Vitamin D influences asthmatic pathology through its action on diverse immunological pathways

*Paul E Pfeffer, *Elizabeth H Mann, Eve Hornsby, Emma S Chambers, Yin-Huai Chen, Louise Rice, and Catherine M Hawrylowicz

* denotes equal contribution

Address: MRC and Asthma UK Centre for Allergic Mechanisms in Asthma, King's College London, London SE1 9RT UK

Corresponding author:

Catherine Hawrylowicz,

Department of Asthma, Allergy and Respiratory Science,

5th Floor Tower Wing,

Guy's Hospital, King's College London,

London SE1 9RT, UK.

Phone: +44 (0) 207 188 0598; Fax: +44 (0) 207 403 8640

catherine.hawrylowicz@kcl.ac.uk

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Abstract

The prevalence of vitamin D insufficiency and deficiency has increased markedly in recent decades to current epidemic levels[1]. In parallel there has been an increase in the incidence of a range of immune-mediated conditions ranging from cancer to autoimmune and respiratory diseases, including chronic obstructive pulmonary disease and asthma[2,3]. There is also an association with increased respiratory infections, which are the most common cause of asthma exacerbations[3]. Together, this has resulted in considerable interest in the therapeutic potential of vitamin D to prevent and improve treatment of asthma and other respiratory diseases. To this end, data from clinical trials involving supplementation with active vitamin D, or more commonly a precursor, are starting to emerge. This review considers mechanisms by which vitamin D may act on the immune system to dampen inappropriate inflammatory responses in the airway whilst also promoting tolerance and anti-microbial defence mechanisms that collectively maintain respiratory health.

Asthma

Heterogeneity in asthma phenotypes (referred to as endotypes) has been described and these endotypes demonstrate differential responsiveness to treatment, underpinned by distinct pathogenic mechanisms[4-7]. Asthma is classically a steroid-sensitive Th2-type immune pathology[8] and the symptoms of asthma are well-controlled by β2-adrenergic agonists and inhaled corticosteroids in the majority of patients. However, these long-term treatments do not 'cure' the disease, and the daily regimen of inhalers and avoidance of asthma triggers has a significant negative impact on many patients' quality of life. Furthermore, there are endotypes of asthma in which corticosteroid treatment is clinically ineffective (steroid insensitive/refractory/resistant asthma)[9]. These steroid-refractory patients suffer considerable morbidity, and are both expensive and challenging to manage clinically.

Vitamin D biology

Vitamin D is primarily synthesised by ultraviolet-B radiation from sunlight photolysing skin resident 7-dehydrocholesterol into vitamin D3, but it is also be ingested in small amounts through the diet (e.g. in oily fish or dietary supplements). Vitamin D then undergoes step-wise metabolism, first by the enzyme CYP27A1 in the liver into 25-hydroxyvitamin D3 (25(OH)D), the major form of vitamin D in the body, and then by the mitochondrial enzyme CYP27B1 to the active metabolite (1α,25-dihydroxyvitamin D3 ($1,25(OH)_2D3$))[10]. Circulating $1,25(OH)_2D3$ is principally formed by CYP27B1 in the kidney and acts to regulate calcium-phosphate

homeostasis. However, high levels of local tissue 1,25$(OH)_2$D3 can be produced by CYP27B1, which is also expressed by a diverse range of parenchymal and immunological cell types, including epithelial cells, macrophages and dendritic cells. In vitro studies show these cell types are able to produce $1x10^{-9}$M to $6x10^{-8}$M 1,25$(OH)_2$D3 from 25(OH)D in culture[11-14]. The active form of vitamin D, 1,25$(OH)_2$D3, principally regulates cellular responses by altering gene transcription - it binds to the vitamin D receptor (VDR), altering the binding of the VDR to genomic vitamin D response elements (VDREs) leading to changes in transcription of VDRE regulated genes. The VDR is expressed by essentially all cells of the immune system and parenchymal cells, particularly upon activation, which enables them to respond to 1,25$(OH)_2$D3[10]. Furthermore, VDREs are present throughout the genome, including in the promoter regions for many genes associated with autoimmune diseases[15]. The actions of 1,25$(OH)_2$D3 are however limited due to rapid catabolism by CYP24A1, an enzyme that is also expressed by structural and immune cells[16].

Measurement of circulating 25(OH)D, as the major form of vitamin D in the body, is used to assess an individual's vitamin D status. There is an ongoing debate as to what comprises sufficiency, although in general terms serum 25(OH)D of less than 20ng/ml (50nmol/l) defines deficiency and 20-30ng/ml (50-75nmol/l) indicates insufficiency[17]. Factors including genetic diversity, skin colour, obesity, season and lifestyle all influence vitamin D status, but in most populations studied, insufficiency is a problem[17].

Vitamin D and Asthma – Epidemiological Evidence

A large study by Black *et al*. in 2005[18] was one of the first to highlight the positive association between vitamin D status and pulmonary health. Multiple studies have confirmed this finding, and in particular show that, compared to non-asthmatic controls, the incidence of vitamin D insufficiency is greater amongst paediatric patients[19-21] and adults[22] with asthma. Serum 25(OH)D demonstrates a negative correlation with the severity of asthma (e.g. number of exacerbations)[20,23] and the requirement for higher dose corticosteroids[22,24,25], an effect which is seen most strongly in paediatric cohorts (reviewed in Gupta *et al.*, 2012[26]). Inverse correlations have also been documented between serum 25(OH)D and airway remodeling, IgE, eosinophil numbers as well as airway hyper responsiveness[20,22,25] (recently reviewed in Mann *et al.*, 2014[27]). Furthermore, a remarkable number of asthma-associated genes are known to be vitamin D regulated and polymorphisms within the VDR have been associated with an increased risk of asthma[28-31].

There are many mechanisms through which vitamin D status may influence asthma and pulmonary health, and which would explain the above epidemiological and genetic

associations. The discussion in the following sections reveals the diversity of pathways influenced by vitamin D. However asthma itself may influence vitamin D status (reverse-causation). Conceptually asthmatics, especially those with pollen sensitivity, may spend a reduced amount of time outside exposed to sunlight, however, limited available evidence does not suggest that this is a significant explanation[32]. Inflammation itself is now known to affect vitamin D metabolism and a large inflammatory response can cause deterioration in vitamin D status[33]. One of the severe asthma phenotypes is characterised by obesity[34], which can itself cause decreased circulating vitamin D levels possibly due to sequestration of vitamin D in fat[35,36]. Furthermore corticosteroids, the corner-stone of asthma treatment, affect vitamin D metabolism and in particular increase VDR and CYP24A1 expression[37-39].

It is most likely that the association between asthma and vitamin D insufficiency is multi-factorial, and underpinned by both mechanisms of causation and reverse-causation. The current epidemic of vitamin D insufficiency may predispose the immune system to excessive immunological responses such as seen in asthma, which may in turn cause further worsening of vitamin D status (and in turn further worsening of asthma). Issues of reverse-causation do not mechanistically exclude vitamin D therapies as treatments for asthma in relevant patient groups.

Mechanisms by which vitamin D maintains respiratory health and regulates pulmonary immune responses

Effects on innate immunity relevant to asthma

Respiratory tract infections (RTIs) are a major cause of asthma exacerbations[40], and chronic infection may contribute to the development of severe asthma[41]. Cross sectional, case-control and cohort studies have repeatedly shown an inverse correlation between serum 25(OH)D and the incidence of acute RTIs[42,43]. There are multiple mechanisms by which vitamin D may enhance anti-microbial responses and beneficially modulate the inflammatory response to bacteria, viruses and fungi[44] (Figure 1).

Pathogen challenge by antigen presenting cells triggers toll-like receptors (TLRs), and TLR stimulation has been shown to enhance CYP27B1 expression in a variety of cell types resulting in increased expression of vitamin D-dependent pathways[14,45]. Many allergens can also stimulate cells through TLRs. In particular, a prominent response to the combination of TLR stimulation and vitamin D in monocytes, macrophages and epithelial cells is the induction of anti-microbial peptides such as cathelcidin and β-defensin-2. These molecules contribute to host defence through direct anti-microbial activity, as well as by modulating innate and

adaptive immunity in addition to wound repair. Although the focus of studies regarding anti-microbial peptide actions has been in bacterial infections, they also appear to be important for anti-viral and anti-fungal immunity[46,47]. The enhancement of anti-microbial peptide expression by vitamin D is accompanied by modulation of the production of pro-inflammatory cytokines which is hypothesised to lead to enhanced pathogen clearance without an excessive inflammatory response[48]. Furthermore, vitamin D stimulates autophagy and autophagosome activity which are important in the anti-microbial response[49].

Effects on the adaptive immune system

The primary, but complex, role of vitamin D in dendritic cell (DC) function is modulation of antigen presenting cell function[50,51]. For example, vitamin D decreases MHC and costimulatory molecule expression by myeloid DCs[52]. Since the magnitude and nature of T cell responses is directly influenced by antigen presenting cells, vitamin D-mediated modulation of DC functions has a major effect on downstream lymphocyte responses. Furthermore, vitamin D has direct effects on T cells, impacting on T cell expansion, phenotype, and cytokine profiles.

Vitamin D consistently inhibits Th1-associated cytokine synthesis in culture and in animal models[53]. However, the effects on Th2 responses relevant to allergic and asthmatic disease are more complex although particularly important because Th2 cytokines play a central role in driving IgE synthesis. Vitamin D has been reported to both inhibit or promote Th2 responses in different animal models and human T cell culture systems[54], a significant concern in early studies investigating the potential of vitamin D to improve asthma control. We have reported a non-linear dose effect of $1,25(OH)_2D3$ in culture on Th2 and Th1 responses[55] (Figure 2), the relevance of which has become more apparent following epidemiological observations relating IgE levels to serum vitamin D status. Studies by Wjst and colleagues suggested that high levels of vitamin D in early life were associated with an increase in allergic disease at 30-years[56]. Subsequently, Hypponen, an author on those earlier studies, demonstrated in a very large cohort that circulating 25(OH)D levels exhibited a non-linear relationship with serum IgE; elevated IgE was only observed at very low or high 25(OH)D levels[57]. We and others, have since reported that serum 25(OH)D levels negatively correlate with total and aeroallergen-specific IgE[20,58]. *In vitro,* $1,25(OH)_2D3$ reduces the production of IgE from peripheral human B cells and increases B cell synthesis of the immunoregulatory cytokine IL-10[59,60]. Notably IL-10 producing B cells synthesise IgG4, an isotype associated with beneficial outcome following allergen desensitization immunotherapy[61]. Together these data suggest both a non-linear relationship of vitamin D status with immune parameters associated with allergic and

asthmatic disease (see model Figure 1), and the existence of compensatory mechanisms to counter vitamin D enhancement of Th2 responses.

IL-17A, and other Th17-associated cytokines, play a central role in defence against bacterial and fungal mucosal infections. However excessive levels of these cytokines, particularly IL-17A, is implicated in severe asthma and other immune-mediated diseases. Many patients with severe asthma have a neutrophilic endotype that is associated with increased IL-17A levels/production[62-67]. In addition to triggering neutrophilia, IL-17A promotes airway hyper-responsiveness and remodelling, steroid-resistance, and synthesis of pro-inflammatory cytokines[66-69]. Vitamin D has been shown to reduce IL-17A responses, both in mice and in humans with severe asthma[65,70].

Effects on regulatory T lymphocyte responses

Regulatory T cells (Tregs) are essential to prevent and control inappropriate and excessive, immune responses including those associated with allergy and asthma (reviewed in[71]). However, the frequency and action of Tregs in addition to levels of anti-inflammatory IL-10 are diminished in asthma patients[72,73]. For example, bronchial lavage from severe therapy-resistant paediatric asthmatics contains lower levels of IL-10 compared to non-asthmatic subjects, whilst the production of IL-10 by PBMCs/T cells from both paediatric and adult severe asthma patients is reduced[74,75].

Vitamin D enhances multiple facets of Treg actions[76] – including promoting distinct CD4+Foxp3+ and CD4+IL-10+ (Tr1/IL-10-Treg) populations *in vitro* and upregulating expression of the inhibitory costimulatory molecules CTLA-4 and PD1[77-80]. Interestingly, the nature of the regulatory T cells enhanced by vitamin D is strongly dependent on the concentration of vitamin D and local cytokine milieu; for example varying vitamin D concentrations in different cytokine environments enhance IL-10 expressing Tregs and FoxP3+ Tregs in a mutually exclusive manner[79,81]. In support of these observations, a positive correlation exists between serum 25(OH)D and expression of Foxp3 on peripheral CD4+ T cells, numbers of circulating Foxp3+ T cells as well as levels of IL-10 in the BAL[24,74,82,83].

In addition to enhancing distinct Treg subsets, vitamin D also affects many other immune regulatory pathways, for example lymphocyte ATP metabolism and CD200 expression. CD200 suppresses the pro-inflammatory activity of local innate immune cells and $1,25(OH)_2D3$ has been shown to increase expression of CD200 on human peripheral and nasal airway human T cells[84]. ATP is a proinflammatory molecule that can be broken down into immunosuppressive adenosine via the action of 5'-ectonucleotidases CD39 (ATP/ADP into AMP) and CD73 (AMP into adenosine)[85]. Both CD39 and CD73 have been proposed as

Treg markers and CD39 expression at least is downregulated in severe asthmatics[65,86]. We have shown that *in vitro*, 1,25(OH)2D3 upregulates CD39 expression and that this contributes to suppression of IL-17A[65].

Effects on corticosteroid responses

Early studies from our laboratory investigated immunological actions of glucocorticoids that were relevant to clinical efficacy. We demonstrated that peripheral blood CD4+ T cells from steroid refractory patients were unable to increase secretion of anti-inflammatory IL-10 *in vitro* when treated with dexamethasone (a synthetic corticosteroid), unlike cells from steroid sensitive individuals and healthy controls[75]. However, vitamin D, either by addition of $1,25(OH)_2D3$ to cultures or by oral supplementation of the steroid refractory asthmatics themselves for 7 days, restored the dexamethasone induction of IL-10[75]. In addition to directly enhancing IL-10 production, we reported that $1,25(OH)_2D3$ can overcome downregulation of the glucocorticoid receptor by dexamethasone. This work led directly to a small proof of concept placebo-controlled clinical study demonstrating clinical steroid-enhancing effects of calcitriol in steroid refractory asthma[87]. A steroid-enhancing role for vitamin D in severe asthma is strongly supported by observational studies, particularly in paediatric cohorts[25,26]. In addition to our T cell studies, Zhang *et al.*[88] have pioneered studies of the anti-inflammatory and corticosteroid-enhancing actions of vitamin D in monocytes in both steroid refractory and sensitive asthma patients.

We recently reported that dexamethasone upregulates IL-17A from PBMC and CD4+ T cells *in vitro*, and demonstrated a positive association between inhaled corticosteroid dose (beclomethasone equivalent) and IL-17A synthesis in culture. These data suggest that corticosteroids may contribute to progression in severe asthma by heightening the Th17 responses. Notably, vitamin D strongly inhibited the corticosteroid induced IL-17A in culture[65].

Effects of vitamin D on airway remodelling

Airway remodelling in asthma irreversibly reduces lung function, and in general is poorly-controlled with current therapies[89]. Vitamin D has been shown to reduce airway smooth muscle (ASM) mass, subepithelial deposition and goblet cell hyperplasia[90,91] (Figure 1). Similarly, an inverse correlation exists between ASM mass and serum 25(OH)D in paediatric asthmatics[20], whilst addition of vitamin D derivatives to cultures of human ASMs impairs proliferation of cells[92]. Furthermore, vitamin D has been shown to reduce production of

extracellular matrix proteins from fibroblasts and reduce expression of enzymes implicated in airway remodelling, namely ADAM33 and MMP9[93-96].

Vitamin D and Fetal Development

The majority of asthmatic patients develop the disease within the first decade of life, often following on from the development of food allergies and hayfever in the first years of life – the atopic march[97]. This strongly suggests that events in the fetal environment and first months of life have a major effect on whether individuals develop asthma. Lower maternal intake of vitamin D and maternal vitamin D deficiency have been found to be associated with increased risk of wheeze/asthma during childhood, both in individual studies and a meta-analysis[98-102]. However some studies show no affect[102-105] others even an adverse affect[106,107]; disparity in outcomes from clinical studies could be due to a variety of factors summarized in Figure 3. Importantly, maternal vitamin D intake is not equivalent to maternal or fetal vitamin D status as diet is only a minor source of vitamin D and different vitamin D supplements contain very different amounts of vitamin D. Furthermore assessment of the response to vitamin D supplements is complicated by supplements often containing other constituents such as vitamin A that have their own actions on the developing immune system[108-110]. The importance of maternal vitamin D status is underpinned by the fact that the placenta is a major site for the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$[111,112]. In addition to its actions on the developing immune system, vitamin D has an important role in fetal lung development[113,114].

Clinical Trials of Vitamin D Therapies – Early Evidence

Given current disagreements as to the definition of vitamin D sufficiency and deficiency, it is not surprising that there is also debate on how to design trials of vitamin D therapies. Different molecular forms of vitamin D may be more or less effective as therapeutic supplements, as well as different dosing regimens. Whether all individuals would benefit from vitamin D supplements or only those with levels below certain thresholds, or only those with certain diseases (and certain endotypes of those diseases) is uncertain. Few studies so far published have investigated vitamin D therapies in asthma and given the uncertainties above, it is not surprising these trials have not reached unanimous conclusions.

In one randomized, double-blind placebo-controlled (RCT) trial 48 children (5-18 years) with newly diagnosed asthma received either budesonide with placebo or budesonide with 500IU vitamin D daily, a comparatively low dose[115]. The authors observed that vitamin D supplementation in the period from September to July prevented declining serum

concentrations of 25(OH)D and reduced the risk of asthma exacerbation triggered by acute respiratory tract infection. In addition to such studies in children, we recently reported in a RCT that 1,25$(OH)_2$D3 led to a modest improvement in the clinical response to oral steroids in a small cohort of adult steroid resistant asthma patients[87].

The large VIDA trial of vitamin D supplementation in vitamin D insufficient asthmatics recently reported its outcomes[105] The primary outcome, time to first asthma treatment failure, was not significantly affected by vitamin D supplementation. There was however a trend towards a reduced overall rate of exacerbations in the vitamin D treated group (P=0.05) and significantly greater reduction in inhaled corticosteroid dose. Possible reasons that the primary outcome measure did not show a significant benefit of vitamin D supplementation include the study being underpowered, some vitamin D treated participants not attaining vitamin D sufficiency and that vitamin D may only be beneficial in particular asthma endotypes.

Data from other large trials in asthma are awaited.

Conclusions and viewpoint

Evidence from clinical initiatives is starting to emerge, in both asthma and respiratory infections. However the data are likely to be complex and reflect the heterogeneity in trial design. It seems probable based on findings to date, and when placed in the context of experimental data, that beneficial effects are most likely to be observed in individuals who are profoundly vitamin D deficient. It should however be considered that the body has numerous measures to compensate for low levels of vitamin D intake via mechanisms including parathyroid hormone. Consequently, there may well be a non-linear relationship between intake of vitamin D and its biological effects, an observation that is supported by certain immunological findings.

In summary there are many pathways pertinent to asthma that are strongly influenced by vitamin D. We strongly believe that future trials of vitamin D therapies in asthma need to consider the above issues. In our opinion smaller trials of vitamin D in specific asthma endotypes with asthma-relevant endpoints suggested by clinical science is the way forward.

Figure legend

Figure 1. The immunomodulatory properties of vitamin D relevant to asthma

Vitamin D modulates immune activity in a multitude of ways. Generally a more regulatory environment is promoted by upregulation of Treg numbers and activity, as well as reduced

pro-inflammatory cytokine production. Clinically the incidence of infections is reduced, airway remodelling halted and corticosteroid responsiveness improved.

Figure 2. Non-linear association of vitamin D levels with immune parameters of relevance to allergic and asthmatic disease.

This cartoon illustrates the non-linear (U-shaped) association of serum 25-hydroxyvitamin D3 levels with IgE; and a non-linear dose effect of 1alpha,25-dihydroxyvitamin D3 in culture on the inhibition of Th2 and enhancement of IL-10 responses[55,57].

Figure 3. Factors that differ between vitamin D clinical studies and may account for observed differences in results.

Differences in the participant cohort and study design are evident in vitamin D clinical studies and are likely to contribute to variation in the results. Summarised here are the key factors that need to be taken into consideration.

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