Vitamin D and atopy and asthma phenotypes in children: a longitudinal cohort study

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Running title: Vitamin D and asthma risk in children

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ABSTRACT

Background: Vitamin D has been linked in some studies with atopy- and asthma-associated phenotypes in children with established disease, but its role in disease inception at community level is less clear.

Objective: To investigate associations between vitamin D status and biological signatures indicative of allergy and asthma development in children aged 6 and 14 years in Perth, Western Australia (32°S).

Methods: Serum vitamin D was assayed in 989 6-year-olds and 1380 14-year-olds from an unselected community birth cohort; 689 were assessed at both ages. Vitamin D levels were assessed as a risk modifier for respiratory and allergic outcomes at both ages, utilising previously ascertained phenotypic data. The predictive value of vitamin D levels at age 6 for development of clinical phenotypes at age 14 was also examined.

Results: Serum vitamin D levels in children at both ages were negatively associated with concurrent allergic phenotypes; gender stratification revealed that this association was restricted mainly to boys. Further, vitamin D levels at age 6 were significant predictors of subsequent atopy/asthma-associated phenotypes at age 14.

Conclusion: In a non-selected community setting, children (particularly boys) with inadequate vitamin D are at increased risk of developing atopy, and subsequently bronchial hyperresponsiveness and asthma. Words 198
Key Messages:

• In a longitudinal community-based study in Perth, Western Australia (32°S), serum vitamin D levels in children at ages 14 and 6 years were inversely correlated with measures of atopy and bronchial hyperresponsiveness.
• After stratification these effects were seen to be restricted to males
• Vitamin D levels at age 6 were predictive of atopy, rhinoconjunctivitis and asthma at age 14.

Capsule Summary:

In a large unselected cohort, males with inadequate vitamin D at 14 and 6 years had increased atopy and bronchial hyperresponsiveness. Low vitamin D at age 6 was a predictor of atopy and asthma at 14. (36 words)

Key Words:

asthma, atopy, bronchial hyperresponsiveness, male bias, Raine Study, vitamin D.

Abbreviations:

BHR: bronchial hyperresponsiveness
HDM: house dust mite
OR: Odds ratio
INTRODUCTION

There is increasing awareness of the importance of vitamin D for maintenance of general immune and respiratory health[1,2]. Humans obtain greater than 80% of their vitamin D via exposure to the UVB components of sunlight, consistent with findings of significant seasonal variation in the circulating levels of 25-OH vitamin D₃ in individuals living in non-equatorial locations. The levels of vitamin D required may vary for different biological needs but >75 nmol/L (30 ng/mL) is currently considered optimal, whilst 50-75 nmol/L may be insufficient, and <50 nmol/L deficient[3-5]

A relationship between inadequate vitamin D and development and severity of allergy and asthma has been proposed[2-4,6,7]. In adults, reduced vitamin D levels associate with impaired lung function, increased airway hyperresponsiveness and reduced responsiveness to glucocorticoids[1,8]. Furthermore, vitamin D has been used as a therapy to treat glucocorticoid resistance in some asthmatic patients[9,10], and an association has recently been reported in asthmatic children between serum vitamin D levels and increased glucocorticoid use[5]. Data from rodent models suggest that vitamin D can control sensitisation to allergens by both regulation of dendritic cell development[11] and induction of IL-10-producing CD4+CD25+ T-regulatory cells[10,12] .

Vitamin D status may affect the development of allergies and asthma earlier in life[13]. In a large study of cord blood mononuclear cells from children of parents with allergic disease or asthma[14], the strongest correlate of responsiveness to innate and adaptive immune stimuli was season of birth, which may reflect vitamin D status. High maternal intake of vitamin D during pregnancy or by infants in the first year of life has been associated both negatively[15,16] and positively[17,18] with wheeze or asthma in their children.
Previous studies of vitamin D levels and expression of allergy and asthma phenotypes have concentrated on children already diagnosed with asthma. For example, low vitamin D was significantly associated with markers of allergy and asthma in asthmatic Costa Rican children between the ages of 6 and 14 years[19] but not asthmatic children from North American cities[20]. In a different study of 100 asthmatic children[6], vitamin D levels inversely correlated with IgE levels and sensitisation to aeroallergens. These reports highlight the need for further analysis of the links between vitamin D and allergy and asthma in different populations. Further, the possibility that vitamin D may modulate the development of wheezing-related phenotypes prior to asthma diagnosis must be considered. In this longitudinal community based study of children conducted in Perth, Western Australia (32°S), associations were sought between vitamin D status (serum 25-OH vitamin D levels) determined at 6 and 14 years of age, and biological signatures indicative of allergy and asthma development. This study was able to capture links between vitamin D levels and the progression of children into a phenotype characterised by atopy, bronchial hyperresponsiveness and asthma.
METHODS

Study subjects
Subjects were from the West Australian Pregnancy Cohort (Raine Study), which is a longitudinal birth cohort; mothers were not selected on any criteria other than having enrolled for antenatal care at the main local tertiary maternity hospital[21]. Analyses were based on clinical and immunological data collected at the age 6 and age 14 follow-ups. The study was approved by the Princess Margaret Hospital Ethics Committee with consent given by parents and subjects.

Vitamin D levels
Vitamin D which is stable in long term storage[22], was measured in thawed serum cryobanked at age 6 (989 subjects) and 14 years (1380 subjects). Serum vitamin D (25 (OH)) levels were measured by enzyme immunoassay kit from Immunodiagnostic Systems Ltd (Scottsdale, Arizona U.S.A.). Twelve subjects at each age also had 25 (OH) vitamin D₃ measured by isotope-dilution liquid chromatography-tandem mass spectrometry by RMIT Drug Discovery Technologies (Melbourne, Australia) according to published methodology[23]. As noted in Figure E1 (Online Repository), correlation between the methods at age 14 was strong (R²=0.933) whereas the immunoassay method appeared to overestimate the vitamin D levels at age 6, suggesting the presence in serum of vitamin D metabolites that cross-react with the immunoassay antibodies. Notwithstanding this, as shown below, relationships between estimated vitamin D levels and clinical phenotypes were comparable at both ages.
Clinical and immunological phenotyping at ages 14 and 6 years

**Current respiratory and allergic conditions**

Current asthma was defined as wheeze plus use of any asthma medication in the last 12 months, in children with a prior doctor diagnosis of asthma. Current rhinoconjunctivitis was assessed via parental response to a standardised questionnaire regarding the child's symptoms (runny, blocked or itchy nose in the presence of runny or itchy eyes) over the preceding 12 months.

Total and specific IgE were measured by ImmunoCap (Phadia AB, Sweden) as described previously[24] and in the Online Repository. Subjects were considered atopic if they had any measured specific IgE $\geq 0.35$ kU/L and/or total IgE $\geq 300$ kU/L for age 14 or $\geq 100$ kU/L for age 6.

**Respiratory assessment**

Lung function was assessed by spirometry and BHR was assessed by methacholine challenge, as detailed previously[24,25] and in the Online Repository.

**Measurement of cellular markers at age 14**

Haematological profiles were recorded from fresh samples on the day of blood collection, with the remainder cryopreserved as viable PBMC[24]. PBMC samples were thawed and cultured either with HDM allergen, LPS or poly(I:C); cytokine responses were subsequently assayed in culture supernatants by time-resolved fluorometry[24].
Statistical analyses

See Online Repository for additional detail. Prevalence of respiratory and allergic outcomes at age 14 was compared between subjects sufficient for vitamin D at age 14 (vitamin D >75 nmol/L) and remaining subjects using chi-square testing. Log_{10}-transformed actual vitamin D was used in univariate linear regression with continuous variables, and in univariate logistic regression to predict binary clinical outcomes. A sinusoidal model incorporating month of blood collection was fitted to the actual vitamin D concentration for each subject to calculate “de-seasonalised vitamin D”[26], which was log_{10}-transformed and entered into regression analyses as for actual vitamin D. Bivariate relationships between vitamin D and immunological variables were assessed using Spearman's correlation.
RESULTS

Vitamin D status at age 14 years

Levels of vitamin D were initially measured serum samples collected at 14 years. Blood collection from these subjects took place over three-years approximating their 14th birthdays, and Figure 1A shows the mean vitamin D levels per collection day over this period. Levels were lowest in mid-Winter (July) and in Spring, then rose to peak around mid-Summer. Overlaying plots of mean HDM-specific IgE and serum vitamin D levels per collection month (Figure 1B) revealed an apparent inverse correlation between these parameters (Spearman correlation: Rho= -0.092; p= 0.001). This and related observations were explored in more detail in the analyses below.

At age 14 only 59.3% of the 1380 subjects had what is currently considered sufficient levels of vitamin D (above 75 nmol/L); 4.4% could be considered vitamin D-deficient (below 50 nmol/l) while 36.3% of subjects had insufficient vitamin D (50 nmol/L to 75 nmol/L) (see Table E1). Vitamin D-deficiency was most common in subjects whose blood was collected in Winter (34/362, 9.4%) followed by Spring (22/399, 5.5%). Only 5/391 subjects with blood collected in Autumn were vitamin D-deficient, and none of the subjects bled in summer were deficient.

Preliminary chi-square analyses were performed to examine relationships between vitamin D status and the asthma- and atopy-associated phenotypes previously ascertained at age 14[24]. As illustrated in Table E2, subjects without sufficient vitamin D at 14 had significantly higher prevalence of BHR and atopy, in particular HDM-sensitisation which is the strongest marker of the atopic phenotype in this population[24]. There was also a trend for increased
prevalence of rhinoconjunctivitis. Several conditions were more prevalent in males than females including atopy (65.2% vs 54.4%, p<0.001), HDM-sensitisation (43.6% vs 34.4%, p<0.001) and poor lung function (10.0% vs 4.8%, p<0.001). Inverse relationships between vitamin D status and prevalence of clinical conditions were seen only amongst males; compared to males with sufficient vitamin D, those without sufficient vitamin D had increased frequency of BHR (19.6% vs 13.3%, p=0.031), atopy (72.2% vs 61.1%, p=0.003) and HDM-sensitisation (50.2% vs 39.8%, p=0.007). There were similar trends for asthma (13.5% vs 9.4%, p=0.094) and poor lung function (FEV1/FVC<80; 12.5% vs 8.5% p=0.087).

**Associations between current vitamin D level and respiratory and allergic phenotypes at age 14**

Our previous studies on this cohort at age 14 demonstrated strong associations between atopy and a range of asthma-related phenotypes[24], and accordingly relationships were examined between these outcomes and vitamin D levels. We performed initial multivariate regression analyses adjusting for the potential confounders sex and collection month; both these variables showed significant association with one or more outcomes (Table E3) and accordingly follow-up analyses included gender stratification and (where appropriate) deseasonalisation of vitamin D data. Univariate logistic regression analyses (Table 1) demonstrated that low serum vitamin D levels were associated with increased risk of BHR and atopic sensitisation, particularly in males; furthermore, this association extended to intensity of these clinical phenotypes, as demonstrated by linear regression analyses (Table 2). Vitamin D level at age 14 was not associated with risk for asthma (p=0.306), rhinoconjunctivitis (p=0.501), poor lung function (p=0.540) or exercise-induced wheeze (p=0.555) in univariate logistic regressions within the whole population, or in single-sex
analyses (not shown). There was likewise no association found between FEV\textsubscript{1}/FVC and vitamin D level at age 14 by linear regression (not shown).

Some of the clinical outcomes of interest in these 14-year-olds, such as asthma and rhinoconjunctivitis, were defined by symptoms over the 12 months prior to assessment, while other outcomes such as atopic status and BHR reflect the results of tests performed on the day of assessment. Given that serum vitamin D status is strongly related to date of sample collection, we repeated these analyses employing de-seasonalised vitamin D data. Comparable relationships (including the same sex stratification) were observed (Tables E4 and E5).

We examined more closely how vitamin D related to clinical conditions by looking at predicted probabilities of outcomes amongst subjects stratified by sex and vitamin D level. Males and females were separately divided into ascending quartiles based on serum vitamin D concentration and mean predicted probabilities for atopy were calculated by logistic regression. Figure 2 shows that the probability of atopy amongst males is highest in quartile 1 and decreases as vitamin D levels increase, whereas the risk for females remains similar across the quartiles.

**Associations between current vitamin D level and clinical phenotypes at 6**

In light of these findings we analysed serum samples collected earlier at the 6-year follow-up (see Table E1). We observed fluctuations in serum vitamin D with season of collection (not shown), similar to those observed at age 14. There was a significant correlation between vitamin D levels at the two ages using unadjusted vitamin D (Spearman’s rho=0.442 p≤0.0001) or de-seasonalised vitamin D (Spearman’s rho=0.454 p≤0.0001). Cross-sectional
analyses of the relationships between vitamin D levels and clinical phenotypes at age 6 were carried out as for age 14, with the exception that BHR data at age 6 was collected on only a subset of the cohort (n=354) and analysed only as a binary measure.

Univariate logistic regression (Table 3) and linear regression analyses (Table 4) indicated inverse associations between (unadjusted) serum vitamin D and expression of BHR and sensitisation which were significant at age 6, albeit weaker than those observed at age 14. Of note, the stronger effects observed in males at age 14 were also found for 6-year-old boys. Likewise, vitamin D level at age 6 was not associated in the whole population with risk for asthma (p=0.506), rhinoconjunctivitis (p=0.311), poor lung function (FEV$_1$/FVC<80; p=0.601) or exercise-induced wheeze (p=0.118). A marginal inverse relationship at age 6 between vitamin D and exercise-induced wheeze was seen amongst males (OR=0.143, p=0.057) but not females (p=0.775). No association was found between FEV$_1$/FVC and vitamin D by linear regression (not shown). Similar associations were again observed employing de-seasonalised vitamin D data (Tables E6 and E7).

**Vitamin D levels at age 6 as a predictor of subsequent clinical phenotypes at 14**

Of the 989 cohort members assessed at age 6, 693 were part of the 14 year follow-up, and we performed longitudinal analyses on this subgroup. We determined by chi-square analysis that there were not significant biases due to loss to follow-up of 307 6-year-old subjects. De-seasonalised vitamin D levels at age 6 were used in logistic regression to predict clinical outcomes at 14. As shown in Table 5, in the overall population low vitamin D levels at age 6 were associated with increased risk for atopy but not for BHR at age 14. In addition, predictive associations were evident between vitamin D at age 6 and subsequent asthma and to a lesser extent rhinoconjunctivitis. Moreover, gender stratification demonstrated that
effects relative to asthma and atopic sensitisation were confined essentially to males, whereas the observed risk association for rhinoconjunctivitis appeared stronger in females.

**Correlation between vitamin D and immunological variables at age 14**

Additional immunological biomarkers were measured at age 14 as previously[24], including levels of circulating granulocytes and PBMC cytokine responses to adaptive and innate immune stimuli. Table 6 shows the results of Spearman correlation analyses comparing vitamin D levels with cytokine responses and peripheral granulocytes at age 14. Vitamin D at age 14 showed a significant positive correlation with HDM-induced IL-10 and IFN$_\gamma$ measured from PBMC cultures, but not with Th2 cytokines. Vitamin D was also significantly positively correlated with levels of IL-10 and TNF produced in response to innate stimuli at age 14. In contrast, vitamin D showed an inverse correlation with numbers of circulating eosinophils, basophils and neutrophils.
**DISCUSSION**

The major strength of this study is its prospective nature, integrating data on vitamin D levels in children at two different ages in conjunction with concurrent clinical and immunological phenotypes. Further, as 693 of the children were studied at both time points, vitamin D levels in the sera of children at aged 6 were tested as a predictor of atopy and the developing clinical indicators of asthma at 14. This was a community cohort of children; there was no selection of highly atopic mothers[27] or children with pre-diagnosed asthma[5,19,20]. Without selection for asthmatic children in the study population, this study was more sensitive to any links between vitamin D levels and signs and symptoms of the developing allergy and asthma signatures. In previous studies, the authors have shown that BHR is a major risk factor for asthma, and high IgE levels to perennial allergens are a risk factor for BHR in the 14-year-olds[24].

Perth at 32°S has a typical Mediterranean climate. Despite the Australian outdoor lifestyle, only 41.4% of children at age 14 were vitamin D sufficient (>75 nmol/L) during the winter months of June-August. During winter 49.2% had insufficient vitamin D levels (50-75 nmol/L) and 9.4% were deficient (<50 nmol/L). The prevalence of vitamin D insufficiency in children is an international problem, with similar rates reported for US asthmatic children[5,21]. Even in equatorial Costa Rica, 24.6% and 3.4% of children were found to be vitamin D insufficient and deficient respectively[19]. The majority of subjects studied were classified into the same vitamin D status categories at ages 6 and 14 (see Table E8). While we have applied the cut-offs most commonly published for classification of vitamin D status this issue is controversial and it has been suggested that current cut-offs may greatly underestimate levels vitamin D required for optimal function of vitamin D-related processes[28, 29]. For this reason we used continuous measures in all regression analyses for
clinical outcomes.

At age 14, the strongest relationship between vitamin D and clinical phenotypes observed was for atopy, as exemplified by HDM sensitisation, followed by BHR, an important risk factor for asthma development[24]. The inverse correlations were mainly amongst males and it is noteworthy that the probability of atopy and HDM sensitisation was also greater in the boys (Figure 2)[24]. This contrasts with the significantly stronger immunomodulatory effects of vitamin D previously reported in adult females compared to males, including a stronger relationship between serum vitamin D and the activity of IL-10-secreting regulatory T-cells[30]. A functional synergy has been proposed between 1,25 (OH)2 vitamin D3 and 17-β estradiol, mediated through estrogen receptor α, to cause effects on vitamin D receptor expression, vitamin D3-inactivating enzyme (CYP24A1) and vitamin D3-binding protein[30,31], and this may make females more resilient against limiting 25(OH) vitamin D.

With further sexual maturation and ageing of subjects in this longitudinal study, it will be interesting to see in future follow-ups if this correlation in the males persists, and/or whether the links between vitamin D levels and asthma and allergy susceptibility in females increase over time. It is notable that the stronger inverse correlation between vitamin D levels and atopy and allergy phenotypes in the males was seen at both 6 and 14 years, when sex hormone concentrations would have varied enormously. The possibility that the atopy phenotypes were sensitive to, and controlled by, lower levels of vitamin D in the females was considered; however further supportive data are required. It is possible that our study lacked sufficient power to detect some associations between and clinical outcomes after stratifying the population by sex.
For children studied at 14, vitamin D levels were also compared with responses by PBMC exposed to innate stimuli and to HDM allergen. Analysis of the HDM responses suggested that although the production of the Th2 cytokines did not fluctuate with vitamin D levels, cytokines produced by human regulatory cells (notably the combination of IL-10 and IFNγ[32]) were significantly and positively correlated. Many laboratories have shown that dendritic cell function can be modulated by vitamin D in vitro, and more recently, this has been shown in vivo[13,33]. Both induction[10,34] and activation of regulatory T-cells[12] by 1,25 (OH)₂ vitamin D₃ have been reported and may explain the correlations observed, possibly indirectly via effects on dendritic cells. The profile of PBMC responses to the TLR4 and TLR3 ligands LPS and poly(I:C) respectively suggest that in subjects with increased vitamin D levels, there was reduced production of pro-inflammatory TNF which was balanced by increased production of the regulatory cytokine IL-10. This pattern accords with predicted homeostatic control by 1,25 (OH)₂ vitamin D₃ of the functions of myeloid cells (reviewed in[2], which include both dendritic cell and macrophage populations within PBMC. Mechanistically, the vitamin D receptor/1,25 (OH)₂ vitamin D₃ complex dose-dependently interferes with the signalling of transcription factors which may include the NFkB-driven maturation pathway in macrophages and dendritic cells[35]. These data thus suggest that vitamin D “sufficiency” supports myeloid cell differentiation along the less inflammatory M2 pathway, which is characterised by increased production of IL-10 and decreased production of TNF (reviewed in[36]). Ligand binding to the vitamin D receptor has also been shown to enhance responses to endogenous cortisol by enhancing IL-10 production by PBMC[5,10], and this mechanism may also contribute to the observed variations in TLR-induced cytokine production.
Vitamin D levels have not been associated with allergy markers in all previous studies of asthmatic children. In the CAMP study of North American asthmatic children [20] low vitamin D levels were associated retrospectively with increased odds of hospitalisation during the previous year, and prospectively with severe asthma exacerbations over the next 4 years but not HDM-IgE and eosinophil counts. In CAMP 13% of children were of African-American descent [21] whereas our study cohort comprised >95% Caucasians. In the previous studies of asthmatic children, the frequency of skin test reactivity to any allergen was approximately 80-90%. However, as ours was a community-based study, this percentage was lower (60% at age 14, 54% at age 6). Our study did not find a positive correlation between vitamin D levels and spirometry measures as reported [5]; this may reflect the low number of asthmatics studied and the nature of this community cohort.

A growing body of evidence suggests that vitamin D deficiency in utero can impact on development of the fetal lung [37] and immune system. We were unable to address this issue and it is possible that such developmental effects may have reduced the associations observed in this study between vitamin D levels and asthma development in childhood. Future studies with longitudinal birth cohorts are needed to determine how vitamin D status in early life and throughout childhood modifies risks for asthma, allergy and related conditions.

ACKNOWLEDGEMENTS

We acknowledge with thanks the skilled technical assistance of Jenny Tizard, and the study families and members of the Raine Study team who took part in the study.
REFERENCES


Table 1. Univariate logistic regression with vitamin D for respiratory and allergic conditions: age 14

<table>
<thead>
<tr>
<th>Outcomes at age 14</th>
<th>Whole population</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>n</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>BHR</td>
<td>1286</td>
<td>236</td>
<td>0.35 (0.13 - 0.95)</td>
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<tr>
<td>Atopy</td>
<td>1379</td>
<td>827</td>
<td>0.49 (0.23 - 1.06)</td>
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<tr>
<td>HDM sensitisation</td>
<td>1379</td>
<td>540</td>
<td>0.36 (0.16 - 0.77)</td>
</tr>
</tbody>
</table>

Actual vitamin D at age 14 (nmol/L) was log10-transformed and included in univariate logistic regression analyses for outcomes at age 14. N: total subjects in regression; n: subjects in regression with outcome. FEV1/FVC below 80 is the criterion used to define poor lung function at age 14.
Table 2. Linear regression between vitamin D and continuous respiratory and allergic measures at age 14

<table>
<thead>
<tr>
<th>Variable at age 14</th>
<th>Whole population</th>
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<tr>
<td></td>
<td>N</td>
<td>β</td>
<td>SE</td>
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<tr>
<td>BHR-dose-slope</td>
<td>1271</td>
<td>-0.069</td>
<td>0.009</td>
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<td>HDM-IgE</td>
<td>1379</td>
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<td>0.003</td>
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<td>Food mix-IgE</td>
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<td>0.005</td>
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<tr>
<td>Phadiatop-IgE</td>
<td>1379</td>
<td>-0.067</td>
<td>0.003</td>
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</table>

All variables except for BHR-dose-slope were log10-transformed; vitamin D was measured in nmol/L, IgE was measured in kU/L. N: subjects in analysis; β: standardised correlation coefficient; t: β divided by SE.
<table>
<thead>
<tr>
<th>Outcomes at age 6</th>
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<td>N</td>
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<td>OR (95% CI)</td>
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<td>BHR</td>
<td>354</td>
<td>184</td>
<td>0.17 (0.04 - 0.81)</td>
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<td>Atopy</td>
<td>989</td>
<td>525</td>
<td>0.79 (0.32 - 1.98)</td>
</tr>
<tr>
<td>HDM sensitisation</td>
<td>989</td>
<td>237</td>
<td>0.35 (0.12 - 1.01)</td>
</tr>
</tbody>
</table>

Actual vitamin D at age 6 (nmol/L) was log₁₀-transformed and included in univariate logistic regression analyses for outcomes at age 6. N: total subjects in regression; n: subjects in regression with outcome.
<table>
<thead>
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<td>N</td>
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<tr>
<td>HDM-IgE</td>
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<td>Food mix-IgE</td>
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<td>Phadiatop-IgE</td>
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<td>-0.068</td>
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All variables were log_{10}-transformed; vitamin D was measured in nmol/L, IgE was measured in kU/L. N: subjects in analysis; β: standardised correlation coefficient; t: β divided by SE.
<table>
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<th>Females</th>
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<tr>
<td></td>
<td>N</td>
<td>n</td>
<td>OR (95% CI)</td>
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<tr>
<td>Current asthma</td>
<td>667</td>
<td>63</td>
<td>0.11 (0.02 - 0.84)</td>
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<tr>
<td>Rhinoconjunctivitis</td>
<td>669</td>
<td>275</td>
<td>0.17 (0.05 - 0.59)</td>
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<tr>
<td>BHR</td>
<td>646</td>
<td>109</td>
<td>0.28 (0.06 - 1.37)</td>
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<tr>
<td>Atopy</td>
<td>693</td>
<td>417</td>
<td>0.14 (0.04 - 0.47)</td>
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<tr>
<td>HDM sensitisation</td>
<td>693</td>
<td>276</td>
<td>0.18 (0.05 - 0.60)</td>
</tr>
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</table>

De-seasonalised vitamin D at age 6 was calculated using a sinusoidal model, \( \log_{10} \)-transformed and included in logistic regression analyses for outcomes at age 14. N: total subjects in regression; n: subjects in regression with outcome.
Table 6. Spearman's correlation of vitamin D at age 14 with immunological variables collected at the same age.

<table>
<thead>
<tr>
<th>Variables</th>
<th>N</th>
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</thead>
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<tr>
<td><strong>Adaptive immunity</strong></td>
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<td></td>
</tr>
<tr>
<td><em>T-cell responses</em></td>
<td></td>
<td></td>
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<tr>
<td>HDM-induced IL-4</td>
<td>1342</td>
<td>-0.011</td>
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<td>HDM-induced IL-5</td>
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<td>HDM-induced IL-9</td>
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<td>1374</td>
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<td><strong>Innate immunity</strong></td>
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<tr>
<td><em>TLR responses</em></td>
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<tr>
<td>LPS-induced IL-10</td>
<td>1368</td>
<td>0.071</td>
<td>0.009</td>
</tr>
<tr>
<td>LPS-induced TNF</td>
<td>1368</td>
<td>-0.069</td>
<td>0.011</td>
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<tr>
<td>poly(I:C)-induced IL-10</td>
<td>1367</td>
<td>0.072</td>
<td>0.007</td>
</tr>
<tr>
<td>poly(I:C)-induced TNF</td>
<td>1367</td>
<td>-0.097</td>
<td>&lt;0.001</td>
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<td><strong>Baseline granulocytes</strong></td>
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<tr>
<td>Eosinophils</td>
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<td>-0.054</td>
<td>0.045</td>
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<tr>
<td>Neutrophils</td>
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<td>-0.084</td>
<td>0.002</td>
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<tr>
<td>Basophils</td>
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<td>-0.084</td>
<td>0.002</td>
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N=number subjects in analysis.
FIGURE LEGENDS

Figure 1. Seasonal variation of serum vitamin D levels. Vitamin D (nmol/L) was measured by enzyme immunoassay from serum, which was collected from 1380 14-year-olds over a three year period. A. Mean vitamin D levels measured for each day of blood collection are shown. B. Mean vitamin D levels for blood collection months combined over the three year period are shown; mean HDM-IgE titers from these subjects were similarly calculated and presented.

Figure 2. Probability of atopic sensitisation decreases as vitamin D increases in males only. Males and females were separately divided into ascending quartiles by vitamin D level at age 14. Predicted probabilities of atopy or HDM-sensitisation were calculated separately for male
and female subjects by univariate logistic regression with actual vitamin D levels, and mean predicted probabilities for each quartile are shown.