ After 3pm  Estimate time in direct sunlight: _____ minutes
                Estimate time in shade: _____ minutes

H 12.2 When outdoors, does your baby wear sun screen?

☐ Always  ☐ Sometimes  ☐ Never (go to H 12.4)

H 12.3 What factor sunscreen?

☐ < Factor 30  ☐ Factor 30  ☐ > Factor 30  ☐ Unknown
H 12.4 Amount of skin exposed
   □ Face and hands   □ Face, hands and arms
   □ Face, hands, arms and legs

H 13 Infant biological samples
   H 13.1 Blood collected?
   □ No, specify reasons (check one only):
      □ Unsuccesful   □ Refused   □ Other specify: _____________________
   □ Yes, time of collection: ___:___ (use 24 hr clock) Volume: ___.__ ml

H 14 Trial products
   Detector clip collected   □ Yes □ No, reason________________
   Stool sample collected   □ Yes □ No, reason________________
   Diary card collected      □ Yes □ No, reason________________

Information provided on the following
   □ Advised caregiver that they will be contacted to cease supplement if blood
tests are outside of normal range
H 15  Eczema Assessment *(complete at the same time as anthropometrics)*

**SCORAD INDEX**

**EUROPEAN TASK FORCE ON ATOPIC DERMATITIS**

**A: EXTENT** Please indicate the area involved

**B: INTENSITY**

**C: SUBJECTIVE SYMPTOMS**

**PRURITUS + SLEEP LOSS**

**CRITERIA**

<table>
<thead>
<tr>
<th>Erythema</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oedema/Papulation</td>
<td></td>
</tr>
<tr>
<td>Oozing/crust</td>
<td></td>
</tr>
<tr>
<td>Excoration</td>
<td></td>
</tr>
<tr>
<td>Lichenification</td>
<td></td>
</tr>
<tr>
<td>Dryness*</td>
<td></td>
</tr>
</tbody>
</table>

**MEANS OF CALCULATION**

INTENSITY ITEMS

| (average representative area) |
| 0 = absence |
| 1 = mild |
| 2 = moderate |
| 3 = severe |

Visual analog scale (average for the last 3 days or nights)

**PRURITUS (0 to 10)**

**SLEEP LOSS (0 to 10)**

Derived from SCORAD INDEX, Dermatology 1993: 186:23-31

Appointment completed by:

Name: __________________________

Signature: __________________________

Date: _____ / _____ / ____

VITAL study  Section H: 6 month appointment  Version 1, 01/08/12