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Editorial from Guest Editor

Vitamin D and Respiratory Health

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This Hot Topic edition of *Current Respiratory Medicine Reviews* considers the literature suggesting that vitamin D status is a key determinant of respiratory health. At first sight, the proposition that deficiency of a single micronutrient could be implicated in the pathogenesis of conditions as diverse as asthma, allergy, respiratory infection and lung cancer may seem biologically implausible. However, a large and growing body of evidence from both the laboratory and the clinic suggests that associations between vitamin D deficiency and susceptibility to these conditions may well be causal.

Humans acquire vitamin D *via* cutaneous synthesis as a result of the action of solar ultraviolet B radiation on 7-dehydrocholesterol in the skin, or from the diet, principally by consumption of oily fish or dietary supplements. Vitamin D from either source is metabolised by the liver to form 25-hydroxyvitamin D (25[OH]D), the major circulating vitamin D metabolite whose serum concentration defines vitamin D status. 25(OH)D is metabolised by the enzyme 25(OH)D-1 α -hydroxylase (CYP27B1) to form 1,25-dihydroxyvitamin D (1,25[OH]₂D), the steroid hormone and active metabolite of vitamin D. 1,25(OH)₂D induces biological actions by ligating vitamin D receptor (VDR), which acts as a transcription factor, regulating the expression of vitamin D-responsive genes [1].

The clinical features of vitamin D deficiency were first described in 1651, when Glisson, Bate and Regemorter published 'A treatise of the rickets: being a disease common to children' [2]. For many years, bone disease was regarded as the only clinical manifestation of inadequate vitamin D status; CYP27B1 was thought to be expressed predominantly in the kidney and 1,25(OH)₂D was regarded primarily as a hormone of calcium homeostasis. This perception changed in 1981, when Barbour and colleagues reported a case of hypercalcaemia in an anephric patient with sarcoidosis who was found to have an elevated serum 1,25(OH)₂D concentration [3]; this observation provided the first evidence that 1-alpha hydroxylation of 25(OH)D could occur extra-renally. CYP27B1 activity has subsequently been demonstrated in macrophages [4], dendritic cells [5], lymphocytes [5] and pulmonary epithelial cells [6]. In the

same year, Abe and colleagues reported expression of VDR in a murine myeloid leukaemia cell line [7], raising the possibility that vitamin D might possess immunomodulatory activity. VDR has subsequently been shown to be expressed in human monocytes, macrophages, B and T lymphocytes [8], in dendritic cells [9] and in both normal and malignant lung tissue [10]; moreover, 1,25(OH)₂D has been shown to exert antimicrobial, anti-inflammatory and anti-proliferative actions on these cells *in vitro* [11]. Taken together, these observations indicate that 1,25(OH)₂D can be synthesised by, and can exert biological activity in, many cell populations in the lung.

The articles presented here encompass the effects of vitamin D status on respiratory health throughout the lifespan. Steven Goldring, John Warner, Seif Shaheen and Robert Boyle open with a review of the clinical evidence linking antenatal vitamin D status with subsequent risk of asthma and atopy. Virender Rehan and John Torday proceed to enlarge on the molecular mechanisms by which perinatal vitamin D deficiency may contribute to asthma pathogenesis in the developing lung, before Graham Devereux and James Wagner discuss insights into the actions of vitamin D provided by animal models and consider the evidence that vitamin D status may influence incidence and severity of asthma in children and adults. Abigail Jackson and Nicholas Hopkinson then review the potential implications of vitamin D deficiency for respiratory, musculoskeletal and cardiovascular health in patients with COPD. Emerging evidence of the potential importance of vitamin D deficiency in susceptibility to acute respiratory infection is then reviewed by Yasmeen Hanifa and Robert Walton, before Kirsten Mitchell, Chris Griffiths and myself provide an update of the evidence linking vitamin D deficiency and susceptibility to tuberculosis. Helga Rhein presents the case for conducting trials to determine whether vitamin D supplementation may prolong survival in patients with lung cancer, before Anna Coussens wraps up the issue with an overview of vitamin D metabolism and of the immunomodulatory actions of 1,25(OH)₂D that underpin its many functions.

The evidence reviewed above is tantalising, but much of it comes from *in vitro* studies and observational epidemiology; randomised clinical trials are now needed to determine whether this scientific promise will translate into a clinical benefit. The challenges in conducting trials of vitamin D supplementation are significant: prevention trials, in particular, will require large sample sizes and prolonged

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follow-up in order to demonstrate clinically plausible effect sizes. Even modest effects are worthy of detection however: vitamin D supplementation is safe, simple to administer and inexpensive, and if it is eventually shown to benefit patients with common and debilitating respiratory disease then the benefits to public health will be very significant.

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Early Life Vitamin D Status and Lung Development

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Abstract: There is evidence of an association between early life vitamin D insufficiency and future risk of developing asthma. Given the high prevalence of vitamin D insufficiency in women during pregnancy when developmental programming is occurring, this may be of critical public health importance. There are plausible biological mechanisms for an association. Vitamin D is the pro-hormone of calcitriol, a secosteroid hormone with widespread pleiotropic actions. It is a powerful immune modulator and has been shown in animal and *in vitro* work to have a role in early lung development. Calcitriol may influence lung development through expression of the vitamin D receptor on lung and immune cells, and through epigenetic mechanisms. If the association between early life vitamin D status and childhood respiratory disease is shown to be causal, then this could have significant implications for public health policy. This hypothesis is currently being tested in a number of prospective intervention trials. The aim of this article is to review the evidence that vitamin D status influences early lung development, with a focus on early life mechanisms.

Keywords: Asthma, diet, lung development, pregnancy, vitamin D.

INTRODUCTION

Abnormal early lung development is seen in respiratory diseases that present in childhood or later life [1]. For asthma in particular, there is a large body of evidence suggesting that early life influences are important in disease pathogenesis, and micronutrient status may be particularly important. Changing environmental factors acting on genetically susceptible individuals are likely to explain the increase in asthma prevalence in the last century; however the precise environmental factors which increase asthma risk remain unidentified [2]. Early life vitamin D status is one such proposed environmental factor. As discussed elsewhere in this supplement, there are intriguing parallels in the prevalence of asthma and vitamin D deficiency, and mechanistic reasons why the relationship may be causal. Apparently contradictory hypotheses argue that either high [3], or low [4] vitamin D status, at critical times in development have contributed to the asthma epidemic. The nature of calcitriol's influence on early immune and lung development, and the dose dependency of this influence illustrate how these hypotheses may not be mutually exclusive, and how such biological effects may be possible. The purpose of this article is to review the potential role of vitamin D in early lung development, on the basis that vitamin D status is an easily modifiable variable which might influence lung development and specifically asthma risk.

EARLY LIFE NUTRITIONAL INFLUENCES ON LUNG DEVELOPMENT

The respiratory system originates as an outgrowth of the primitive foregut between the 4th and 7th week of human

gestation [5]. Through timely orchestration of a complex array of transcription factors, growth factors and physical forces, [6] epithelial tubules invaginate the surrounding splanchnic mesenchyme to form the bronchial tree. Key processes are branching morphogenesis, angiogenesis, epithelial cell differentiation, sacculization, surfactant production and alveolarisation, the latter being predominantly a postnatal event in humans [1]. Epithelial-mesenchyme interactions play a crucial role [7].

Early life influences on this process include both genetic and environmental factors, and minor alterations during foetal and perinatal life may have significant postnatal consequences. For example they may alter responses to early life infections and other airway insults that are implicated in the aetiology of asthma [8, 9]. Several long term cohort studies have established that infant lung function tracks into later life, suggesting that successful modification of early lung development may influence lung function in the long term [10, 11].

Known environmental, and nutritional factors which may influence lung development [12, 13] include cigarette smoking [14], polyunsaturated fatty acids [15, 16] milk fat [17], vitamin E [18], and vitamin A [19]. The data regarding vitamin A are particularly relevant to this article, due to the common signalling pathway shared by the activated forms of vitamins A and D. The active metabolite of vitamin A, retinoic acid (RA), is a critical signalling molecule in early lung development with maternal deficiency causing profound abnormalities of the infant respiratory system, including tracheoesophageal fistula, lung hypoplasia and lung agenesis [20]. Mechanisms include RA effects on TGF- β and FGF10 pathways at prospective sites of lung formation [21]. RA also influences alveolarisation [1] so that postnatal treatment with RA increases the number of pulmonary alveoli in rats [22]. Results of a recent randomized controlled trial of vitamin A supplementation in a population of married women of childbearing age with chronic vitamin A deficiency in rural Nepal confirm the relevance of these

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mechanistic findings to respiratory health in humans. At age 9 to 13 years, offspring of mothers who received vitamin A before, during and for 6 months after pregnancy had significantly improved lung function compared with a placebo group (mean increase in FEV₁ 46mls, 95% CI 6-86mls) [19]. All children in the trial received supplemental vitamin A from 6 months of age, suggesting that vitamin A status from preconception through to age 6 months influences long term lung development. It should be noted that data regarding potential confounders such as later nutritional status were not reported in this trial. However the study does provide evidence that maternal micronutrient status can influence fetal/infant lung development with significant consequences for longer term respiratory health in humans.

VITAMIN D

Vitamin D is a pro-hormone obtained from either diet or photosynthesis in the skin by the action of ultraviolet B light on 7-dihydrocholesterol. To become metabolically active vitamin D undergoes two hydroxylations. The first occurs primarily in the liver to form 25-hydroxyvitamin D (25(OH)D₃) and the second, more tightly regulated hydroxylation occurs mainly in the kidney to form the biologically active hormone 1,25-dihydroxyvitamin D (1,25(OH)₂D₃), also known as calcitriol, *via* the action of a mitochondrial P450 hydroxylase, CYP27B1. Many extra-renal cells also express both the vitamin D receptor (VDR) and CYP27B1 so that 1,25(OH)₂D₃ is able to act as an intracrine and paracrine hormone in many tissues. This is the basis of calcitriol's widespread pleiotropic actions including effects on the pulmonary and immune systems [23].

The actions of 1,25(OH)₂D₃ are mediated *via* two pathways, a slow genomic response and a rapid, non-genomic response [24, 25]. Both involve binding to its nuclear receptor, the vitamin D receptor (VDR). In the genomic pathway VDR functions as a heterodimer with the retinoid X receptor (RXR). After binding, VDR undergoes a conformation change that promotes RXR-VDR heterodimerization. The bound heterodimer translocates to the nucleus where VDR binds to vitamin D responsive elements and ultimately modifies gene transcription [26, 27]. The expression of over 200 genes are controlled *via* 1,25(OH)₂D₃/VDR dependant pathways acting on vitamin D responsive elements [28]. In the non-genomic pathway, 1,25(OH)₂D₃ binds to VDR present in the caveolae of the plasma membrane, forming ligand bound VDR which activates signalling cascades including protein kinase C and phospholipase C [29]. Rapid responses triggered by this pathway include intestinal absorption of calcium and the secretion of insulin by pancreatic β -cells [25].

VITAMIN D METABOLISM IN EARLY LIFE

The vitamin D status of a pregnant women directly influences that of her foetus/infant [30]. During the course of pregnancy, maternal levels of 25(OH)D₃ modestly decline and at delivery, cord 25(OH)D₃ levels are typically \leq 20% lower than maternal levels [31, 32]. The decidua and placenta have large amounts of CYP27B1 enzyme activity resulting in generation of 1,25(OH)₂D₃ [33]. During

pregnancy methylation of placental CYP24A1, the enzyme responsible for catabolism of 1,25(OH)₂D₃ to inactive metabolites, represses transcription of this gene. Thus maternal 1,25(OH)₂D₃ levels increase as high as two fold in late pregnancy compared to postpartum or non pregnant controls [34].

1,25(OH)₂D₃ does not readily cross the placenta and combined with foetal suppression of renal CYP27B1 by low PTH and phosphorous levels, circulating foetal 1,25(OH)₂D₃ concentrations are significantly lower than maternal values in humans [31]. Postnatally, foetal dependency on maternal vitamin D may manifest in infants of deficient mothers with hypocalcaemia and rickets. Breast milk has a low vitamin D and 25(OH)D₃ content unless the mother takes supplements [31] and so exclusively breastfed infants are largely dependent on their prenatally acquired vitamin D stores or supplementation. For this reason infant formula is supplemented with vitamin D, and many countries have a policy of routine vitamin D supplementation for breastfed infants [35].

PERINATAL VITAMIN D DEFICIENCY – PREVALENCE AND IMPACT ON RESPIRATORY OUTCOMES

Early life hypovitaminosis D is common and increasingly recognized as a global public health issue [36]. The optimum level for health is not known but a consensus statement has identified concentrations \geq 75nmol/L as ideal for healthy bones [37]. Table 1 lists recent studies describing the prevalence of hypovitaminosis D amongst pregnant women and their newborn infants in different populations. At risk groups include ethnicities with pigmented skin, wearing concealing clothing [38-40], poor uptake of vitamin D supplementation [36, 41-44] and prolonged breast feeding [35]. Hypovitaminosis D is also common in pregnant white women with no additional 'risk factors' [45]. A recent genome wide association study has identified common genetic variants that identify individuals at increased risk of vitamin D insufficiency [46].

Current recommendations for vitamin D supplementation vary worldwide. In the UK, pregnant women and at risk groups are advised to take 400IU daily. However, it is acknowledged that current guidance from health care providers to pregnant women is inconsistent and a barrier to effective primary prevention of hypovitaminosis D [45]. A recent editorial has called for urgent intervention to address early life hypovitaminosis D [47].

A number of studies have shown a relationship between early life vitamin D status and respiratory outcomes (Table 2). Studies of maternal dietary intake of vitamin D in pregnancy have suggested a protective effect of a higher intake on early childhood wheezing [48-50], asthma and allergic rhinitis [51], and eczema [50]. In contrast, in another birth cohort study, the highest maternal blood 25(OH)D₃ concentrations were associated with an increased risk of asthma at 9 years of age in the offspring [52]. It should be noted that the gold standard measure of vitamin D status is the blood concentration of 25(OH)D₃, as it captures vitamin D exposure from diet and sunlight combined. In contrast, dietary intake of vitamin D makes a relatively small contribution to overall vitamin D status, and apparent effects

Table 1. Vitamin D Status of Pregnant Women and their Offspring: Recent Studies

Setting	Population	Cord Blood 25(OH)D3 Levels	Maternal 25(OH)D3 Levels
US national population survey [100]	928 unselected pregnant women	NA	33%<50nmol/L
US antenatal clinic [101]	433 unselected pregnant women; 376 newborns	38%<37.5nmol/L	23%<37.5nmol/L
US antenatal clinic [102]	80 pregnant black adolescents	NA	21%<37.5nmol/L
UK antenatal clinic [52]	466 pregnant Caucasian women	NA	49.5% <50nmol/L
UK antenatal clinic [103]	263 pregnant adolescents	NA	30% <25nmol/L
UK antenatal clinic [104]	180 pregnant women at 27 weeks gestation 45 Asian 45 Middle Eastern 45 Black 45 Caucasian	NA	% <25nmol/L: 47% 64%; 58%; 13%
Netherlands antenatal clinic [44]	86 pregnant women: 48 'high risk' (dark skin or veiled), 38 'controls' (light skin, not veiled)	%<25nmol/L 63% 16%	NA
US antenatal clinic [41]	400 pregnant women 200 black 200 white	<37.5nmol/L 46% 10%	<37.5nmol/L 45% 2%
Australian antenatal clinic [43]	971 unselected women at 28 weeks gestation; 901 neonates	11%<25nmol/L	15% <25nmol/L [Veiled 71%; Non veiled 11%]
New Zealand primary care antenatal clinic [105]	90 unselected pregnant women		61% deficient

All values have been converted to nmol/L where 1 ng/ml = 2.5 nmol/L.

of vitamin D intake may be confounded by other correlated nutrients. Finally, vitamin D supplementation with 2000IU during infancy in Finland was associated with an increased risk of atopy and allergic rhinitis in adulthood [53]. The finding of an increase in serum total IgE at both low (<25nmol/L) and high (>135nmol/L) blood concentrations of 25(OH)D3 in a recent large British adult cohort suggests a possible mechanism by which both high and low vitamin D status could be associated with increased asthma risk [54].

MECHANISMS THROUGH WHICH EARLY LIFE VITAMIN D STATUS MIGHT INFLUENCE LATER RESPIRATORY HEALTH

Effects of Vitamin D on Early Lung Development: Animal Data

A number of separate observations from animal work suggest that alveolarisation may be influenced in part by a complex growth axis which involves 1,25(OH)₂D3. First, an early study of the offspring of vitamin D deprived rats showed a significant decrease in lung compliance (CL) and CL to dry lung weight ratio compared with the offspring of vitamin D sufficient rats, suggesting vitamin D deficiency may decrease lung distensibility through an effect on interstitial tissue modelling [55]. Second, it is known that the VDR is expressed during late intrauterine life in rat pulmonary tissues [56, 57]. This is during the period of maturation of alveolar type two (ATII) cells which synthesise and release surfactant [58, 59]. 1,25(OH)₂D3

stimulates DNA synthesis in ATII cells [60], and the discovery that lung fibroblasts are capable of metabolising 25(OH)D3 to 1,25(OH)₂D3 identified a paracrine system for vitamin D in the developing rat lung [61].

Other data suggest a role for 1,25(OH)₂D3 in lung fibroblast development, which may in turn influence alveolarisation. 1,25(OH)₂D3 significantly increases proliferation of rat perinatal lung fibroblasts, an effect which is greatest in combination with RA on postnatal day 4 (PN4) [62]. The same authors showed that this effect is dependent on platelet derived growth factor-AB (PDGF-AB). The PDGF null mouse has a bronchopulmonary dysplasia (BPD) like picture with an absence of fibroblasts and abnormal septation, and PDGF appears to be essential for normal alveolarisation of the developing lung [63]. PDGF contains a transcriptional control region containing response elements to 1,25(OH)₂D3 [64] and vitamin A [65]. Thus a proposed mechanism of action for 1,25(OH)₂D3 in promoting lung development is *via* a synergistic effect with RA in stimulating growth of immature fibroblasts in the postnatal lung, with PDGF as the critical effector [62]. Further experiments have demonstrated a role for the vitamin D analogue EB1089 on fibroblast proliferation but suggested, contrary to other work, that relatively high doses of this vitamin D analogue may disrupt alveolarisation [66]. Histological findings in EB1089-treated rats include increased alveolar chord length and prominent regions of fibroblast hypercellularity. These stained strongly for alveolarisation growth factors PDGF-AA and vascular endothelial growth factor (VEGF) and the overall effect was

Table 2. Observational Associations Between Early Life Vitamin D Status and Respiratory Health

Setting	Design	Follow Up	Method	Outcome	Result	Comment
Boston, USA [49]	Birth cohort n=1194	Age 3	Maternal diet FFQ	Recurrent wheezing	OR 0.39 (95%CI 0.25, 0.62) highest vs lowest quartile	Protective
Aberdeen [48]	Birth cohort n=1212	Age 5	Maternal diet FFQ	Ever wheezing Wheeze in previous year Persistent wheeze	OR 0.48 (95%CI 0.25, 0.91) highest vs lowest quintile OR 0.35 (95%CI 0.15, 0.83) OR 0.33 (95%CI 0.11, 0.98).	Protective
Finland [51]	Birth cohort n=1669	Age 5	Maternal diet FFQ	Asthma Allergic rhinitis	HR 0.80 (95%CI 0.64, 0.99) HR 0.85 (95%CI 0.75, 0.97)	Protective
Japan [50]	Birth cohort n=764 mother child pairs	Age 16-24 months	Maternal diet FFQ	Wheeze	OR 0.64 [95%CI 0.43, 0.97]	Protective
Southampton [52]	Birth cohort n=178	Age 9	Maternal serum 25(OH)D	Asthma	OR 5.40 (1.09, 26.65) For children of mothers with vitamin D >75nmol/L vs <30nmol/L	Increased risk
Finland [53]	Infant cohort n=12,058	Age 31 (63% f/up)	Infant vitamin D supplement	Allergic rhinitis Asthma Atopy	OR 1.66 (1.10, 1.60) OR 1.35 (0.99-1.80) OR 1.46 (1.4-2.0)	Increased risk

to promote fibroblast proliferation but disrupt alveolarisation [66]. Whether this disruptive effect is dose dependant or related to other effects of the vitamin D analogue in question is not known.

A third mechanism through which 1,25(OH)₂D₃ may influence alveolarisation is *via* the inhibition of apoptosis in lipid laden interstitial lung fibroblasts (LF) [67]. LF cells become highly apoptotic during the first 1-2 weeks of postnatal life in rats, a developmental process that contributes to alveolar wall thinning and alveolarisation [68]. Both *in vitro* treatment (at embryological day 19, [ED19]) and *in vivo* treatment (postnatal days 0 to 14, [PN0-14]) with 1,25(OH)₂D₃ resulted in dose-dependent increases in LF and ATII cell proliferation and decreased apoptosis, accompanied by increases in the expression of epithelial mesenchyme differentiation markers [67]. These data suggest a role of 1,25(OH)₂D₃ in alveolar thinning. 1,25(OH)₂D₃ may have a specific role in transcriptional regulation of Lgl1 (late gestation lung 1) during alveolarisation [69]. Lgl1 is a mesenchymal protein found in foetal lung that regulates epithelial airway branching and is maximally expressed in late gestation and early postnatal life [70]. In Lgl1 null mice, absence of Lgl1 is lethal prior to lung formation. Heterozygotes also display an abnormal respiratory phenotype. *In vitro* experiments in rat lung fibroblasts from E18 to PN14 suggest 1,25(OH)₂D₃ has a complex interplay with glucocorticoid (GC) and RA in the control of Lgl1. 1,25(OH)₂D₃ directly inhibits Lgl1 mRNA production, and inhibits GC induction of Lgl1. When combined with RA there was no effect on GC induction of Lgl1 gene transcription. Lgl1 may be a mesenchymal mediator of 1,25(OH)₂D₃ effects on epithelial maturation, with regulation of the target gene promoter by GC and RA as well as 1,25(OH)₂D₃ [69].

Further, less direct, evidence of a role for vitamin D status in foetal lung development come from studies of VDUP1 (vitamin D up regulated protein 1) expression in the

ovine foetal lung. The basal level of lung expansion is known to be an important determinant of airway epithelial cell phenotype [71]. VDUP1 is an intracellular protein whose expression is upregulated by vitamin D₃ administration [72]. In sheep lung, VDUP1 is localized to airway epithelium in small bronchioles, AEC's and mesenchymal cells. Expression of VDUP1 mRNA increase significantly during the alveolar stage of lung development [73]. Furthermore, VDUP1 mRNA correlate with different levels of lung expansion, with increased levels in under expanded lungs and decreased levels in over expanded lungs compared to normal controls [73]. It is well known that pressure effects in the airways induced by respiratory movements, contraction and relaxation of smooth muscle in the airway wall and the pressure of amniotic fluid have a potent influence on airway and alveolar development. Reduced amniotic fluid is associated with lung hypoplasia, and reduced foetal respiratory effort (eg opiate-dependent mothers) has a profound effect on post-natal lung function. Thus it is possible that VDUP1 is a vitamin D status dependent moderator of foetal lung growth and development in response to changes in foetal lung expansion [73].

Finally, in mature rats, VDR expression is present in lung fibroblasts and 1,25(OH)₂D₃ appears to have a role. Recent work shows that 1,25(OH)₂D₃ inhibited TGFβ₁-induced fibroblast proliferation, and blunted TGFβ₁-induced upregulation of mesenchymal cell markers and abnormal expression of epithelial cell markers. TGFβ is expressed during branching morphogenesis and has an important role in modelling of the airway wall by stimulating fibroblast collagen production [74]. Thus vitamin D status may have a homeostatic role in the developing lung, preventing some of the effects of excess TGFβ on the developing epithelial-mesenchymal trophic unit, which are thought to lead to changes such as the airway remodelling commonly seen in asthma.

Effects of Vitamin D on Early Lung Development: Human Data

Rachitic respiratory distress has been described very early in infancy, for example in small preterm infants [75]. This suggests that vitamin D deficiency of prenatal origin can have a profound acute effect on early lung function. As with the animal studies, there is also evidence in human studies that 1,25(OH)₂D₃ influences different components of lung development including fibroblast proliferation and alveolarisation. The VDR has been detected in human foetal lung fibroblasts from 16 weeks gestation, [76, 77] and in ATII cells from the second trimester [78]. In ATII cells, VDR expression is largely dependent on induction by 1,25(OH)₂D₃ [78]. After incubation of a human ATII cell line with 1,25(OH)₂D₃, a number of metabolites are also formed including 1,25-(OH)₂-3-epi-VD₃, which can increase surfactant phospholipid synthesis, surfactant SP-B mRNA gene expression and surfactant SP-B protein synthesis in pulmonary ATII cells [79]. However the effects of vitamin D metabolites on human lung development are likely to be complex since decreased, increased and no effect on surfactant protein synthesis have been observed on treating human fetal lung explants and ATII cells with calcitriol [78].

In the mature human lung, data are now emerging as to the cellular targets that may be influenced by vitamin D status. The VDR is expressed in the epithelia of normal and malignant bronchial tissue [80], in lung epithelial cells treated with vitamin D metabolites [81], and in airway smooth muscle (ASM) cells which demonstrate a large array of changes in gene expression in response to stimulation by 1,25(OH)₂D₃ [82]. In ASM cells that were passively sensitized with serum from an asthmatic, 1,25(OH)₂D₃ suppressed proliferation and significantly down regulated expression of matrix metalloproteinases-9 (MMP-9) and a disintegrin and metalloprotease 33 (ADAM33); both of these being significant promoters of airway remodelling [83-85]. Polymorphisms of ADAM 33 are associated with a higher risk of asthma particularly in relation to bronchial hyper-responsiveness. In British Bangladeshi adults, 25(OH)D₃ status has been found to be inversely associated with plasma MMP-9 levels, with supplementation in vitamin D deficient individuals leading to reduced MMP-9 levels *in vivo* [86]. *In vitro* 1,25(OH)₂D₃ inhibits MMP-9 expression by M. Tuberculosis in cell culture [87]. Therefore any effects of vitamin D status on lung development may be mediated through effects on MMP-9 levels in plasma or lung tissue. Finally, in bronchial ASM samples taken during surgery, 1,25(OH)₂D₃ decreased PDGF induced cell proliferation [88].

Whilst speculative, if such relationships are present in the developing lung this could explain potential benefits of vitamin D metabolites on future lung health. In light of current concepts that molecular regulators originally associated with developmental processes may be implicated in adult diseases such as chronic obstructive airway disease [1] this is of great interest, and suggests a potential role for maintaining adequate vitamin D repletion to ensure 25(OH)D₃ and 1,25(OH)₂D₃ are available to the tissues for treatment, or prevention, of airway remodelling in asthma [89].

Effects of Vitamin D Status on Early Immune Development

1,25(OH)₂D₃ is now widely recognized as having a key role in immune signalling, with well defined actions as a potent promoter of tolerogenic dendritic cells and regulatory T cells, and an inducer of antimicrobial peptide secretion in response to innate immune stimuli [23]. These effects occur through the local conversion of 25(OH)D₃ to the biologically active 1,25(OH)₂D₃ [90] by CYP27B1 in immune cells [90]. It is possible that global deficiency or excess of 25(OH)D₃ in the whole organism may lead to adverse consequences on immune development. Healthy immune development is in turn critical for lung development, since immune deficiency increases the risk of damaging pulmonary infections, and allergic airway inflammation is strongly associated with the development and persistence of asthma [91]. In animal models the principle that vitamin D deficiency at a critical time point may adversely affect immune development has been supported - developmental deficiency of vitamin D in rats induces alterations in immune organ morphology and function in adult offspring, with an increase in both immune organ size and in the pro-inflammatory IL-2 response by lymphocytes [92]. Other animal work has shown decreased allergen-induced airway hyper-responsiveness (AHR) in mice deficient in VDR, and VDR knockout mice fail to develop experimental allergic airway disease suggesting that VDR expression may be necessary for lung inflammation to occur [93, 94]. However other authors have reported that 1,25(OH)₂D₃ inhibits airway inflammation and decreases IL-4 levels in bronchoalveolar lavage (BAL) fluid and may do this through impairing T cell migration [95]. In separate experiments irradiation with a single minimal erythral dose of UVB light prior to sensitization with antigen blunted airway hyper-responsiveness and inflammation [96]. In a murine model of allergic airway disease, treatment with calcitriol during immunizations enhanced IL-4 and IL-13 responses, whereas later treatment inhibited IL-5 and airway eosinophilia [97]. These data illustrate the complexity of 1,25(OH)₂D₃'s role in inflammatory responses involved in the pathogenesis of asthma. Their contradictory nature may relate to the differences between mouse models of allergic asthma and the human disease, or to the critical importance of dose, type of vitamin D supplement and timing of administration for optimal immune effects.

CURRENT VITAMIN D INTERVENTION TRIALS

Five prospective randomized controlled trials of vitamin D supplementation during pregnancy are currently in progress, and will shed some light on the relevance of the mechanisms described above to human health. In the United States, the 'Maternal Vitamin D Supplementation to Prevent Childhood asthma' (VDAART) study, will randomise 870 women carrying a foetus at high risk of asthma, to receive 4000IU of cholecalciferol vitamin D daily, or placebo from 10-18 weeks gestation to delivery. The primary outcome measure is asthma or recurrent wheeze at 1 year and 3 years. Also in the United States, Hollis *et al.* are conducting a three arm randomised trial of either 400, 2000 or 4000IU of vitamin D per day from 12 weeks gestation in healthy women (NCT00292591). Primary outcomes are 25(OH)D₃

levels throughout pregnancy and bone mineral density in mother and infant. To our knowledge this trial is not evaluating respiratory or atopic outcomes in the offspring. In the Copenhagen Vitamin D Supplementation During Pregnancy for Prevention of Asthma in Childhood (ABCvitaminD; ISRCTN NCT00856947), 600 women will be randomised to receive either 2400IU daily of cholecalciferol vitamin D or placebo in addition to 400IU of vitamin D daily from 24 weeks gestation to 1 week after delivery. The primary outcome is recurrent wheeze in the first 3 years. This is a factorial randomised controlled trial with a second intervention of prenatal fish oil or no fish oil. In Southampton the Maternal Vitamin D Osteoporosis Study (MAVIDOS; ISRCTN 82927713) is randomising 1074 pregnant women with a 25(OH)D3 concentration of 25-100nmol/l during pregnancy who receive vitamin D supplementation at a dose determined in pilot work, or placebo from 14 weeks gestation to delivery. Primary outcome measures relate to bone health, but the trial may also yield information on pulmonary and atopic outcomes at follow up. In our own study (ISRCTN 68645785) 180 women in London with a high prevalence of vitamin D deficiency, were randomised to no additional vitamin D in pregnancy, 200,000IU of calciferol once at 27 weeks gestation or 800IU daily of ergocalciferol from 27 weeks to delivery. The primary outcome measure is history of wheezing during the first 3 years of life, and this study is due to report in 2011.

CONCLUSIONS

The precise relationship between vitamin D status and asthma, and the impact of dietary vitamin D supplementation on this risk, are unclear. Potential mechanisms through which perinatal vitamin D may influence asthma or lung development include increased immune regulation preventing atopic immune responses, increased microbial killing capacity limiting the host consequences of viral infections, and direct effects on lung growth and development particularly on alveolarisation, fibroblast proliferation and airway smooth muscle. Interactions with correlated nutrients such as vitamin A, which interacts with vitamin D metabolites in essential ways but can antagonise in excess, may complicate this relationship [98, 99].

Current intervention trials evaluating the effects of early life vitamin D supplementation at a range of doses, in a range of populations and using a range of treatment schedules will play a critical role in establishing the possible role that vitamin D may play in this regard.

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Perinatal Vitamin D Deficiency and Childhood Asthma: A Molecular Perspective

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Abstract: There is a large body of literature suggesting that the recent increase in the incidence of childhood asthma might be associated with vitamin D deficiency during lung development. There are also strong experimental animal data showing that vitamin D is one of the local alveolar paracrine factors that spatiotemporally modulates perinatal pulmonary maturation. However, the mechanistic link between vitamin D deficiency during pregnancy and childhood asthma is not known. In this review, we demonstrate how perinatal vitamin D deficiency could mechanistically drive both the proximal and distal airways to a myogenic phenotype, molecularly and structurally, predisposing the offspring to asthma. More specifically, we will review how perinatal vitamin D deficiency results in an increased abundance of mesenchymal myofibroblasts, a feature that is highly consistent with the pathophysiology of asthma. We also provide evolutionary insights as to how vitamin D deficiency might (re)activate the atavistic host defense mechanisms that could precipitate a lung phenotype consistent with asthma. Since vitamin D deficiency seems to alter the normal homeostatic epithelial-mesenchymal signaling pathways in the developing lung, it offers a distinct translational opportunity to prevent this process through targeted molecular manipulations. While we wait for the results of on-going trials of vitamin D supplementation during pregnancy to get some definitive answers on its role in the pathogenesis of childhood asthma, we advocate studies to discover new vitamin D analogs and/or metabolites with optimal respiratory effects and without any significant side effects.

Keywords: Vitamin D, asthma, pregnancy, epithelial-mesenchymal interactions, PTHrP, PPAR γ , Wnt signaling.

INTRODUCTION

Asthma is the most common chronic disease of childhood in the world [1], resulting in a significant medical burden and healthcare costs [2]. The burden of this disease is increasing rapidly, with over 300 million people affected worldwide [1]. A large body of literature suggests that the recent increase in the incidence of asthma might be associated with vitamin D deficiency during lung development. For example, many recent studies demonstrate a high prevalence of vitamin D deficiency among women of child-bearing age, and in pregnant women, resulting in a large number of infants being born deficient in vitamin D [3-8]. Additionally, a number of recent studies have demonstrated that a higher maternal vitamin D intake during pregnancy or early life is inversely correlated with risk of asthma and allergy in later life [9-12]. However, the mechanism by which vitamin D deficiency during gestation and early lung development is linked to childhood asthma is not well established. And, in fact, some studies also suggest a detrimental effect of vitamin D supplementation on the development of allergy [13, 14], suggesting that a more detailed understanding of vitamin D's role in lung development and asthma is necessary.

If there is a real causal relationship between perinatal vitamin D deficiency and childhood asthma, then we would

expect that vitamin D would promote signaling pathways that are known to be important for normal lung development, and its deficiency would dysregulate these pathways, leading to a lung molecular and structural phenotype which predisposes to asthma. In this review, we demonstrate how perinatal vitamin D deficiency could mechanistically drive both the proximal and distal airways to a myogenic phenotype, molecularly and structurally, predisposing the offspring to asthma. More specifically, we will describe how perinatal vitamin D deficiency results in an increased abundance of mesenchymal myofibroblasts, a feature that is highly consistent with the pathophysiology of asthma [15, 16]. Fig. (1) outlines the proposed effects of vitamin D on lung development that might modulate asthma. Understanding the molecular mechanisms underlying vitamin D-associated childhood asthma could be crucial in designing effective preventive and therapeutic strategies against childhood asthma.

Vitamin D's role in Proximal Airway Development:

During lung development, mesodermal fibroblasts, which provide the scaffolding for both the proximal and distal airways, are dominated by the Wnt pathway, which is the default program for the muscle phenotype [17]. The Wnt pathway actively down-regulates CCAAT/enhancer-binding protein α (C/EBP α) and its downstream targets [18]. The functionally relevant molecular intermediates in the Wnt pathway are glycogen synthase kinase β (GSK3 β), β -catenin, lymphoid enhancer-binding factor 1/T cell protein (LEF-1/TCP), C/EBP α and peroxisome proliferator-activated receptor γ (PPAR γ). These molecules interact canonically, including phosphorylation, proteolysis, and ubiquitination. This cascade culminates in the expression of myogenic genes

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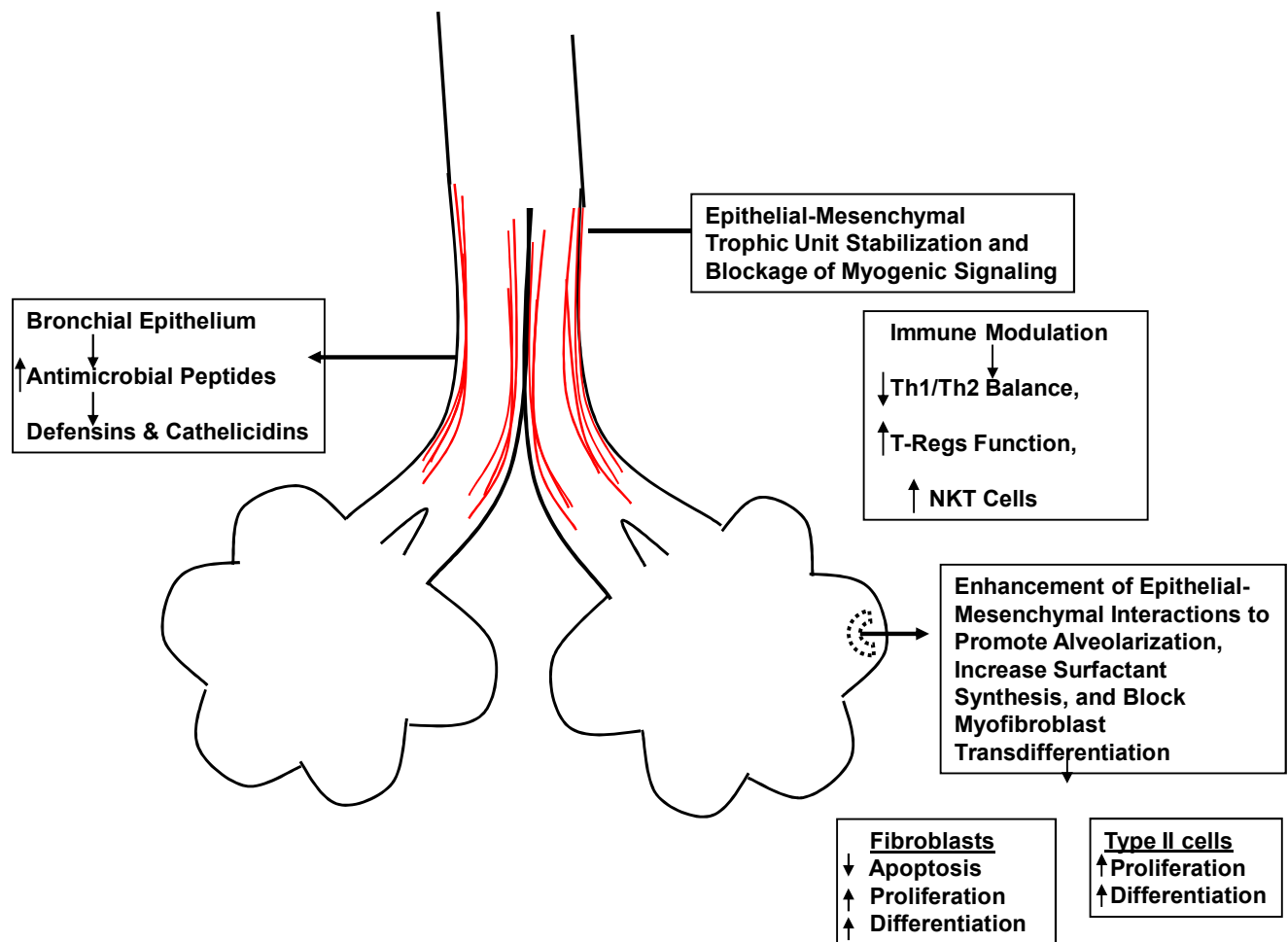


Fig. (1). Proposed effect of vitamin D that might modulate asthma.

such as myosin heavy chain, α smooth muscle actin and calponin. Recently, the vitamin D receptor (VDR) has been shown to be expressed in airway smooth muscle, and its activation inhibits smooth muscle proliferation [19]. Persistent expression of some of the early markers of myogenic differentiation has also been reported in VDR knockout mice [20], suggesting that vitamin D deficiency during development could lead to an enhanced myogenic phenotype of the proximal airways. Furthermore, the epithelium of the asthmatic airways is known to be structurally and functionally abnormal, with increased susceptibility to injury by endogenous and exogenous exposures and an impaired ability to repair [21].

Vitamin D's Role in Distal Airway Development:

Alveolar epithelial-mesenchymal interactions play a critical role in perinatal pulmonary maturation, which is characterized by an increase in surfactant synthesis and alveolar wall thinning. Adepithelial lipid-laden interstitial lung fibroblasts, or lipofibroblasts (LIFs), which are normally present in abundance at birth, and which undergo apoptosis during the perinatal period, play a significant role in both surfactant synthesis and alveolar septal thinning [22, 23]. Recently, vitamin D has been shown to spatio-temporally coordinate alveolar epithelial and LIF proliferation and differentiation, leading to increased

surfactant synthesis and alveolar septal wall thinning [24]. These effects are mediated by vitamin D's stimulatory effect on homeostatic Parathyroid Hormone-related Protein/Peroxisome Proliferator-Activated Receptor γ (PTHrP/PPAR γ) epithelial-mesenchymal signaling, and inhibitory effect on myogenic Wnt signaling.

During normal lung development, under the influence of Sonic Hedgehog, the developing endoderm expresses PTHrP, along with its receptor, on the adjacent mesenchyme [25]. PTHrP binding to its receptor on the mesenchyme activates cAMP-dependent PKA signaling [26], which down-regulates the mesenchymal default Wnt pathway [27, 28], and up-regulates the adipogenic pathway through the key nuclear transcription factor PPAR γ [29] and its downstream regulatory genes such as Adipocyte Differentiation-Related Protein (ADRP) [30] and leptin [31, 32]. ADRP is necessary for the transit of neutral lipids from the LIF to the alveolar type II (ATII) cell for surfactant phospholipid synthesis [30]. Lipofibroblasts, in turn, secrete leptin, which acts on its receptor on ATII cells, stimulating both surfactant phospholipid and protein synthesis [32]. Since PTHrP stimulates leptin production by LIFs, it provides a complete growth factor-mediated homeostatic paracrine loop for the synthesis of pulmonary surfactant. Hence, alveolar PTHrP/PPAR γ signaling, by inhibiting Wnt signaling,

inhibits the default myogenic phenotype, and induces the lipogenic phenotype, which, through its autocrine effect on LIFs and its paracrine effect on ATII cells, is necessary for alveolar development and homeostasis. This mechanism appears to apply to both rodents [33] and primates [34] alike. The net result of alveolar vitamin D epithelial-mesenchymal paracrine signaling is increased substrate for surfactant phospholipid synthesis, enhanced epithelial and mesenchymal differentiation and septal wall thinning [22, 24].

These data clearly demonstrate vitamin D's critical role in the development of both the proximal and distal airways, and show how its deficiency during pregnancy could interfere with homeostatic lung signaling pathways, resulting in the up-regulation of the default Wnt signaling-driven myogenic pathway in both the proximal and distal airways, thereby predisposing offspring to bronchial hyper-responsiveness. The association of Wnt signaling genes and impaired lung function in two childhood asthma cohorts reported in a recent study further validates the link between vitamin D deficiency/Wnt signaling and childhood asthma [35].

Vitamin D's Role in the 'Evolution' of Asthma: Even deeper insights into the etiology of asthma come from the all-encompassing evolutionary overlap between PTHrP/PPAR γ , Wnt signaling and environmental factors. We have previously shown how and why lung evolution has been driven by the interaction between external and internal factors that can be traced all the way back to the swim bladder of fish [36], raising the question as to how and why vitamin D evolved to promote lung development and homeostasis. The mechanisms of lung development, environmental selection pressure and vitamin D intersect functionally through vitamin D's roles in maintaining epithelial host defense by stimulating both surfactant proteins and antimicrobial peptides. In vertebrates, antimicrobial peptides are inhibited by salinity, and in fish, the salinity of the water environment activates epithelial vitamin D by increasing its hydroxylation [37], counterbalancing the loss of antimicrobial peptide activity. As is the case for many such counterbalancing selection pressures, vitamin D hydroxylation has evolved as a constitutive cis regulatory mechanism through selection pressure. Therefore, vitamin D deficiency may (re)activate the atavistic antimicrobial peptide mechanism, promoting host defense, but may inadvertently precipitate dysmorphogenesis, as we have observed with the cytokine agonist lipopolysaccharide, which disrupts normal cell-cell interactions [38]. The recrudescence of such deep evolutionary homologies in complex diseases has been termed decanalization [39], referring to the reversal of the mechanism of canalization that Waddington used to describe evolution [40]. Such evolutionary insight to the genetic 'history' of asthma offers novel, counterintuitive ways to effectively preempt the asthma phenotype using the evolutionary strategy. As a note in proof of the overlap between vitamin D, host defense and asthma, antimicrobial peptides expressed in airway epithelial cells and skin are regulated by vitamin D [41]. These antimicrobial peptides fail to up-regulate in atypical dermatitis [42], which is closely associated with asthma. Therefore, vitamin D

deficiency may precipitate both atopy and asthma through its effect on antimicrobial peptide production in lung and skin.

In conclusion, this review introduces a novel concept that vitamin D deficiency during pregnancy and the perinatal period interferes with normal developmental, homeostatic lung signaling pathways, resulting in up-regulation of the default myogenic pathways of both the proximal and distal airways, thereby predisposing offspring to bronchial hyper-responsiveness. This structural predisposition to asthma due to perinatal vitamin D deficiency is fundamentally different from the previously proposed allergy hypothesis, which, with or without increased frequency and/or severity of respiratory infections, has been linked to vitamin D deficiency and childhood asthma [43, 44]. In experimental allergic models of asthma, either no benefit [13], some benefit [45, 46], or even harm [13, 45] has been reported with vitamin D supplementation, undermining the validity of the allergy hypothesis. However, it is important to emphasize that the concept proposed in this review and the previously proposed allergy hypothesis may not be mutually exclusive, but may act in tandem.

The hypothesis that vitamin D deficiency alters the normal homeostatic epithelial-mesenchymal signaling pathways in the developing lung affords a unique molecular/mechanistic perspective on asthma pathogenesis, and provides a direction for translational research to prevent this process through targeted molecular manipulations. Though the results of on-going trials of vitamin D supplementation during pregnancy are likely to provide some definitive answers on the role of vitamin D deficiency in the pathogenesis of childhood asthma, its optimal dose may still not be known in the near future. Moreover, the discovery of a vitamin D analog or metabolite that exerts beneficial respiratory effects without inducing adverse effects associated with the physiologically active hormone dihydroxycholecalciferol is a research priority. Lastly, since asthma is both genetic and environmental in origin, it is possible that genetic variants in PTHrP/PPAR γ /Wnt signaling might influence the effects of vitamin D on childhood asthma phenotype.

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Vitamin D and Asthma: Scientific Promise and Clinical Reality

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Abstract: Widespread vitamin D insufficiency and vitamin D supplementation (even low dose rickets prophylaxis) have been hypothesised as contributory factors to the recent increase in asthma. These hypotheses are supported by reports of immunomodulatory effects of vitamin D on antigen presenting cells, regulatory T cells and T-helper cells and evidence that vitamin D influences fetal lung differentiation and epithelial-mesenchymal function. Studies of vitamin D in animal models confirm complex effects of vitamin D on asthma immunopathogenesis. In humans a majority of epidemiological studies support the hypothesis that vitamin D insufficiency during pregnancy increases the likelihood of childhood wheeze and possibly asthma, although some studies do report the converse. In children and adults with asthma, reduced serum vitamin 25-hydroxyvitamin D levels have been associated with parameters of increased asthma severity. Clinical trials are underway addressing whether maternal vitamin D supplementation during pregnancy reduces the likelihood of childhood asthma and if there is a role for vitamin D supplementation in established asthma.

Keywords: Vitamin D, asthma, pregnancy, T cells, lung development, prevention, treatment.

INTRODUCTION

The recognition that changing environmental exposures underlie the recent increase in asthma and allergic disease has stimulated research attempting to identify responsible factors with the ultimate aim of implementing primary preventive interventions. In the last five years there has been increasing interest in the possible association between changes in vitamin D status and asthma prevalence, with a remarkably rapid transition from basic science and observational studies to the setting up of clinical trials investigating the potential of vitamin D supplementation to prevent asthma and to complement conventional asthma treatment. In this review we will outline the hypotheses relating vitamin D to asthma and allergy, summarise the biological properties of vitamin D considered to be pertinent, discuss studies of vitamin D in rodent models and detail epidemiological studies of vitamin D and asthma.

VITAMIN D AND ASTHMA HYPOTHESES

Three somewhat contradictory but not irreconcilable hypotheses link vitamin D to wheezing symptoms, asthma and allergy:

In 1999 Wjst and Dold hypothesised an adverse effect of vitamin D on the likelihood of asthma and allergy [1]. They observed that the increase in asthma and allergy prevalence followed, to some extent, the geographical and temporal use of vitamin D supplementation as rickets prophylaxis from the 1950s onwards in many westernised countries in the form of cod liver oil and fortification of milk, cereal and margarine [2]. It was suggested that the lower prevalence of asthma and allergy reported in anthroposophic communities and farms could be explained by the lifestyle-associated

avoidance of vitamin D supplements. A further suggestion was that the vitamin D fortification policies in the USA, UK and Germany may have contributed to the higher prevalence of asthma in these countries [2].

In 2007 Litonjua and Weiss hypothesised that the recent increase in asthma and allergy was a consequence of the widespread vitamin D insufficiency that has been well documented in westernised countries [3]. This insufficiency is believed to be a consequence of an increasing tendency to stay indoors for the purposes of recreation and employment and the health promotion message of covering up and using sun block whenever exposed to direct sunshine in order to reduce the risk of melanoma.

In 2009, Camargo and colleagues [4] questioned whether there is any association between vitamin D status at physiologic levels and the risk of asthma. They suggested that the reported associations between reduced vitamin D status and increased childhood wheezing illness (see below) reflect adverse effects of reduced vitamin D status on innate immune responses with consequential increases in the frequency and/or severity of acute respiratory infections. It was speculated that any associations with asthma reflected the diagnostic difficulties associated with recurrent wheezing episodes consequent upon an increased susceptibility to respiratory infections because of vitamin D insufficiency.

All of these hypotheses are supported by studies that have investigated the effects of vitamin D on innate and adaptive immune processes.

IMMUNOMODULATORY EFFECTS OF VITAMIN D

Asthma and allergic diseases are inflammatory conditions initiated and perpetuated by CD4⁺ T-helper (Th) cells of the Th2 phenotype. Regulatory T cells that can directly inhibit both Th1 and Th2 responses have been implicated in the immunopathogenesis of asthma and allergic disease with several studies highlighting IL-10

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secreting Tr1 regulatory T cells and CD25⁺ regulatory T cells [5]. Given that effector Th-cells and regulatory T cells are critical to the immunopathogenesis of asthma and allergy it seems likely that any environmental factor contributing to the increase in these conditions will have immunomodulatory properties to alter the balance of Th1/Th2 differentiation, either directly and/or indirectly *via* effects on antigen presenting cells and/or regulatory T cells.

Vitamin D has potent immunomodulatory properties affecting cells of the innate and adaptive immune system. 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) induces expression of antimicrobial peptides such as defensins and cathelicidin by myeloid-derived cells [6] and bronchial epithelial cells [7]. The most well-established effects of vitamin D on the adaptive immune system are its actions on Th-cells and regulatory T cells. 1,25(OH)₂D₃ directly inhibits Th1 interferon (IFN)- γ responses in murine [8] and human [9] Th1 cells and in the presence of the corticosteroid dexamethasone the inhibitory effect of 1,25(OH)₂D₃ on IFN- γ release is augmented [10]. The effect of 1,25(OH)₂D₃ on Th2 responses is more variable with reports of negligible effects on IL-4 release from human Th2 clones [9] and inhibition of IL-4, IL-13 and IFN- γ by 1,25(OH)₂D₃ in *ex vivo* studies of cord blood T cells obtained from neonates [11]. An increase in IL-5 and IL-13 release upon the addition of 10⁻⁶M 1,25(OH)₂D₃ to *ex vivo* Th-cells has also been reported in the absence and presence of dexamethasone [10]. A more recent study has demonstrated dose-dependent inhibition of both Th1 (IFN- γ) and Th2 (IL-13) responses with 1,25(OH)₂D₃, except at the highest concentration tested (10⁻⁶M), where less suppression but no enhancement of either Th1 or Th2 responses was observed [12]. These studies suggest that vitamin D exerts variable dose-dependent effects on Th1/Th2 balance, possibly augmenting Th2 differentiation at higher concentrations.

The effects of 1,25(OH)₂D₃ on the immune response are more complex than direct influences on Th-cells alone, and include actions on regulatory T cells and antigen presenting cells. When stimulated in the presence of dexamethasone, human regulatory T cells usually secrete high levels of IL-10, which is a potent anti-inflammatory and immunosuppressive cytokine [10]. In contrast, the *ex vivo* addition of dexamethasone to regulatory T cells from subjects with corticosteroid resistant asthma fails to induce the IL-10 response observed in cells from normal subjects [13]. However, IL-10 responsiveness to dexamethasone is nearly completely restored by treatment of the regulatory T cells from corticosteroid-resistant asthmatics with 1,25(OH)₂D₃ either *in vitro* or by the oral administration of 1,25(OH)₂D₃ to subjects [13]. More recently it has been reported that 1,25(OH)₂D₃ at concentrations $\leq 10^{-7}$ M induces dose-dependent changes in *ex vivo* cytokine responses with suppression of Th1 (IFN- γ) and Th2 (IL-13) responses but stimulation of IL-10 secretion. However, at a higher concentration (10⁻⁶M) of 1,25(OH)₂D₃, IL-10 responses declined substantially [12]. Vitamin D has been shown to promote a tolerogenic phenotype in human dendritic cells with *in vitro* 1,25(OH)₂D₃ treatment of dendritic cells leading to the induction of Foxp3⁺ regulatory T cells with suppressive activity [14]. The complex effects of vitamin D on immune responses encompass effects on antigen presenting cells, regulatory T cells and Th-cells that appear

to be dose- and possibly age-dependent. In addition to its immunomodulatory properties, vitamin D has also been implicated in fetal lung development and interactions between bronchial epithelial and mesenchymal elements involved in asthma pathogenesis.

VITAMIN D AND AIRWAY EPITHELIAL-MESENCHYMAL DEVELOPMENT AND FUNCTION

Although central to asthma pathogenesis, immunologically driven inflammation alone does not explain many features of asthma [15]. Holgate has highlighted evidence that the epithelium of asthmatic airways is structurally and functionally abnormal, with an increased susceptibility to injury by endogenous and exogenous exposures and an impaired ability to repair [15, 16]. The intrinsic airway epithelial abnormality, particularly the interaction with smooth muscle cells, is believed to contribute to airway remodelling and also increases the likelihood of asthmatic airway inflammation, particularly if there is a parallel development of a Th2-biased immune response [15, 17]. It is likely that intrinsic airway epithelial abnormalities develop in early life, probably *in utero* [17]. Vitamin D has been implicated in fetal airway development, the processes of airway remodelling and airway defence - processes known to be abnormal in asthma. In fetal rat lung explants there is expression of vitamin D receptor (VDR) sites during the process of type II pneumocyte differentiation [18, 19]. In addition, in fetal rat lung 1,25(OH)₂D₃ has been reported to play a critical role in perinatal lung maturation by stimulating epithelial-mesenchymal differentiation and proliferation of lipofibroblasts and type II pneumocytes [20]. Further evidence for an effect of vitamin D on epithelial-mesenchymal function comes from the demonstration that VDR is present in human airway smooth muscle cells and that stimulation of these cells by 1,25(OH)₂D₃ upregulates genes implicated in morphogenesis and cell growth as well as genes encoding proteins implicated in airway remodelling such as vascular endothelial growth factor (VEGF) and fibronectin FN1 [21]. Vitamin D also regulates airway epithelial cell secretion of antimicrobial peptides such as defensins and cathelicidin [22], the secretion of which is known to be reduced in asthma [23]. Clearly vitamin D has the potential to influence the immunological and airway developmental processes central to the airway inflammatory and remodelling processes characteristic of asthma. However, it is not possible to predict reliably whether vitamin D adversely or beneficially influences the development of asthma and allergic disease in humans. These issues have been addressed in a series of investigations in rodent models of asthma that induce eosinophilic airway inflammation by sensitisation and airway challenge with ovalbumin as an antigenic stimulus.

VITAMIN D RECEPTOR KNOCKOUT MICE

VDR-dependent modulation of experimental asthma has been described in a series of studies using VDR knockout (KO) mice, which are deficient in the nuclear receptor that binds and mediates 1,25(OH)₂D₃ effects. Ovalbumin sensitization and challenge induced airway hyper-reactivity, mucus production and inflammation in wild type (WT), but not VDR knockout, mice [24]. This lack of airway response

occurred despite significant production of IgE in KO mice compared to allergic WT mice. Even adoptive transfer of activated splenocytes from allergic WT mice could not induce the allergic airway phenotype in VDR KO mice [25]. Conversely, immune-activated splenocytes derived from VDR KO mice were able to induce allergic airway inflammation when transferred to WT mice. These results suggest that, despite appropriate immune activation of T cells in VDR KO mice, their recruitment to the lung is compromised. Interestingly, a similar deficit in T cell homing is proposed to underlie the susceptibility of VDR KO mice to develop experimental inflammatory bowel disease [26]. In these later studies, the severity of disease was associated with lack of recruitment and homing of CD4 and CD8 cells to gut epithelium. Notably, deficits in airway recruitment in VDR KO mice are not limited to Th2 signals, but are also compromised in response to airway exposure to lipopolysaccharide [25].

STUDIES OF VITAMIN D DEFICIENCY AND SUPPLEMENTATION IN RODENTS

Few studies of allergic responses have examined naturally occurring deficiency by feeding mice vitamin D depleted diets. Wittke and co-workers [25] reported that mice fed vitamin D deficient diets started in late gestation could still respond with an allergic airway phenotype after ovalbumin sensitization and challenge in adolescence. However these responses in deficient mice were not compared with allergic mice fed a normal vitamin D sufficient diet. In rodent models of developmental vitamin D deficiency, wherein females are placed on deficient diets well before breeding, the offspring display altered immune function. For example, contact hypersensitivity in male (but not female) BALB/c mice is enhanced in animals derived from vitamin D deficient colonies [27]. In similar studies of 10 week-old Sprague Dawley rats derived from gestational vitamin D deficiency, spleen and thymus weights were increased and cytokine production from blood mononuclear cells were enhanced compared to rats derived from normal gestational diets [28]. These dietary regimens more closely model the reported associations of an increased likelihood of wheeze and asthma in children from mothers with maternal dietary vitamin D deficiency (see below). However the effects of vitamin D deficiency *in utero* have yet to be tested in rodent models of eosinophilic airway inflammation.

INTERVENTION WITH VITAMIN D IN ALLERGIC MICE

Several animal studies have investigated the effects of vitamin D supplementation in established asthma and allergy. Mice require approximately 125 ng of vitamin D₃ (cholecalciferol) daily [29], of which approximately 50% is absorbed [30]. Most treatment studies in rodents however, have used the VDR-binding metabolite 1,25(OH)₂D₃, or calcitriol. These intervention studies in allergic mice typically employ doses of 100-200ng of calcitriol, which is similar to the ingested amount of the nonactive parent compound, and is usually delivered by bolus injection by subcutaneous (s.c.) and intraperitoneal (i.p.) routes. As such these protocols rely on pharmacologic doses of the bioactive, VDR-binding hormone, rather than addressing food intake of

vitamin D₃. For example daily administration of 100 ng calcitriol can decrease allergic pulmonary inflammation and histopathological lesions when given to mice by i.p. [31] or s.c. injections [32]. Conversely, daily ingestion of 200ng calcitriol in rodent chow has no effect on the development of allergic airway disease in the ovalbumin sensitized BALB/c mouse [24]. However this same dietary regimen is effective in IBD mouse models [33]. Given that these doses of calcitriol are on the order of 150-200 times greater than therapeutic doses in humans, the translational value of these studies to understand allergic airway responses is limited. Meanwhile, approaches that modify vitamin D₃ (cholecalciferol) intake, *via* either supplements or food choices, have not been reported in experimental asthma models. As such, while the results from animal studies corroborate in part the immunomodulatory ability of vitamin D demonstrated *in vitro*, significant problems of pharmacokinetics, molecular formulations, and toxicity need to be addressed.

OBSERVATIONAL STUDIES OF VITAMIN D AND ASTHMA IN HUMANS

Epidemiological investigations of the association between vitamin D and asthma lag somewhat behind scientific investigations of the effects of vitamin D on immune function and airway cell biology *in vitro* and in animal models. Epidemiological studies of vitamin D and asthma can be broadly divided into those investigating associations between asthma and early life vitamin D status during fetal development and infancy, and those that have investigated vitamin D status in subjects with asthma.

Early Life Vitamin D Status

Five birth cohort studies have reported associations between maternal vitamin D status during pregnancy and childhood wheeze and asthma outcomes [34-38]. Whilst the studies have been conducted prospectively in populations that differ in dietary patterns, smoking habits, socio-economic profiles and probably genetic susceptibility all have methodological weaknesses. Four studies did not fully assess maternal vitamin D status during pregnancy, reporting associations with maternal vitamin D intake, thus failing to account for the substantial contribution of sunlight derived vitamin D [34-37]. Project Viva in the US reported reduced maternal vitamin D intake during pregnancy to be associated with an increased likelihood of wheezing outcomes in 1194 children aged 3 (e.g. recurrent wheeze, highest vs lowest quartile of maternal intake OR 0.39, 95% confidence interval 0.25-0.62) [34]. In Scotland reduced maternal vitamin D intake during pregnancy has been reported to be associated with an increased likelihood of wheezing outcomes in 1212 children aged 5 (e.g. current wheeze highest vs lowest quintile of maternal intake OR 0.35, 95% confidence interval 0.15-0.83); however this study was notable for reporting no association between maternal vitamin D intake and asthma, lung function or exhaled nitric oxide in children aged 5 years [35]. A Japanese birth cohort study similarly reported reduced maternal vitamin D intake during pregnancy to be associated with an increased likelihood of wheezing in 762 children aged 16-24 months (e.g. current wheeze highest vs lowest maternal intakes (divided at 25th centile) OR 0.64,

95% confidence interval 0.43-0.97); however it is likely that this association reflects the isolated finding of a markedly reduced likelihood of wheezing in children born to mothers in the second quartile of vitamin D intake [36]. Reduced maternal vitamin D intake during pregnancy has been reported to be associated with an increased likelihood of asthma in 1669 Finnish children aged 5, hazard ratio 0.76, 95% confidence interval 0.59–0.99 [37]. However this study quantified maternal diet postnatally and the children were selected for their increased risk of type I diabetes because of their HLA-DQB1 genotype. A weakness of these studies is that vitamin D intake alone was measured, failing to quantify vitamin D generated from sunlight exposure. Serum 25-hydroxyvitamin D (25[OH]D) concentration is considered to be the best circulating biomarker of vitamin D status, reflecting contributions from dietary and sunlight exposure. An adverse association between maternal serum 25(OH)D concentration during the third trimester of pregnancy and asthma in 178 children aged 9 years has been reported in a UK birth cohort study (maternal serum 25(OH)D >75 nmol/l vs <30 nmol/l OR 5.40, 95% confidence interval 1.09-26.65); however the loss to follow up (62%) was substantial [38]. High dose (2000 IU/day) vitamin D supplementation during infancy has also been adversely associated with allergic outcomes (atopic sensitisation, allergic rhinitis) in 7,648 adults aged 31 years participating in a Finnish birth cohort survey [39]. The likelihood of asthma in these adults was also increased in those supplemented during infancy (OR 1.33, 95% confidence interval 0.97-1.82), but this was not statistically significant ($p=0.08$). Taken as a whole these studies suggest that reduced maternal vitamin D intake during pregnancy is associated with a higher likelihood of childhood wheezing and maybe asthma. However, despite methodological limitations it should be noted that the likelihood of childhood allergic and asthma outcomes has been adversely associated with high maternal serum 25(OH)D during pregnancy and high-dose (2000 IU/day) vitamin D supplementation during infancy. The possible dose-dependent associations with vitamin D are analogous to those reported in some immunological studies [12].

Vitamin D in Asthma

The immunomodulatory properties of vitamin D justify studies of vitamin D status in people with asthma because of the potential use of vitamin D supplements to complement conventional asthma therapy; conversely, an adverse association could lead to a re-evaluation of osteoporosis vitamin D prophylaxis in asthma. A number of studies have investigated the association between serum 25(OH)D concentration and parameters of asthma severity in adults and children. Brehm and colleagues reported low serum 25(OH)D concentrations to be associated with elevated total IgE and eosinophil counts and increased likelihood of methacholine airway responsiveness, asthma-related hospitalization and use of anti-inflammatory medication in 616 Costa Rican children with asthma aged 6-14 [40]. Brehm and colleagues have also quantified the serum 25(OH) D concentrations of the 1024 children with mild to moderate asthma participating in the Childhood Asthma Management Program [41]. In cross-sectional and prospective analyses, vitamin D insufficiency (25(OH)D \leq 75 nmol/L) was associated with an increased likelihood of an

asthma exacerbation necessitating hospitalisation or a visit to the Emergency Department in the year prior to (OR 1.7, 95% CI 1.2-2.7), and the four years after (OR 1.4 95% CI 1.0-1.9) measurement of serum 25(OH)D concentration. Similar associations between reduced serum 25(OH)D concentration and increased use of inhaled corticosteroids, use of oral corticosteroids, raised serum IgE and number of positive aeroallergen skin prick tests have also been reported in 100 children with asthma aged 0-18 years (median age 7 years, interquartile range 4-10 years) [42]. The same study also reported positive associations between increased serum 25(OH)D concentration and increased FEV1 and FEV1:FVC ratio. The interpretation of this study is likely to be complicated by a number of design features. Some very young children with asthma were included; furthermore there is a high likelihood of significant selection bias because the studied children were identified by the fact that they had had a serum 25(OH)D measurement as part of their clinical management. In 54 adults with asthma reduced serum 25(OH)D concentration has been reported to be associated with reduced FEV1, and in the same study vitamin D insufficiency (serum 25(OH)D <75 nmol/L) was associated with an increased likelihood of methacholine airway responsiveness [43]. Whilst these studies suggest an association between severity of asthma and vitamin D, it is not possible to conclude from these studies that people with asthma have a reduced vitamin D status because the studies outlined above did not include control subjects. A recent case-control study of 80 subjects with asthma aged 15-50 years and 80 closely matched control subjects reported no difference in serum total 25(OH)D between subjects with asthma (mean 10.1 ng/ml, 95% CI 8.3-11.9) and those without (mean 10.1 ng/ml, 95% CI 8.4-11.7) [44]. In the subjects with asthma serum total 25(OH)D was not associated with FEV1 or asthma severity. This study suggests that serum 25(OH)D concentration is not reduced in asthma, and does not support the widespread use of vitamin D to supplement conventional asthma therapy. A notable feature of this study were the very low 25(OH)D levels (mean 10.1 ng/ml 95% CI 8.9-11.3) and this may have limited the ability of the study to detect a difference; of note, there was no difference in serum total 25(OH)D concentrations between cases and controls in the two participating centres, one in the North East of Scotland (mean 7.4 ng/ml, 95% CI 5.9-9.0) and the South East of England (mean 14.1 ng/ml, 95% CI 12.6-15.5). It is worth noting that an association between maternal vitamin D intake during pregnancy and childhood wheeze has been reported in women of the same age living in the same Scottish Region [35]. Although the very limited data to date suggest that universal vitamin D supplementation of people with asthma is not currently justified, the possibility remains that vitamin D supplementation may be beneficial in select groups of people with asthma e.g. corticosteroid resistant, severe, and indeed randomised controlled trials are underway to directly address these issues.

Two studies of vitamin D in adults, whilst not directly relevant to asthma warrant discussion. In NHANES III the highest quartile of serum 25(OH)D (>85.7 nmol/l) was associated with an increased FEV1 (106ml) and FVC (142ml) in 14,091 adults [45]. The absence of any association between serum 25(OH)D and FEV1:FVC ratio

suggests that in adults vitamin D is not associated with airflow obstruction, but rather the associations are in keeping with an effect of vitamin D on lung growth and/or rate of decline of lung function. A non-linear association between serum 25(OH)D and serum IgE has been reported in 7288 adults aged 45 participating in the UK 1958 birth cohort study [46]. Serum IgE was elevated by 29% in those with a serum 25(OH)D in the lowest quartile and by 56% in those in the highest quartile. This non-linear “U” shaped association is analogous to the dose-dependent associations with vitamin D status reported in some immunological studies.

CONCLUSIONS

The increasing interest in vitamin D and asthma mirrors a generalised increase in interest in vitamin D in relation to many conditions such as cancer, infection, cardiovascular disease, schizophrenia, multiple sclerosis, inflammatory bowel disease and diabetes [47]. The scientific debate surrounding vitamin D and asthma is somewhat unusual in that beneficial, adverse and null effects have been hypothesised, with all being supported to some extent by studies of cellular immunology, airway cell biology, animal models and epidemiological studies. Currently the major questions in the field are whether vitamin D supplementation during pregnancy reduces the likelihood of childhood asthma, and whether there is a role for vitamin D supplementation in asthma. The available evidence suggest that whilst people with asthma appear not to be more deficient in vitamin D than normal subjects [44] there is some evidence suggesting that children and adults with severe asthma have reduced vitamin D status when compared with those with less severe asthma [40-43]. Trials are currently underway to ascertain whether vitamin D supplementation increases the therapeutic effectiveness of corticosteroids in asthma and is beneficial in severe asthma.

Based on the results of epidemiological studies and consideration of biological properties, vitamin D supplementation during pregnancy has been advocated for pregnant women as a preventative for childhood asthma [48]. Intervention trials of vitamin D supplementation during pregnancy with the intention of primary asthma prevention raise a number of difficult scientific, ethical and regulatory issues. Whilst indeed a majority of observational studies suggest that vitamin D supplementation during pregnancy should reduce the likelihood of childhood wheeze and possibly asthma, it is important to highlight the significant minority of studies suggesting that perhaps vitamin D supplementation during pregnancy and infancy may have an adverse effect on asthma. In mice, higher doses with the precursor vitamin D₂ can result in malformed fetuses [49]. It has only been recently described that the endometrium during pregnancy expresses VDR and synthesizes 1,25(OH)₂D [50, 51]. That vitamin D can suppress signals of normal cell cycling [52] and inhibit myometrial cell growth *in vitro* [53], suggests the *in utero* environment during pregnancy could be sensitive to high concentrations of vitamin D, and therefore defining the therapeutic index for adverse versus beneficial effects is critical. Despite these concerns the evidence to date is that high dose vitamin D supplementation (up to 4,000 IU/day) during pregnancy is

safe [54]. It still remains to be seen whether safety in a relatively small trial of women is translated into safety for millions of pregnant women and fetuses.

Further observational, immunological, cellular and animal studies are unlikely to resolve the issues surrounding the potential for vitamin D supplementation to prevent asthma and therefore scientific, ethical and economic considerations justify the interventional trials of vitamin D supplementation in pregnant women that are underway in the US, Denmark and New Zealand. The results of these trials are likely to provide definitive answers in the near future as to whether childhood asthma is reduced, increased or unaffected by maternal vitamin D supplementation during pregnancy.

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Vitamin D in COPD - A Pleiotropic Micronutrient in a Multisystem Disease

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Abstract: Chronic Obstructive Pulmonary Disease (COPD) is a debilitating disease affecting an estimated 3 million people in the United Kingdom. It is characterised by progressive and irreversible airway obstruction and lung parenchymal damage, and by multisystem involvement including skeletal muscle impairment, systemic inflammation, and an increased prevalence of osteoporosis, cardiovascular disease and lung cancer.

Patients with COPD have reduced dietary intake of vitamin D, spend a reduced amount of time outdoors and have been shown to have lower levels of 25-hydroxyvitamin D (25[OH]D) than age-matched subjects without COPD. The active metabolite of vitamin D, 1,25-dihydroxyvitamin D (1,25[OH]₂D), is a pleiotropic hormone with effects on lung development and function, the immune system and musculoskeletal function; vitamin D deficiency also associates with increased prevalence of cardiovascular disease and cancer. This article focuses on the evidence that vitamin D deficiency is highly prevalent in patients with COPD and reviews associations between vitamin D status and lung function, muscle function and risk of osteoporosis, cardiovascular disease and cancer. The potential pathological mechanisms which may be involved are also discussed.

Keywords: COPD, immune system, lung function, osteoporosis, skeletal muscle, vitamin D.

INTRODUCTION

COPD is a chronic and under-diagnosed disease affecting an estimated 1.4 million people in England [1] and it is predicted to be the 3rd leading cause of death worldwide by 2020 [2]. It is predominantly caused by cigarette smoking and is characterised by progressive and irreversible airflow obstruction due to airway inflammation and destruction of lung parenchyma. Although classified as a disease of the respiratory system, COPD has important systemic manifestations. Patients with COPD have a high prevalence of osteoporosis and osteopenia [3], leading to an increased risk of fracture. Skeletal muscle weakness is a common feature of the condition [4], which is associated with decreased quality of life and an increase in mortality [5, 6]. There is systemic as well as lung inflammation [7], and an increased susceptibility to infection with recurrent exacerbations causing rapid decline in lung function [8] and body mass index (BMI) [9]. COPD patients also have an increased prevalence of lung cancer [10] and cardiovascular disease [11].

Vitamin D is a pro-hormone which is produced by the action of sunlight on the skin, although it can also be ingested in the diet. It is metabolised in the liver to 25(OH)D, which is transported and stored in the blood bound to vitamin D binding protein (DBP). 25(OH)D is metabolised to the active form, 1,25(OH)₂D, by the enzyme 1 α -hydroxylase which is produced predominantly in the kidney, although it has now been identified in a number of other target organs where 1,25(OH)₂D is thought to work in a paracrine fashion. In the kidney, it has been demonstrated

that 25(OH)D bound to DBP is delivered to the site of the enzyme 1 α -hydroxylase by megalin/cubilin mediated receptor endocytosis, although it is not clear whether this process occurs in all tissues [12]. Vitamin D deficiency has been implicated in the development of a wide range of medical conditions associated with COPD as well as decline in lung function itself. Considering that in the mouse, vitamin D directly or indirectly regulates over 3% of the genome [13] and the widening areas of research into vitamin D, the growing interest in the role of vitamin D in respiratory disease is not surprising [14]. This review will focus on the evidence that vitamin D deficiency occurs in COPD, and review associations between vitamin D deficiency and lung function, muscle strength and risk of osteoporosis as well as summarising other important actions of vitamin D on the immune and cardiovascular systems which are likely to be relevant in patients with COPD.

25-HYDOXYVITAMIN D LEVELS IN COPD

Over half of the elderly population in the UK and USA have vitamin D deficiency depending on the definition used [15, 16]. Debate is still ongoing about the optimal definition of vitamin D deficiency, which could be based on levels required to suppress parathyroid hormone (PTH), levels required for optimal calcium absorption in the intestine and levels required to maintain bone mineral density (BMD) and prevent falls and fractures. Current consensus is that levels greater than 75 nmol/l are required for optimal bone health [17]. Factors associated with 25(OH)D levels in normal subjects are sunlight exposure, latitude and season, skin type, dietary intake of vitamin D, BMI [16] and genetic influences, in particular polymorphisms in the vitamin D binding protein (DBP) [18-20].

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Vitamin D can be obtained from exposure to sunlight or in the diet. In the United Kingdom, sunlight is only sufficiently strong to produce vitamin D between April and November [21]. A recent study in city-dwelling subjects in the UK suggests that current recommendations for brief episodes of sun exposure in the summer months will place most people in the 'sufficient' range of 25(OH)D, but not the optimal range above 75nmol/l. COPD patients are less active [22] and spend less time outdoors [23, 24] compared to healthy elderly subjects due to breathlessness and leg fatigue. They are therefore less likely to have adequate sunlight exposure although this has not been measured directly. They can also have poor nutritional intake, particularly with severe disease. One study in Spain showed that only 4% of COPD subjects consumed the recommended daily intake of 10µg of vitamin D [25]. These factors combined put them at increased risk of having vitamin D deficiency.

One published cross-sectional study has investigated 25(OH)D levels specifically in COPD patients compared to a control population [26]. This study reported that COPD patients had significantly lower levels of 25(OH)D than controls, and that 25(OH)D levels decreased with increasing GOLD Stage of disease. The control population consisted of smokers and ex-smokers who were matched for age and sex, and no subjects were taking any vitamin D supplementation. This evidence is supported by an earlier study that investigated 25(OH)D levels in patients with severe lung disease who were referred for lung transplantation, of whom approximately 50% had a diagnosis of COPD [27]. Although there was no control group, vitamin D deficiency was common: 52% of underweight patients and 55% of those with normal weight had serum 25(OH)D levels < 37.5 nmol/L.

There is an inverse relationship between BMI and serum 25(OH)D concentration [16, 28], which is dependent on fat mass rather than fat free mass (FFM) [29, 30], and which is more pronounced in women than men [31]. This is important in the subset of patients with COPD who are obese; however COPD subjects are often cachectic, which is likely to be due to a combination of decreased calorific intake, increased work of breathing, systemic inflammation and increased resting energy expenditure. Loss of FFM rather than loss of fat is an important prognostic indicator [5]. Even in underweight subjects with lung disease though, fat mass is an important determinant of 25(OH)D levels [27].

25(OH)D and 1,25(OH)₂D levels are affected by genetic factors. DBP is the main transport and storage protein for vitamin D. The DBP wildtype allele is Gc1F, which over time has undergone 2 mutations to produce a Gc1S allele and a Gc2 allele. Both of the latter alleles have a lower affinity for 25(OH)D and 1,25(OH)₂D and are associated with lower circulating levels of both vitamin D metabolites [18]. Interestingly, in COPD the Gc2 allele has been shown to have a protective effect on the development of COPD due to cigarette smoking [32], and the frequency of Gc1F homozygotes has been found to be increased in COPD versus control populations in two other studies [33, 34]. In one of these studies, Gc1F homozygotes had a faster decline in lung function and more severe changes of emphysema seen on CT scan [34]. A more recent study however, found

that Gc1S homozygotes had an increased risk for COPD, and this risk appeared to be due to the effects of the Gc1S allele in reducing 25(OH)D levels [26]. Large genetic association studies have not confirmed a clear-cut relationship between DBP polymorphisms and COPD [35, 36]. Studies looking at the effects of polymorphisms in the vitamin D receptor (VDR) [37, 38] or the 1α-hydroxylase enzyme CYP27B1 [39], have not shown any consistent relationship with serum 25(OH)D or 1,25(OH)₂ levels.

STUDIES OF VITAMIN D AND LUNG FUNCTION

The 3rd National Health and Nutrition survey (NHANES) was a large US population-based study involving over 14,000 people. It demonstrated convincing evidence that 25(OH)D levels are independently associated with both FEV₁ and FVC [40]. Between the lowest and highest quartiles of serum 25(OH)D concentration there was a difference of 126mls in FEV₁ and 172mls in FVC, after correcting for confounding factors. No association was seen with FEV₁/FVC however, which might suggest that vitamin D deficiency does not contribute to airflow obstruction. However, when looking at the patients in the survey who had a diagnosis of emphysema or bronchitis, the difference in FEV₁ and FVC across the quintiles of 25(OH)D concentration was larger.

Although there appears to be a clear-cut relationship between vitamin D status and lung function in normal subjects, the evidence in COPD is less extensive. The previously mentioned study by Janssens *et al.* did find a correlation between circulating 25(OH)D concentration and % predicted FEV₁ in COPD patients, but not in controls. However, they do not report on whether 25(OH)D was an independent predictor of FEV₁ or FVC [26]. A Norwegian study involving unselected patients referred for lung transplant found only an association between 25(OH)D and the FEV₁/FVC ratio [27].

POTENTIAL PATHOLOGICAL MECHANISMS

The pathological mechanisms of COPD are complex and poorly understood. There is evidence for alteration of the protease/antiprotease balance mediated by an increase in neutrophils and macrophages [41], increased oxidative stress [42], autoimmune dysfunction [43] and dysregulation of lung development pathways including retinoic acid, notch and hedgehog signalling [44]. Although cigarette smoking is the predominant cause of COPD, not all of those who smoke develop the disease. Genetic influences must therefore be important, as illustrated by the case of DBP.

There has been limited research to date looking at potential mechanisms of vitamin D action in the lung. VDR has been identified in bronchial smooth muscle cells [45] and lung fibroblasts [46]. The enzyme 1α-hydroxylase is expressed in respiratory epithelial cells [47] and in alveolar macrophages where increased expression has been demonstrated in disease states such as lung cancer and sarcoidosis [48]. 1,25(OH)₂D has been shown to inhibit matrix metalloproteinases which are important in lung remodelling and repair [49]. It has also been shown to mediate the pro-fibrotic response to TGFβ in lung fibroblasts and epithelial cells [46]. A potential role in lung

development is also emerging with 1,25(OH)₂D stimulating surfactant production in type II alveolar cells [50], and calcitriol analogues stimulating actin and collagen production in neonatal rat lung fibroblast cultures [51]. However in the latter study the calcitriol analogues impaired the overall alveolarisation process, supporting the hypothesis that excessive vitamin D may have adverse consequences.

VITAMIN D AND THE IMMUNE SYSTEM

There are no studies to our knowledge looking directly at the effects of vitamin D on immune function in COPD. However, vitamin D is an important modulator of the innate and adaptive immune systems. The VDR is expressed in most cells of the immune system including macrophages, dendritic cells, neutrophils, B cells and activated CD4⁺ and CD8⁺ T cells [52]. There are two important roles that vitamin D has in the regulation of the immune system which are likely to have an impact on the development and progression of COPD.

In the innate immune system, 1,25(OH)₂D stimulates the production of cathelicidin. This is an antimicrobial peptide which has broad spectrum activity against bacterial and viral pathogens. It acts as a chemoattractant for various inflammatory cell types, and is involved in epithelial proliferation and repair and angiogenesis [53]. Cathelicidin expression has been demonstrated in a wide variety of cells and tissues including lung, small intestine, liver, monocytes, neutrophils and myeloid cells [54]. A vitamin D response element is present in the cathelicidin gene promoter region, and direct 1,25(OH)₂D-mediated expression of cathelicidin has been demonstrated in keratinocytes, neutrophils, monocytes, lung adenocarcinoma cells, and in airway epithelia and serous and mucous cells of the airway submucosal glands [55]. Vitamin D induces activity against *Mycobacterium tuberculosis*, an action which may be partially mediated by cathelicidin [56]. More importantly, for subjects with COPD, cathelicidin has also been shown to have activity against *Pseudomonas aeruginosa* and *Escherichia coli* [55]. There are very few studies looking at the role of cathelicidin in COPD. One study in Polish farmers with COPD found higher levels of cathelicidin in induced sputum compared to farmers without COPD and urban dwellers [57]. Another study also found higher levels of cathelicidin in induced sputum in patients with COPD and cystic fibrosis compared to control subjects, whilst an asthmatic subgroup had the lowest levels [58]. Despite the limited evidence available, cathelicidin, through its multiple actions, may play a role in the development and progression of COPD and further work is required in this area.

Another important role of vitamin D in the immune system is that of T cell regulation. 1,25(OH)₂D alters transcription of IL-2, IFN- γ and IL-4 and has been shown to directly influence Th1/Th2 balance in CD4⁺ cell populations [59, 60]. More recently it has been demonstrated that 1,25(OH)₂D suppresses production of IFN γ , IL-17 and IL-21 by CD4⁺ cells and induces the development of adaptive regulatory T cells [61]. These latter actions may be relevant to the pathogenesis of COPD. The lungs of subjects who smoke but have normal lung function have been shown to have higher levels of regulatory T cells than those who have never smoked, and compared to smokers who have developed COPD [62]. Studies in mice have demonstrated an inflammatory airway response mediated by Th17 cells

and characterised by increased neutrophilic inflammation and B cell influx, which was suppressed by the co-transfer of regulatory T cells [63]. Taken together, it appears that functional regulatory T cells may protect against the development of COPD by controlling the T cell response to immune stimuli.

RESPIRATORY INFECTIONS

The actions of vitamin D on the innate immune system are likely to be of relevance to the occurrence of respiratory infection in COPD. The disease is characterised by recurrent exacerbations which are associated with increased breathlessness and reduced quality of life. Recovery back to baseline levels after an exacerbation is often incomplete [64], and recurrent exacerbations are associated with a decline in lung function. Sputum is characterised by an increase in neutrophils which is related to exacerbation severity, and in 78% of cases in one study, bacterial and/or viral pathogens were isolated [65].

COPD exacerbations more commonly occur in the winter months, and a connection between 25(OH)D levels, which are lower in the winter, and susceptibility to upper respiratory tract infections (URTIs) in the population in general has been postulated. This is supported by data from the NHANES study which found an association between recent self-reported URTI and lower 25(OH)D levels [66]. This relationship was stronger in subjects with asthma than in those with COPD. It is possible that the mechanism involved in this protective effect of vitamin D is related to the production of cathelicidin [67].

It also appears that DBP influences the induction of cathelicidin through its effects on the bioavailability of 25(OH)D to monocytes. Monocytes cultured with serum containing low affinity Gc1S or Gc2 DBP had a nearly 3-fold higher induction of cathelicidin compared to monocytes cultured in serum with high affinity Gc1F DBP [68]. This finding could help to explain the protective effects of the low affinity Gc2 allele seen in COPD. DBP is itself immunologically active when it is metabolised to macrophage activating factor by enzymes released from lymphocytes, and it also has been shown to enhance neutrophil chemotaxis [69].

A trial in school children has shown a decrease in the relative risk of influenza A, but not influenza B or rapid diagnostic test negative flu-like symptoms, with vitamin D supplementation [70]. An interesting secondary outcome in this study was a reduction in asthma exacerbations in the intervention arm. Viral infection may contribute to 50% of acute COPD exacerbations attending the emergency department [71], although influenza A is not the most commonly identified pathogen. However, if a simple measure such as vitamin D supplementation can reduce the exacerbation rate in patients with COPD, this could improve quality of life and delay disease progression.

OSTEOPOROSIS IN COPD

Vitamin D has well known actions on bone which are beyond the scope of this review. Essentially, low levels of 25(OH)D contribute to the development of osteopenia and

osteoporosis as there is reduced calcium absorption from the gut, and both PTH and 1,25(OH)₂D increase bone resorption to maintain calcium levels [72]. Vitamin D supplementation in elderly people, when given with calcium, has been shown to improve bone mineral density (BMD) and reduce the risk of falls and fracture [73-75]. The reduction in fracture risk may be due to a combination of increases in BMD and indirectly through increases in muscle strength and a reduction in falls.

Recent studies have shown a consistently high prevalence of osteoporosis and osteopenia in patients with COPD. A systematic review showed that osteoporosis occurred in 9 to 69% of patients, and osteopenia in 27 to 67%, with an overall mean prevalence of osteoporosis from 13 studies, involving 772 patients, of 35.1% [3]. Four studies included in this review compared COPD patients and age-matched healthy control subjects and there was a significant difference in prevalence: 32.5% in COPD vs. 11.4% in healthy controls. Interestingly, the prevalence of osteoporosis in COPD appears to be higher than in some other chronic lung diseases, including those involving chronic corticosteroid use such as idiopathic pulmonary fibrosis [76-78]. Factors which are consistently related to osteoporosis in multivariate analysis are age, sex, BMI and other anthropometric measurements, lung function and corticosteroid use [79-81]. As well as the morbidity and mortality associated with hip and wrist fractures, vertebral compression fractures are of particular relevance in COPD patients. These cause kyphosis which has a detrimental effect on lung function [82, 83].

Only one study has investigated the association between 25(OH)D levels and BMD in COPD patients: it found no relationship between them. BMD was compared in 49 COPD and 40 healthy control subjects and found to be significantly lower in COPD patients in the lumbar spine, femoral neck and total femur. FEV₁ (l) and weight were independently associated with BMD in the multivariate model which included corticosteroid use and 25(OH)D [84]. Again, this is an area which needs further research.

VITAMIN D AND SKELETAL MUSCLE FUNCTION IN COPD

Vitamin D has a significant role in skeletal muscle function. Patients with osteomalacia develop a myopathy which is usually proximal, and skeletal muscle biopsies in these patients have shown a reduction in size and number of type II muscle fibres [85]. VDR has been demonstrated in skeletal muscle [86] and interestingly the amount of receptors has been shown to decrease with age [87]. VDR knockout mice have reduced type I and type II fibre diameter, and abnormal expression of myogenic regulatory factors which is corrected by the addition of 1,25(OH)₂D *in vitro* [88]. Other demonstrated actions of vitamin D in muscle include regulation of calcium transport [89] and phospholipid metabolism [86].

A number of cross-sectional studies have been carried out in elderly subjects demonstrating an association between vitamin D status and different measures of muscle strength [90-92] and a meta-analysis shows a reduction in falls with supplementation of vitamin D [93]. Supplementation with

vitamin D has also been shown to increase the size and number of type II fibres [94, 95].

Approximately a quarter of patients with COPD develop skeletal muscle weakness [96] which is thought to be due mainly to inactivity, with other factors such as systemic inflammation and oxidative stress also being important [97]. The myopathy is characterised by a switch from type I to type II fibres with a subsequent reduction in the size of type II fibres. No studies to date have looked at vitamin D levels in relation to muscle function in COPD. One study by our group has looked at polymorphisms in the VDR and found an association between the *FokI* polymorphism and muscle strength in COPD patients and control subjects. People with the C allele, which produces a shorter protein product, had a reduced quadriceps strength when compared to those with one or more T alleles, and this supports previous findings in healthy elderly men [98]. Interestingly, the shorter allele has been associated with an increased risk of type I diabetes, and human monocytes homozygous for the short VDR protein product express higher levels of IL-12 protein and mRNA [99]. Thus it is possible that the reduced muscle strength associated with the C allele is due to an exaggerated response to inflammatory stimuli in muscle. Further studies are required to demonstrate the interaction of 25(OH)D and 1,25(OH)₂D levels and polymorphisms in the VDR and effects on muscle strength in COPD.

OTHER CO-MORBIDITIES

Vitamin D is also implicated in other co-morbidities which are more common in COPD such as cardiovascular disease and cancer. In 1 α -hydroxylase knockout mice, increased blood pressure, activation of the renin-angiotensin system, myocardial hypertrophy and decreased cardiac function are seen which are prevented by the administration of 1,25(OH)₂D [100]. Low levels of 25(OH)D have been associated with increased risk of hypertension [101] and cardiovascular disease [102], and 8 weeks of vitamin D and calcium supplementation have been shown to reduce systolic blood pressure compared to calcium alone [103].

Several epidemiological studies have associated low levels of vitamin D with increased risk of colorectal, breast and prostate cancer risk [104]. However its effects in lung cancer have not been clearly demonstrated. VDR polymorphisms have been associated with the incidence of lung cancer [105] and *in vitro* studies have shown that vitamin D has anti-cancer effects such as suppression of angiogenesis and cell proliferation [104]. Mouse studies have shown a protective effect of 1,25(OH)₂D against metastases in lung cancer [106]. However epidemiological data has not shown an association between low 25(OH)D and lung cancer [107].

SUMMARY

In summary, COPD is a common and debilitating disease for which there is currently no cure, and little benefit from available treatment strategies. The widespread effects of vitamin D are peculiarly relevant to the co-morbidities associated with vitamin D deficiency, although current evidence in this field is limited. It is interesting to note that a number of the effects of vitamin D deficiency, such as

osteoporosis and skeletal muscle weakness, seem exaggerated in subjects with COPD compared to those with other lung diseases. Whether this is due to coincidence, to vitamin D deficiency, or to defects in the vitamin D pathway and/or genetic influences requires further research as most of the current evidence is cross-sectional and associative, and causality of vitamin D deficiency in the pathogenesis of COPD has not yet been proven. In addition, and regardless of any causal relationship, supplementation studies are required to see whether any important clinical effect related to COPD might be obtained, in both preventive and therapeutic strategies.

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Vitamin D and Acute Respiratory Tract Infection

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Abstract: Acute respiratory tract infections (ARTI) generate a major disease burden worldwide, disproportionately affecting the elderly and the very young. A growing body of evidence supports an important immunomodulatory function for the biologically active metabolite of vitamin D, 1,25-dihydroxyvitamin D (1,25[OH]₂D). Respiratory epithelial cells and leucocytes express both the vitamin D receptor and CYP27B1, the enzyme which converts 25-hydroxyvitamin D to 1,25(OH)₂D. Observational and ecological studies report associations between inadequate vitamin D status and susceptibility to ARTI, and vitamin D deficiency has been proposed as the seasonal stimulus for influenza epidemics. In children, associations between profound vitamin D deficiency and susceptibility to lower respiratory tract infection have been reported in a variety of settings. In adults, particularly amongst those with asthma and COPD, inadequate vitamin D status has been reported to be associated with susceptibility to upper respiratory tract infection. Vitamin D supplementation trials for the prevention of ARTI report conflicting results. This may reflect variation in study populations and methodology, or factors such as genetic heterogeneity within the vitamin D metabolic or signalling pathways. Further trials of vitamin D supplementation in different settings, with measurement of participants' vitamin D status and evaluation of genetic factors which might modify the effect of vitamin D supplementation are needed.

Keywords: Vitamin D, acute lower respiratory tract infection, upper respiratory tract infection, influenza, cold, immune function.

1. INTRODUCTION

Acute respiratory tract infections (ARTI) are a major global cause of morbidity and mortality. They disproportionately affect the elderly [1-4] and the very young [5, 6] and impose a significant economic burden in terms of health care usage [7]. A number of observational studies have reported an association between inadequate vitamin D status and susceptibility to ARTI in both adults and young children [8-13]. These findings are complemented by a rapidly evolving body of evidence which demonstrates that vitamin D metabolites exert immunomodulatory actions *in vitro* [14]. However, in spite of the existence of plausible biological mechanisms whereby vitamin D could enhance antimicrobial immunity [15-18], clinical trials of vitamin D supplementation for the prevention of acute respiratory infection report conflicting results [19-24]. This review article will discuss current evidence for the role of vitamin D deficiency as a risk factor for ARTI, and for vitamin D supplementation as a strategy to prevent ARTI.

1.1. Vitamin D Metabolic and Signalling Pathways

1.1.1. Metabolism of Vitamin D

Vitamin D is predominantly generated through the action of solar ultraviolet B (UVB) radiation (wavelength, 290 to 315 nm) on vitamin D precursors within the skin; dietary sources are limited. Vitamin D undergoes two successive hydroxylation steps to generate the biologically active metabolite, 1,25-dihydroxyvitamin D₃ (1,25[OH]₂D) [14]. The first hydroxylation step, 25-hydroxylation by CYP27A1

and CYP2R1 enzymes, occurs predominantly in the liver and generates 25-hydroxyvitamin D (25[OH]D). 25(OH)D is the major circulating vitamin D metabolite and the accepted measure of vitamin D status. The second step, 1- α hydroxylation, is catalysed by CYP27B1 and generates 1,25(OH)₂D which is transported to target cells bound to vitamin D binding protein (DBP) or deactivated by 24-hydroxylation by CYP24A1 enzyme. 1- α hydroxylation occurs mainly in the kidney, but respiratory epithelial cells [15] and leucocytes also express CYP27B1 and are therefore also potentially capable of generating 1,25(OH)₂D [25]. 1,25(OH)₂D exerts its biological effects by binding a nuclear receptor, the vitamin D receptor (VDR), which acts as a transcription factor either inducing or repressing the transcription of genes possessing a vitamin D response element (VDRE) in their promoter region. VDR is expressed in a wide range of cells including respiratory epithelial cells and leucocytes [15, 25] (Fig. 1).

1.1.2. Genetic Polymorphisms in the Vitamin D Metabolic and Signalling Pathways

VDR, DBP, CYP27A1, CYP2R1, CYP27B1 and CYP24A1 are all polymorphic. DPB and VDR polymorphisms have been studied extensively in the context of tuberculosis, and have been reported to influence susceptibility to the disease both independently [26, 27] and in association with vitamin D deficiency [28, 29] and response to antimicrobial therapy [30-32]. An association between VDR polymorphisms and susceptibility to viral bronchiolitis in young children has also been reported [33, 34].

The DBP gene is highly polymorphic and three common alleles (Gc1F, Gc1S and Gc2) result in six common phenotypes. Gc1F allele frequency is common amongst black Americans and black Africans and Gc1F protein has

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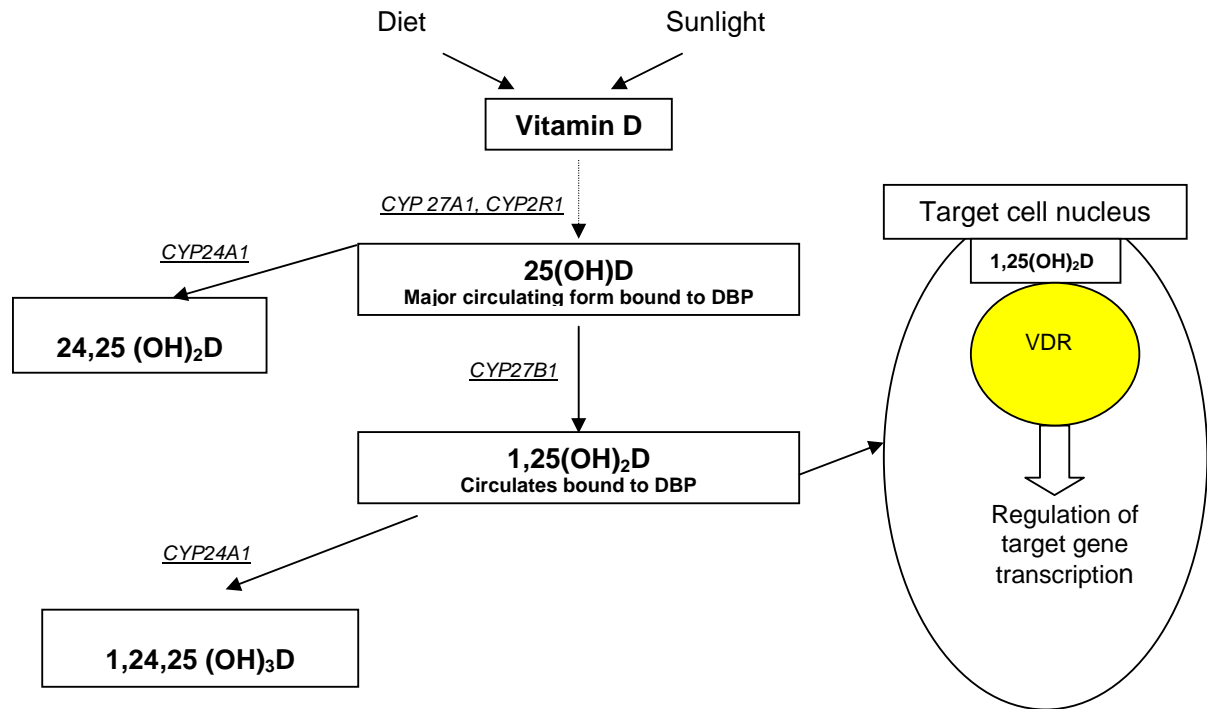


Fig. (1). Key elements in the vitamin D metabolic and signalling pathways. DBP=Vitamin D binding protein; VDR= Vitamin D receptor.

greater affinity for, and is a more efficient transporter of, vitamin D metabolites than other variants [35]. Gc2 protein has the lowest affinity for vitamin D metabolites [36] and is metabolised more quickly than Gc1 [37]. DBP polymorphisms associate with serum 25(OH)D concentrations [38, 39] and influence the 25(OH)D response to vitamin D supplementation [38]. Hence DBP genotype might influence the amount of 25(OH)₂D reaching target tissues, and predict which individuals would most benefit from supplementation.

There are four well characterised single nucleotide polymorphisms (SNP) in the VDR gene. Three SNPs (*TaqI*, *ApaI* and *BsmI*) are synonymous (i.e. they do not change the amino acid sequence of translated protein), [32] but are in linkage disequilibrium with a poly(A) length polymorphism in the 3' untranslated region (UTR) of the VDR gene. Carriage of the *t* allele of the *TaqI* polymorphism has been reported to associate with an increase in VDR gene expression [40]. Because the 3'-UTR is a major regulator of mRNA half-life [41], this association was originally thought to be mediated *via* an effect on mRNA stability [40]; however, subsequent studies have refuted this hypothesis [42, 43], and the mechanism for the association is not currently understood. In contrast the *f* allele of the *FokI* VDR polymorphism generates a VDR of increased length which is less efficiently transcribed, thus reducing the amount of VDR produced [44]. *In vitro*, *FokI* variants have been found to have differing effects on immune cell proliferation and cytokine synthesis and on the activity of transcription factors that co-regulate immune responses [44]. These findings suggest that VDR genotype could modify immunomodulatory effects of vitamin D supplementation.

1.2. Immunomodulatory Role of Vitamin D in Respiratory Infection

Vitamin D has pleiotropic immunomodulatory functions and *in vitro* data provide plausible biological mechanisms whereby vitamin D could influence immune responses to infection. *In vitro* studies using supra-physiological concentrations of 1,25(OH)₂D demonstrate multiple actions within the innate immune system. Respiratory epithelial cells, activated macrophages, monocytes and dendritic cells all express CYP27B1 and are thus potentially capable of generating the high local concentrations of 1,25(OH)₂D required for exertion of its immune actions [15, 45]. 1,25(OH)₂D directly induces the expression of antimicrobial peptides (AMP) such as cathelicidin by monocytes, neutrophils, and respiratory epithelial cells, and these peptides provide a rapid and broad-spectrum defence mechanism against bacterial, viral and fungal pathogens [17, 18, 46]. Viral infection itself upregulates CYP27B1 expression by respiratory epithelial cells [15] and activation of macrophage Toll-like receptors (TLR) by pathogenic antigens also induces the expression of CYP27B1, VDR and AMPs [16]. Hence 1,25(OH)₂D has the potential to play an important role within the innate immune system –the first line of defence against respiratory pathogens.

In vitro, 1,25(OH)₂D inhibits the synthesis of pro-inflammatory Th1 cytokines such as IFN- γ and TNF- α , modulates expression of inflammatory cytokines induced by viral infection and upregulates the production of anti-inflammatory cytokines such as Interleukin-10 (IL-10) [15, 25, 45]. This anti-inflammatory action has been demonstrated in patients with congestive heart failure, where raised circulating concentrations of pro-inflammatory cytokines are

thought to contribute to disease pathogenesis, and vitamin D supplementation significantly increased the concentration of the anti-inflammatory cytokine IL-10 and prevented an increase in pro-inflammatory TNF- α [47]. In influenza infection, where host inflammatory response is implicated in the pathogenesis of severe disease [48], this anti-inflammatory action might be beneficial. However type I cytokines also play a protective role against some infections: IFN- γ may be protective against hypoxia in acute bronchiolitis [49], and infants with severe RSV bronchiolitis have been shown to have significantly lower levels of IFN- γ in nasopharyngeal aspirates compared with those with less severe disease [50]. Thus, suppression of type I cytokine responses has potential for adverse as well as favourable consequences.

1.3. Epidemiology of Vitamin D Insufficiency and Deficiency

Vitamin D status is determined by measurement of circulating 25(OH)D concentration; liquid chromatography-tandem mass spectrometry is considered the gold standard assay [51-53]. Interpretation of reported 25(OH)D concentrations is complicated by variation in performance of different assays, and inter-laboratory variability has been reported with immunoassays and high performance liquid chromatography [54-56]. Quality assurance schemes have been established in the UK (www.deqas.org) and the US (www.nist.gov) to address this problem. Some experts recommend a target level of >30 ng/ml (75 nmol/l) throughout the year for optimal skeletal and non-skeletal health outcomes, and define vitamin D deficiency and insufficiency as 25(OH)D levels of <20 ng/ml (50 nmol/l) and 20-30ng/ml (50 to 75 nmol/l) respectively [55, 57]; others regard concentration > 20 ng/ml (50 nmol/L) as adequate for optimal health [58]. As dietary sources of vitamin D are limited, the most important determinants of inadequate vitamin D status in otherwise healthy individuals are factors which reduce cutaneous synthesis of vitamin D. These include inadequate sun exposure; season and latitude (over 35 degrees from the equator very little vitamin D is produced in Winter and Spring as fewer UVB photons penetrate the atmosphere); skin pigmentation and use of sunscreen (as both melanin and sunscreen absorb UVB); and aging, which reduces the concentration of vitamin D precursors in the skin [14]. Breast milk of vitamin D deficient mothers contains little vitamin D, hence exclusively breast fed infants of vitamin D deficient mothers are at high risk of developing vitamin D deficiency [14].

Using the aforementioned definitions a current world wide epidemic of vitamin D inadequacy is reported [59]. An estimated 30-77% of European and US populations are either vitamin D insufficient or deficient [8, 55, 60], most notably during the winter and spring months [61] when incidence of ARTI peaks [62, 63]. Deficiency is common in patients with COPD and asthma, amongst whom reduced 25(OH)D levels associate with disease severity [8, 64, 65], and URTIs are common precipitants for acute exacerbations [1, 66, 67].

Profound vitamin D deficiency (serum 25(OH)D concentration <10 ng/ml [25 nmol/l]) which is frequently accompanied by osteomalacia (or rickets in young children) is particularly common amongst the institutionalised elderly, who are also most susceptible to ARTI [59, 68]. Hence observational data certainly support a link between inadequate vitamin D status and acute respiratory tract infection.

1.4. Classification of Upper and Lower Respiratory Tract Infection

The International Classification of Diseases defines acute upper respiratory tract infection (URTI) as acute infection affecting the airway at any level above the bronchi. Influenza and pneumonia with or without upper airway involvement, and acute bronchitis and bronchiolitis are categorised as acute lower respiratory tract infections (ALRI) [69]. It is useful to distinguish between viral and bacterial infections in order to provide appropriate treatment, where required. URTIs are generally viral in origin [70] and ALRI is often caused by bacteria [71, 72]. However this is not a hard and fast rule, as viral bronchiolitis is an important cause of ALRI, influenza can infect both bronchi and lungs, and up to 10% of URTIs are caused by bacteria [70]. Combinations of symptoms have been validated to distinguish between URTI [73], influenza [74] and ALRI [63, 75], but there is overlap between these symptom-complexes, in particular between those used to identify URTI and influenza. It can be difficult to accurately categorise ARTI as URTI or ALRI using symptom criteria alone. In most of the studies discussed below it has been necessary to use symptom complexes, and some studies have investigated the incidence of upper and lower acute respiratory tract infections combined. In a research context, where an intervention is being evaluated for the prevention of ARTI, the gold standard for defining a case of ARTI should ideally involve direct evidence of infection, i.e. detection of bacteria or virus, aided by indirect evidence i.e. radiographic pneumonia; this would remove the need to categorise infections as upper or lower respiratory in origin. Furthermore, the rapid advances in nucleic amplification tests have made it possible to easily directly detect the presence of respiratory virus infection [76]. However, microbiological techniques are not always effective in identifying the causative agents of ARTI, and in resource-constrained settings access to microbiological diagnostics and chest radiography remains limited.

1.4.1. Case Definitions for ALRI

In early childhood the World Health Organization (WHO) clinical case definitions for identifying ALRI have generally been used in studies evaluating the association between ALRI with rickets or inadequate vitamin D status. These are well-validated case definitions, aimed to assist health care workers in the management of ALRI, which focus on the detection of pneumonia and bronchiolitis that are responsible for the majority of ALRI globally in early childhood [77]. Children under 5 years with cough or difficulty breathing are considered to have 'clinical

pneumonia' if their respiratory rates are raised (≥ 60 breaths/min in children ≤ 2 months; ≥ 50 breaths/min in children 2–11 months; ≥ 40 breaths/min in children 1–4 years) or if lower chest wall indrawing is present [78, 79]. Radiological criteria, where available, are considered useful for defining bacterial episodes and chest radiographs are more specific than clinical criteria alone [77]. Laboratory investigations are not considered useful in establishing a diagnosis of ALRI in resource-limited settings, and these have not in general been used to identify cases in the studies discussed below.

In adults the Macfarlane criteria [63], which require the presence of a new or worsening cough together with at least one other lower respiratory tract symptom (sputum production, dyspnoea, wheeze, and chest pain or discomfort) are widely used to identify ALRI cases. These criteria have been validated in a hospital setting, but in a primary care setting, only the presence of focal chest signs was found to reliably identify patients with ALRI, against a gold standard of isolation of respiratory pathogen and/or radiographic features of pneumonia [63].

1.4.2. Case Definitions for URTI

Symptom-complex criteria are widely used to identify URTIs in research settings. The Jackson criteria were validated in young health volunteers to identify experimentally-induced colds [73]. Jackson symptoms comprise sneezing, sore throat, headache, chilliness, malaise, nasal obstruction, nasal discharge and cough which are scored in severity by patients on a scale of 0 to 3. The total symptom score is used to identify URTI [73]. Influenza-like illness is commonly identified in epidemiological studies by the presence of cough, feeling of fever or chilliness and myalgia [74]. The majority of studies discussed below use combinations of the aforementioned symptoms, some also grading severity of infection, in order to assign case definitions for URTI.

2. ACUTE LOWER RESPIRATORY INFECTION (ALRI)

Pneumonia is the leading cause of death in young children worldwide [5] and still remains the leading respiratory killer of the elderly in the UK [1]. RSV bronchiolitis in infants is also a major cause of severe respiratory infections globally and generates substantial morbidity [6, 80]. In early childhood there is a strong association between malnutrition and both incidence and mortality arising from ALRI [75, 80]. Nutritional interventions, in particular zinc supplementation and the promotion of breast feeding are proven to prevent childhood ALRI in developing countries [75], and researchers have called for the evaluation of other nutritional interventions [80] including vitamin D supplementation [75]. In spite of the burden of ALRI in the elderly [1] who are at high risk of vitamin D deficiency [59, 68], studies evaluating the association between vitamin D status and risk of ALRI [10, 11, 13, 34, 81–85] and the effectiveness of supplementation in preventing or treating these infections [23, 86] have to

date only been conducted in the very young. These studies are discussed below.

2.1. Observational Studies

Table 1 summarises key details of observational studies evaluating the association between vitamin D status or nutritional rickets and risk of ALRI. In general observational studies from developing countries describe an association between either vitamin D deficiency or nutritional rickets and risk of ALRI, [10, 11, 13, 81, 84, 85] but findings differ in two Canadian case-control studies [82, 83], most likely reflecting differences in vitamin D status of the populations studied and predominance of viral bronchiolitis in the Canadian studies.

2.1.1. Studies Evaluating the Association Between Rickets and Risk of ALRI

Salimpour *et al.*, who systematically screened children admitted to a hospital in Iran for rickets, found that 43% of children with radiologically-confirmed rickets had bronchopneumonia [13]. A case-control study in Jordan, which also screened hospitalised infants for rickets, reported unadjusted odds of ALRI in rachitic infants three-fold that of non-rachitic infants, but failed to report any multivariable analysis [11]. In both of these studies, although the case definitions for rickets were rigorous and included radiological improvement after vitamin D treatment, the criteria for diagnosis of ALRI were not described. The strongest evidence for an association between rickets and ALRI comes from a large case-control study of young children admitted to a hospital in Ethiopia with pneumonia, which used case definitions incorporating validated clinical criteria and radiological features for pneumonia and rickets [10]. After adjusting for confounding factors, the odds of rickets in their pneumonia cases were 13-fold higher than in matched controls. These findings contrast with a Zambian study in which no evidence of rickets was found in 96 children who were admitted with a clinical and radiological diagnosis of pneumonia [87]. Most of the children in this latter study were malnourished, and in the absence of 25(OH)D measurement, the presence of subclinical vitamin D deficiency cannot be excluded. 25(OH)D levels were also not available in any of the aforementioned studies in which rickets was assumed to be the result of vitamin D deficiency [10, 11, 13, 87]. Although the diagnosis of rickets was supported by radiological improvement after vitamin D supplementation in two of these studies [11, 13], data from Nigeria suggest that in some parts of Africa calcium deficiency, rather than vitamin D deficiency, is the primary cause of rickets [88].

2.1.2. Studies Evaluating the Association Between Vitamin D Status and Risk of ALRI

Five recent case-control studies have explored the association between vitamin D status and susceptibility to ALRI in young children. Three studies from developing countries, in which the majority of both cases and controls were vitamin D deficient (25[OH]D < 50 nmol/l), all found

Table 1. Summary of Published Studies Evaluating an Association Between Vitamin D Status or Nutritional Rickets, and ALRI in Young Children

Study, Country, Type	Study Population	Case Definition	Predominant ALRI	Average Age in Months (Age Range)	Average 25(OH)D Concentration (nmol/L) in Cases	Average 25(OH)D Concentration (nmol/L) in Controls	Key Finding
Salimpour 1975 [13] Iran Survey	Admissions to a children's hospital were systematically screened for radiological rickets over 7 years. N=200 children with radiologically confirmed rickets.	Case definition for ALRI not described All but 8 rickets cases had radiological improvement of rickets with vitamin D treatment.	Pneumonia	(60% between 4 and 12 months)	Not available	Not available	43% of rickets cases had bronchopneumonia
Muhe 1997 [10] Ethiopia Case Control	N=1000 children admitted over 5 years n=500 Cases Children admitted with pneumonia n=500 Controls who were matched for age and admission within 3 months of cases.	Pneumonia was defined using WHO criteria, examination, and CXR Rickets was diagnosed using both clinical and radiological features.	Pneumonia	Cases: 13.6 Controls: 13.4	Not available	Not available	After adjusting for confounding factors including measures of malnutrition the odds of rickets in pneumonia cases was 13-fold higher than among controls (adjusted OR 13.37, [95%CI 8.08–24.22], p<0.001).
Najada 2004 [11] Jordan Case control	N=443 acute admissions were screened for rickets over a 9 month period n=47 Cases with rickets n=396 Controls who did not have rickets	Case definition for ALRI not described Rickets was diagnosed by radiological improvement after vitamin D treatment in infants with suggestive clinical or biochemical features.	Pneumonia	Cases: 8 Controls: 7.9 (3-24 months)	Not available	Not available	In a univariable analysis 40/47 (85.1 %) of rachitic infants were admitted due to ALRI compared with 119/396 (30%) of the control infants (p < 0.01).
Wayse 2004 [85] India Case Control	N=150 recruited in May and June n=80 cases with ALRI n=70 unmatched healthy controls attending for vaccination	ALRI defined using WHO clinical criteria. 25(OH)D measured using RIA (Diasorin)	Not reported	23.9	22.8	38.4	1. Mean 25(OH)D was significantly lower in cases compared to controls (22.8 nmol/l [95% CI 21.0-24.7] vs 38.4 nmol/l [95% CI 34.2-43.1]; p<0.001) 2. After adjusting for factors including measures of malnutrition 25(OH)D > 22.5nmol/l was significantly associated with lower risk of ALRI (adjusted OR 0.09, 95% CI 0.03-0.24; p<0.001)

(Table 1) contd.....

Study, Country, Type	Study Population	Case Definition	Predominant ALRI	Average Age in Months (Age Range)	Average 25(OH)D Concentration (nmol/L) in Cases	Average 25(OH)D Concentration (nmol/L) in Controls	Key Finding
Karatekin 2009 [81] Turkey Case Control.	N=40 recruited over winter and spring n=25 cases newborns with ALRI n=15 healthy newborn controls.	ALRI defined using clinical, radiographic and biochemical findings. 25(OH)D measured using RIA (Biosource).	Not reported	0.3	22.8	40.8	1. Mean 25(OH)D was significantly lower in cases than in controls (22.8 nmol/l vs 40.8 nmol/l; p=0.011). 2. Mean 25(OH)D of mothers of cases was significantly lower than mothers of controls (33.5 nmol/l vs 57 nmol/l; p=0.012). 3. 25(OH)D levels of newborns were highly correlated with their mothers' 25(OH)D.
Roth 2009 [84] Bangladesh Case Control.	N=50 recruited in January and February n=25 cases admitted with ALRI n=25 controls matched for age, sex and village.	ALRI defined using WHO clinical criteria and examination by study physician.		4.2	29.2	39.2	1. Mean 25(OH)D was significantly lower in cases than in controls (29.1 nmol/l vs 39.1 nmol/l; p=0.015). 2. The adjusted odds for ALRI was approximately reduced four-fold for every 10 nmol/l increase in serum 25(OH)D (adjusted OR 0.23, 95% CI 0.06-0.81; p=0.022). This suggests that there may be a threshold of 25(OH)D level beyond which risk of ALRI increases.
McNally 2009 [82] Canada Case Control.	N=175 recruited over winter and spring. n=105 cases admitted with ALRI. n=92 controls matched for age.	ALRI defined using clinical and radiographic features as recommended by Canadian expert consensus. 25(OH)D measured using EIA and validated against Vitamin D External Quality Assessment Scheme (DEQAS)	N=50 pneumonia. N=55 bronchiolitis.	13.6	81 49 (Amongst cases requiring intensive care)	83	1. Mean 25(OH)D levels did not differ significantly between cases and controls. 2. In a subgroup analysis the mean 25(OH)D level was significantly lower in n=16 cases requiring paediatric intensive care compared with cases admitted to general ward (49 ±24 nmol/l vs 87 ± 39 nmol/l, p=0.001); and also compared with controls (49 ±24 nmol/l vs 83 ± 30 nmol/l, p=0.001). 3. After adjusting for confounding factors, cases requiring ICU admission had an 8-fold increased risk of vitamin D deficiency compared with cases admitted to the ward. (adjusted OR 8.23, 95% CI 1.4-48.0, p=0.02) This suggests that D deficiency is associated with more severe disease.

(Table 1) contd.....

Study, Country, Type	Study Population	Case Definition	Predominant ALRI	Average Age in Months (Age Range)	Average 25(OH)D Concentration (nmol/L) in Cases	Average 25(OH)D Concentration (nmol/L) in Controls	Key Finding
Roth 2009 [83] Canada Case Control.	N=129 recruited during winter over 2 years. n=64 cases admitted with ALRI n=65 controls matched for age.	ALRI defined using clinical criteria, and designated as pneumonia if features on CXR. 25(OH)D measured using RIA in year 1, and tandem mass spectrometry in year 2.	N=4 pneumonia N=60 bronchiolitis (81% RSV detected).	13.3	77.2	77.0	Mean 25(OH)D levels did not differ significantly between cases and controls.

Abbreviations: ALRI = Acute lower respiratory tract infection; WHO=World Health Organization; CXR= Chest radiograph.

significantly lower 25(OH)D levels amongst ALRI cases compared with controls [81, 84, 85]. Studies from India [85] and Bangladesh [84], amongst children of mean age 2 years and 4 months respectively, reported differences between controls and cases in mean 25(OH)D of 15.6 nmol/l and 10.0 nmol/l respectively. The Bangladeshi study also reported a substantial reduction in the odds of ALRI amongst cases for each 10 nmol/l increase in serum 25(OH)D concentration (OR 0.23, 95% CI 0.06–0.8; $p=0.02$) [84]. ALRI was diagnosed on the basis of clinical features in both of these studies. Karatekin *et al.*, using a more rigorous case definition for ALRI which included radiographic features, reported a difference in mean serum 25(OH)D concentration of 18 nmol/L between healthy control newborns and newborns admitted with ALRI in Turkey [81]. A strong correlation was found between neonatal and maternal serum 25(OH)D concentrations which were also significantly lower in the mothers of cases compared to the mothers of controls. The majority of mothers of both cases and controls were vitamin D deficient, highlighting the need for vitamin D supplementation during pregnancy in this setting [81].

Two Canadian studies of children (mean age 13 months) admitted with ALRI defined using clinical and radiological features, failed to demonstrate a significant difference in mean 25(OH)D levels between cases and controls [82, 83]. Roth *et al.* documented RSV bronchiolitis in the majority of their cases [83], whereas McNally *et al.* had approximately equal numbers of bronchiolitis and pneumonia cases [82]. In both studies, the majority of cases and controls were vitamin D replete (25(OH)D ≥ 75 nmol/l), most likely reflecting successful food fortification strategies in Canada [89]. The results of subgroup analyses by McNally *et al.* did however yield interesting findings. In particular, 25(OH)D levels were significantly lower in ALRI cases with severe disease requiring intensive care (49 nmol/l) when compared to controls (83 nmol/l) and cases admitted to general wards (87 nmol/l). After adjusting for confounding factors, cases admitted to the intensive care unit (ICU) were 8-fold more likely to have vitamin D deficiency than cases admitted to general wards, although the small sample size is reflected in the wide confidence intervals [82]. Pneumonia cases were twice as likely to have vitamin D deficiency compared with

controls, but this difference failed to reach statistical significance. These findings may indicate that in a developed world setting vitamin D deficiency is associated with more severe ALRI.

It seems likely therefore that the contrasting findings between the Canadian and developing country setting studies reflect key differences in vitamin D status and infectious disease aetiology. The majority of cases and controls in India, Bangladesh and Turkey were vitamin D deficient with mean 25(OH)D levels amongst cases ranging from 22.8 to 29.1 nmol/l [81, 84, 85]. Furthermore in developing countries bacterial pneumonia is a major problem, compared with developed countries where most ALRI is viral in origin [90]. These studies certainly provide evidence for vitamin D deficiency as a risk factor for ALRI, and Roth *et al.* have proposed that there may be a threshold 25(OH)D level below which risk of ALRI increases above baseline [84]. However associations from observational studies do not prove causality, and confounding cannot be excluded as an explanation; in particular vitamin D deficiency may merely reflect malnutrition and socioeconomic deprivation which are known to be risk factors for infectious diseases. However studies which have adjusted for these factors and used validated case definitions for ALRI report strong associations between either rickets or low 25(OH)D levels and risk of ALRI [10, 81, 84, 85].

2.2. Studies Evaluating the Effect of Vitamin D Supplementation for the Prevention or Treatment of ALRI

A double-blind randomised clinical trial of vitamin D supplementation recently conducted in Afghanistan randomly allocated young children diagnosed with pneumonia based on clinical features, to either a single high-dose oral vitamin D supplement (100,000 IU of cholecalciferol) or placebo, in addition to routine pneumonia treatment. Although no difference was observed in time to recovery, supplementation significantly reduced the risk of a repeat episode of pneumonia during the 90 days following supplementation (RR 0.78; 95% CI 0.64, 0.94, $P = 0.01$), and time to a repeat episode was significantly longer in

supplemented children (72 days vs 59 days; HR 0.71; 95% CI 0.53-0.95; $p=0.02$) [5]. 25(OH)D levels were not measured in this study but it is highly likely that most participants were vitamin D deficient, as the prevalence of vitamin D deficiency amongst children in Kabul is known to be very high [91]. It is possible that larger or additional bolus vitamin D doses might have influenced recovery times or further reduced the incidence of repeat pneumonia episodes, but these strategies will need to be evaluated in settings where 25(OH)D and calcium levels can be monitored. In a less rigorously conducted study Rehman treated 47 children with a history of frequent respiratory infection or febrile illness, who were diagnosed with sub-clinical rickets based on raised serum calcium and alkaline phosphatase levels, with both high dose vitamin D and calcium supplements over a 6 week period. Over a 6-month post treatment period there was no recurrence of infection in these children [86]. This study lacked any randomisation or blinding procedures or measurement of 25(OH)D levels, and the criteria used to define cases of infection were not reported. In addition the provision of calcium supplementation makes it difficult to draw any conclusion regarding whether vitamin D supplementation was responsible for preventing further respiratory infections in supplemented children. These studies are summarised in Table 2.

2.3. Vitamin D Receptor Polymorphisms and Risk of ALRI

The association between VDR genotype and the risk of ALRI has been evaluated in the context of RSV bronchiolitis in early childhood. Two case control studies, one a larger genetic association study looking at single-nucleotide polymorphisms (SNPs) in candidate genes involved in

immunity and asthma [33], and the other a smaller study focussing on VDR [34] have found a strong association between *FokI* genotype and risk of severe bronchiolitis requiring hospitalisation. Roth *et al.* found a significantly greater (seven-fold) adjusted odds of ALRI in Canadian children with the *ff* genotype compared with those carrying the *FF* genotype, and a weaker association with the *TaqI* polymorphism [34]. These preliminary findings support a role for vitamin D in determining susceptibility to viral bronchiolitis, and further larger studies are needed to confirm these results.

Hence it appears that in developing countries, where the prevalence of vitamin D deficiency is high and pneumonia is the predominant cause of ALRI, there is a strong association between inadequate vitamin D status and risk of ALRI in young children [10, 81, 84, 85]; whereas in a developed country setting vitamin D deficiency appears to be associated with more severe ALRI [82]. One clinical trial of vitamin D supplementation has demonstrated a significant reduction in the risk of repeat episodes of pneumonia in young children in Afghanistan [23], but larger trials are needed to evaluate the role of vitamin D supplementation both for the prevention and treatment of ALRI. These trials should be conducted in both developed and developing country settings, and should have access to 25(OH)D level measurement as well diagnostic tests to identify the respiratory pathogen. Different dosing regimens should be evaluated and, if it exists, the optimal 25(OH)D level to prevent ALRI needs to be ascertained. Preliminary studies suggest VDR polymorphisms influence susceptibility to ALRI, and studies are needed to evaluate whether they also influence any effect of vitamin D supplementation on incidence of ALRI. There is very little data regarding potential associations between

Table 2. Studies of Vitamin D Supplementation for the Prevention or Treatment of Acute Respiratory Tract Infections in Young Children

Study, Country, Type	Study Population	Case Definition	Main Endpoints	Intervention	Key Findings
Rehman 1994 [86] India Case control	N=47 children Age range 3-12 years n=27 cases with ≥ 6 respiratory or other febrile illnesses needing antibiotics in preceding 6 months who did not have clinical or radiological features of rickets. n=20 age- and sex-matched 'controls' with ≤ 1 infectious episode in preceding 6 months.	No case definitions described.	Frequency of any infection.	Cases were given 60000 IU oral vitamin D/week and daily 650mg calcium for 6 weeks. Both groups were followed up for 6 months.	1. Mean baseline serum alkaline phosphatase level was significantly greater in cases compared to controls (299 ± 41 vs 201 ± 23 ; $P < 0.005$), and this normalised after the intervention, suggesting that cases had subclinical vitamin D deficiency. 2. During the 6 month follow up period no difference was observed in the frequency of infections between the test and control groups of children.
Manaseki-Holland 2010 [23] Afghanistan Double blind, placebo-controlled, randomised clinical trial	N=453 children admitted with ALRI, mean age 13.2 months. n=224 received intervention. n=229 received placebo.	ALRI defined using WHO clinical criteria.	Duration of pneumonia Risk of repeat episode within 3 month follow-up period.	Single dose of 100,000 IU D3 or placebo given in addition to standard antibiotic treatment for pneumonia as per national protocol.	1. There was no difference in mean time to recovery from the index episode of pneumonia in the intervention group compared with the placebo group (4.74 vs 4.98 days respectively, $p=0.17$). 2. The risk of repeat episode of pneumonia was significantly lower in the intervention group compared to the placebo group (RR 0.78; 95% CI 0.64, 0.94; $p=0.01$).

vitamin D status and risk of ALRI in the elderly, and this inequity must be addressed.

3. UPPER RESPIRATORY TRACT INFECTION (URTI), INFLUENZA, AND STUDIES WHICH DO NOT DIFFERENTIATE BETWEEN URTI AND ALRI

An estimated 72% of adults in the United States experience at least one URTI each year which imposes a significant economic impact in terms of health care usage and absenteeism from work [7]. Older adults are particularly susceptible to influenza, which is associated with a high mortality in this population [92]. Viruses are the most common cause of URTI although many of them (including influenza and parainfluenza viruses, rhinovirus and RSV) can cause both URTIs and LRTIs. Observational data, mainly from recent studies in developed countries, describe an association between vitamin D deficiency and risk of URTI or influenza-like-illness (ILI). Five trials of vitamin D supplementation for the prevention of URTI and ILI have been conducted to date, but report contrasting findings which are discussed below.

3.1. Observational Studies

Vitamin D insufficiency is particularly common in winter and spring, particularly amongst older adults [68], when UVB available from sunlight is of insufficient intensity to stimulate cutaneous vitamin D synthesis [61]. Ecological studies demonstrate that the peak in prevalence of vitamin D insufficiency during winter and spring coincides with the peak in incidence of upper and lower respiratory tract infections caused by influenza and parainfluenza viruses and RSV, and vitamin D insufficiency has been proposed as the seasonal stimulus for influenza epidemics [62, 93-95]. A recent cross-sectional analysis of 18,883 participants in the US Third National Health and Nutrition Examination Survey (NHANES III) aged 12 years and older demonstrated an independent association between vitamin D insufficiency and susceptibility to recent URTI (OR 1.36) ascertained by self-report, which was stronger amongst participants with asthma and COPD (OR, 5.67 and 2.26, respectively) [8]. This is an interesting finding as URTIs are known to be common precipitant for acute exacerbations in patients with asthma and COPD [66, 67]. Two further studies have used case definitions for acute respiratory infection which incorporate physician diagnosis with or without microbiological evidence of infection [9, 12]. Amongst Finnish military conscripts followed for 6 months, those with vitamin D deficiency ($25(\text{OH})\text{D} < 40 \text{ nmol/l}$) at baseline had significantly more days of absence from duty due to respiratory tract infection, than those with baseline $25(\text{OH})\text{D} > 40 \text{ nmol/l}$ (median: 4 vs 2 days respectively) [9]. This study used documentation of physician-diagnosed respiratory infection (sinusitis, tonsillitis, otitis, pharyngitis, laryngitis, bronchitis and pneumonia) abstracted from the medical records of participants [9]. Sabetta *et al.* followed a cohort of 198 healthy adults for 5 months and found that amongst participants with a baseline serum $25(\text{OH})\text{D}$ concentration $\geq 95 \text{ nmol/l}$ the incidence of acute viral respiratory tract infection was almost 3-fold lower than in those with levels $< 95 \text{ nmol/l}$ ($p=0.015$) [12].

3.2. Interventional Studies

The findings from five placebo-controlled randomised trials of vitamin D supplementation for the prevention of ARTI have been published to date (Table 3). There are important methodological differences between the trials, including different supplementation regimes which influenced the proportion of participants in intervention arms who attained vitamin D repletion and the time taken to reach repletion. Case definitions for ARTI or URTI also vary, with one trial using detection of respiratory virus [24], and most of the others using patient self-report of infection or antibiotic use [19, 20, 22]. Characteristics of the study populations also vary, in particular according to baseline vitamin D status and reported compliance with study medication. Two trials report secondary analyses of trials of vitamin D supplementation for the maintenance of skeletal health [19-22, 24]. Aloia *et al.* performed a secondary analysis of adverse events reported during a randomised placebo-controlled trial of vitamin D supplementation for the prevention of bone loss in postmenopausal African-American women [19]. Participants in the intervention arm received 800 IU/day of vitamin D3 for 2 years, followed by 2000 IU/day in the final year, and self-reported cold or influenza symptoms were recorded on 6-monthly basis. Mean baseline $25(\text{OH})\text{D}$ levels in both intervention and control arms were $< 50 \text{ nmol/l}$, rising to $> 70 \text{ nmol/L}$ after supplementation [96]. Significantly fewer participants receiving supplementation reported cold and influenza symptoms than those receiving placebo (8/104 vs 26/104, respectively) and supplementation appeared to abolish the seasonality of these infections [19]. The absolute number of infections recorded (34 over 3 years) was much lower than would be expected however. Secondary analysis of a larger UK trial of vitamin D supplementation for fracture prevention in older adults found that supplementation with 800 IU vitamin D3 per day did not have any significant effect on incidence of self-reported infection or antibiotic use [20]. However in a per-protocol analysis based on self-reported pill taking, the odds for infection and antibiotic usage in the D3 group were smaller, although this was of borderline statistical significance; hence poor compliance may have been an issue in this study.

In a follow-up to the study by Aloia *et al.*, Li-Ng conducted a 3 month placebo-controlled trial of vitamin D3 administered at a higher dose (2000 IU/day) in healthy adult volunteers recruited during winter and spring [22]. No difference in the frequency of self-reported URTI was seen in intervention vs control arms of this study. Mean baseline $25(\text{OH})\text{D}$ levels were approximately 64 nmol/l in both groups and rose to 88.5 nmol/l in the intervention group. A longer follow-up period, or supplementation prior to winter might have yielded more significant findings, as with this daily dosing regimen repletion would only have been attained after 3 months. Laaksi *et al.* followed on from their observational study by conducting a trial of 400 IU/day vitamin D3 over a 6 month period in Finnish military recruits [21]. They found no significant difference in the number of days of absence from duty due to ARTI or frequency of self-reported ARTI symptoms between intervention and control groups. Their findings may reflect the very low dose of

Table 3. Interventional Trials Evaluating Vitamin D Supplementation for the Prevention of ARTI, URTI or ILI

Study, Country, Type	Study Population (N)	Main End Point [Case Definitions]	Intervention	Mean Baseline 25(OH)D Concentration (nmol/L)		Outcome
				Baseline	Follow-Up	
Aloia 2007 [19] USA Secondary analysis of adverse events reported during a randomised, double-blind, placebo-controlled trial of vitamin D supplementation for the prevention bone loss.	N=208 calcium replete post-menopausal African-American women. n1=104 received D3 n2=104 received placebo.	Self-reported cold or influenza in previous 6 months, recorded as part of 6-monthly adverse event reporting. Seasonality of cold/influenza symptoms.	800 IU/day D3 for 2 years followed by 2000 IU/day for 1 year, or placebo for 3 years.	n1=46.9 n2=43	n1=70.8 with 3 months of 800IU/d D3; and 86.9 after 3 months of higher dose. N2=no significant change.	1. A significantly greater proportion of participants reporting cold or flu symptoms were in the placebo group compared with the intervention group (26/34 vs 8/34; (p<0.002). 2. Placebo group had symptoms mainly during winter and intervention group had symptoms throughout the year. 3. Only one person reported symptoms whilst taking the higher D3 dose.
Avenell 2007 [20] UK Sub-study of a randomised, double-blind, placebo-controlled trial of vitamin D supplementation for the secondary prevention of osteoporotic fractures in older people (RECORD trial).	N=3444 adults aged 70y+ participating in RECORD trial who responded to a postal questionnaire in March 2002. n1=1740 receiving D3 with or without calcium. n2=1704 receiving calcium or placebo.	Self-reported infection or antibiotic use in the week prior to receiving the questionnaire.	800 IU/day D3, 1000mg calcium/day, 800 IU/day D3 + 1000mg calcium/day, or placebo for 24-62 months.	38 in a sample of 60 participants.	62 after 1 year of D3 in the same participants.	1. Adjusted odds of reported infection or antibiotic use in D3 group was not significantly lower than in placebo group (0.90 [95% CI 0.76-1.07]; p=0.23 and 0.84 [95% CI 0.64-1.09]; p=0.18 respectively). 2. In per protocol analyses based on self-reported pill taking, the odds for infection and antibiotic usage in D3 group were smaller but did not attain statistical significance (0.80, [95% CI 0.64 to 1.01]; p=0.06 and 0.74, [95% CI 0.52 to 1.06]; p=0.10 respectively).
Li-Ng 2009 [22] USA Randomised, Double-blind, placebo-controlled trial of vitamin D supplementation for the prevention of URTI.	N=162 healthy adult volunteers recruited between December '06 and March '07 n1=84 received D3 n=78 received placebo.	Self-reported URTI symptoms recorded in bi-weekly questionnaires. [URTI defined as presence of two or more of: fever, cough, productive sputum or change in sputum colour and quantity, muscle aches, nausea or vomiting in the absence of allergy symptoms].	2000 IU/day D3 for 3 months or placebo.	n1=64.3 n2=63.0	n1=88.5 n2=60.9	1. The frequency of reported URTI was slightly greater in the placebo compared to the D3 group, but did not reach statistical significance (50/363 [13.8%] vs 48/388 [12.4%] reports respectively, p=0.56). 2. There was no difference in severity or duration of URTIs between D3 and placebo groups.

(Table 3) contd.....

Study, Country, Type	Study Population (N)	Main End Point [Case Definitions]	Intervention	Mean Baseline 25(OH)D Concentration (nmol/L)		Outcome
				Baseline	Follow-Up	
Urashima 2010 [24] Japan Randomised, double-blind, placebo-controlled trial of vitamin D supplementation to prevent influenza A in school children.	N=430 children aged 6-15y recruited between November and December 2009, invited to participate by paediatricians at study sites (outpatient clinics). n1=217 received D3 n2=213 received placebo	Influenza A diagnosed by rapid test on nasopharyngeal swab. Secondary outcomes included physician-diagnosed asthma attacks, and non-specific febrile illness.	1200 IU/day D3 for 4 months or placebo.	Not measured	Not measured	<ol style="list-style-type: none"> 1. The risk of influenza A was significantly less in the D3 compared to the placebo group (RR: 0.58; [95% CI 0.34-0.99]; $p=0.04$), and this was more prominent in children who had not been taking any other vitamin D supplements. 2. Influenza A occurred significantly less often in D3 compared to placebo group between days 31 and 60 of supplementation (RR:0.41; [95% CI 0.19-0.86], $p=0.014$). 3. In a subgroup analysis of children known to have asthma, significantly fewer children in the D3 group had asthma attacks compared with those in the placebo group (RR:0.17; [95% CI 0.04-0.73], $p=0.006$).
Laaksi 2010 [21] Finland Randomised, double-blind, placebo-controlled trial of vitamin D supplementation to prevent ARTI in young Finnish men.	N=164 Finnish military conscripts volunteering to participate from 400 entering the unit. n1=80 received D3 n2=84 received placebo.	Number of days absent from duty due to ARTI between October 2005 and March 2006, ascertained from documentation in medical records of diagnosis of ARTI. Secondary outcomes included self-reported ARTI symptoms (any of cough, runny nose, sore throat, fever, or common cold symptoms).	400 IU/day D3.	n1=78.7 n2=74.4	n1=71.6 n2=51.3	<ol style="list-style-type: none"> 1. There was no difference in number of days absent from duty due to ARTI between intervention and control groups. 2. The frequency of self-reported ARTI symptoms did not differ between groups. 3. Cox regression analysis indicated the hazard ratio for absence from duty due to ARTI was lower in the D3 group. 4. In the first 6 weeks of the study there was a trend towards less absence in the D3 compared to control groups, although this did not reach statistical significance (0.7 ± 2.1 days vs 1.4 ± 2.6 days respectively, $p=0.06$).

n1=Intervention arm; n2=control arm; URTI=upper respiratory tract infection; ARTI=acute respiratory tract infection.

vitamin D3 used, and the relatively high baseline vitamin D status of participants (mean serum 25(OH)D > 70 nmol/L). Follow up 25(OH)D levels were lower than at baseline in both intervention and placebo groups, although the difference was greater in the placebo group.

The strongest evidence to date that vitamin D supplementation might prevent URTI comes from a placebo-controlled trial of vitamin D supplementation conducted in Japanese school children for the prevention of influenza A [24]. Participants received 1200 IU/day of vitamin D3 or placebo and were followed up for 4 months. A rigorous case

definition which required detection of influenza from nasopharyngeal swabs was used, but pre- and post-treatment 25(OH)D measurement was not available. The risk of influenza A was almost halved in children in the intervention arm, and this effect was greatest in the second month of supplementation; exacerbations of asthma were also reduced in the intervention arm of this study. No effect of vitamin D supplementation was seen on risk of influenza B however.

Hence on balance, although observational data supports an association between vitamin D insufficiency and susceptibility to acute upper or viral respiratory tract

infection, only two trials provides evidence that vitamin D supplementation prevents these infections [19, 24]. Aloia *et al.*'s findings although significant are limited by a case definition which uses 6-monthly self-reported symptoms, and the findings may not be generalisable as the study population were post-menopausal African-American women [19]. Further trials are needed in populations at high risk of ARTI, utilising bolus dosing regimens to rapidly correct vitamin D deficiency and case definitions which include detection of respiratory pathogen.

4. CONCLUSIONS

In developing countries where vitamin D deficiency is prevalent, high dose vitamin D supplementation in addition to routine pneumonia treatment significantly reduces the recurrence of disease in young children. Case-control studies in these settings demonstrate a strong association between vitamin D deficiency and ALRI, whilst similar studies in developed countries suggest that deficiency is associated only with more severe ALRI. Clinical trials using robust case definitions for ALRI are needed to evaluate the role of vitamin D supplementation in addition to other proven interventions in both children and pregnant women for the primary prevention of ALRI. Further trials of vitamin D supplementation for the prevention of URTI are also required: these should be conducted in populations where baseline vitamin D insufficiency is highly prevalent, and should utilise bolus dosing of vitamin D, which can be directly observed, and which results in rapid correction of vitamin D insufficiency [97].

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Vitamin D and Tuberculosis

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Abstract: Tuberculosis is a major global health problem: the World Health Organisation estimates that there were 9.4 million incident cases and 1.8 million deaths from the disease in 2008. The development of new agents to prevent acquisition or reactivation of latent *Mycobacterium tuberculosis* infection and to allow shortening of antimicrobial therapy regimens for active tuberculosis without loss of efficacy is a research priority. In this article we describe the immunomodulatory actions of vitamin D metabolites in mycobacterial infection and review the growing body of evidence from observational and intervention studies suggesting that vitamin D might have a role in the prevention and treatment of TB.

Keywords: Tuberculosis, vitamin D, immunomodulation, clinical trials.

INTRODUCTION

Tuberculosis (TB) is a major public health problem. The global prevalence of latent *Mycobacterium tuberculosis* (MTB) infection has been estimated at 32% [1], and this carries a 5-20% lifetime risk of reactivation disease in people who are not infected with Human Immunodeficiency Virus (HIV) [2]; reactivation rates higher than 10% per annum have been reported in HIV-infected people [3]. The World Health Organisation estimates that in 2008 there were 9.4 million incident cases of active TB, 11.1 million prevalent cases of TB, 1.3 million deaths from TB in HIV-uninfected people and 0.52 million deaths from TB in HIV-infected people [4]. The development of new agents to prevent acquisition or reactivation of MTB infection and to allow shortening of antimicrobial therapy regimens for active TB without loss of efficacy is a research priority. In this article we will review the growing body of evidence from studies conducted both *in vitro* and *in vivo* suggesting that vitamin D might have a role in the prevention and treatment of TB.

IMMUNOMODULATORY ACTIONS OF VITAMIN D IN MYCOBACTERIAL INFECTION

With the exception of a single report [5], vitamin D and its metabolites have not been shown to possess antimycobacterial activity in the absence of cells. However, the active metabolite of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)₂D), has long been recognised to induce antimycobacterial activity *in vitro* in mononuclear phagocytes, the cells which control growth of MTB [6]. Ligation of macrophage toll-like receptor 2/1 heterodimers by mycobacterial antigens induces expression of the vitamin D receptor (VDR) and the 1-alpha hydroxylase enzyme CYP27B1 [7, 8] which synthesises 1,25(OH)₂D from the principal circulating vitamin D metabolite 25-hydroxy-

vitamin D (25[OH]D). Because extra-renal 1-alpha hydroxylase follows first order kinetics, the rate at which it synthesises 1,25(OH)₂D depends on availability of 25(OH)D substrate [9]. Orally ingested vitamin D is freely converted to 25(OH)D, and this provides the rationale for administering 'parent' vitamin D to induce antimycobacterial responses at the site of disease.

1,25(OH)₂D modulates immune responses by ligating membrane VDR to induce rapid effects (within minutes), or nuclear VDR to induce genomic effects (within hours) [10]. Experiments using selective agonists and antagonists of these two receptors indicate that ligation of nuclear VDR is both necessary and sufficient for induction of antimycobacterial responses by 1,25(OH)₂D *in vitro* [11]. 1,25(OH)₂D modulates the host response to mycobacterial infection by pleiotropic mechanisms including the induction of reactive nitrogen and oxygen intermediates [12, 13], down-regulation of the gene encoding tryptophan-aspartate containing coat protein [14], promotion of phagolysosome fusion [15], suppression of matrix metalloproteinase enzymes implicated in the pathogenesis of pulmonary cavitation [16] and induction of antimicrobial peptides including cathelicidin LL-37 [7, 11] and human beta defensin 2 [17]. Cathelicidin LL-37 possesses antimycobacterial activity [7, 18] and also induces autophagy [19]; 1,25(OH)₂D₃-induced antimycobacterial activity has been reported to be dependent on expression of the gene encoding cathelicidin LL-37 [20].

VITAMIN D AND TUBERCULOSIS: HISTORICAL ASPECTS

In 1849, Chapman reported results of administering cod liver oil to patients with 'consumption', and made the following observation: 'the beneficial effects of the oil were manifested most speedily and most decisively, in the improvement of the appetite, aspect of the countenance, strength and spirits'. He concluded that cod liver oil was 'probably the only remedial agent by which the vital powers may be enabled to struggle successfully against that malady'

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[21]. This report represents the first circumstantial evidence that administration of a preparation containing vitamin D improved clinical outcome in patients with TB, although it should be noted that no control group was studied, and that any beneficial effects of cod liver oil may have been attributable to its content of vitamin A rather than vitamin D [22]. The first TB sanatorium was opened in Gorborsdorf, Germany (today Sokolowsko, Poland) in 1859, and heliotherapy (exposure of TB patients to sunlight, which induces cutaneous vitamin D synthesis) subsequently became common practice, and was credited with improvements in clinical outcome in many cases [23]. In 1903, Niels Finsen was awarded the Nobel Prize in Physiology or Medicine for his discovery that shortwave ultra-violet light was effective in the treatment of cutaneous TB [24]. Vitamin D₂ was purified and crystallised in 1931 [25] and Charpy subsequently pioneered the use of pharmacologic doses (≥ 1.25 mg daily) of vitamin D₂ to treat cutaneous TB [26]. Vitamin D₂ was also used to treat pulmonary TB, both as a single agent and, following the introduction of effective anti-tuberculous chemotherapy, as an adjunct to antibiotic treatment [27].

ASSOCIATION BETWEEN VITAMIN D DEFICIENCY AND SUSCEPTIBILITY TO TUBERCULOSIS

In 1985, Davies observed that people migrating to the United Kingdom from countries with a high incidence of latent MTB infection experienced rates of active TB that exceeded rates in their countries of origin, and that this increased risk coincided with the development of vitamin D deficiency, probably arising as a result of decreased sun exposure [28]. He suggested that vitamin D deficiency may predispose to reactivation of latent MTB infection in this setting, a hypothesis supported by his observation that vitamin D deficiency associated with susceptibility to active tuberculosis [29]. Since then, a further 11 case control studies investigating the association between vitamin D status and susceptibility to active TB have been published. Of these, 7 have reported a statistically significant association between vitamin D deficiency and susceptibility to active TB [30-36], 3 have reported a non-statistically significant trend towards such an association [37-39] and one [40] has reported that active TB was associated with both 'high' and 'low' serum 25(OH)D concentrations (>140 nmol/L and < 75 nmol/L, respectively). Potential explanations for an association between vitamin D deficiency and active TB include both causality (i.e. vitamin D deficiency impairs host immune response to MTB and causes susceptibility) and reverse causality (i.e. active TB causes vitamin D deficiency, due to anorexia, decreased exposure to sunlight in debilitated patients, or MTB-induced dysregulation of vitamin D metabolism [34]).

ASSOCIATION BETWEEN SUSCEPTIBILITY TO TUBERCULOSIS AND POLYMORPHISMS IN THE VITAMIN D RECEPTOR AND VITAMIN D BINDING PROTEIN

Human VDR is encoded by the *VDR* gene located on chromosome 12q. This gene is polymorphic, and numerous single nucleotide polymorphisms have been described. The hypothesis that VDR variants might associate with

susceptibility to active TB was first investigated by Bellamy and colleagues, who reported an association between carriage of the T allele of the *TaqI* VDR polymorphism and susceptibility to active TB in a case control study conducted in Gambian adults [41]. Wilkinson and colleagues subsequently reported that associations between susceptibility to TB and carriage of the T allele of the *TaqI* VDR polymorphism and the ff genotype of the *FokI* VDR polymorphism in Gujarati Asians living in London were restricted to vitamin D-deficient individuals [32]; this study is the first to report that gene: environment interactions may operate to influence susceptibility to active TB. Numerous case control studies investigating the association between VDR variants and susceptibility to active TB have since been published; a recent meta-analysis of 23 such studies reported that in Asian populations, the *FokI* ff genotype associated with susceptibility to active TB (pooled OR 2.0, 95% CI 1.3 to 3.2), and the *BsmI* bb genotype (defined by the presence of two restriction sites for the *BsmI* endonuclease) was associated with protection against active TB (pooled OR 0.5, 95% CI 0.4 to 0.8); no associations were seen in African or Latin American populations however [42].

Further case control studies have investigated associations between polymorphisms in the vitamin D binding protein (DBP) and susceptibility to active TB. DBP is a highly expressed multifunctional 58 kDa serum glycoprotein encoded on chromosome 4. Two common polymorphisms at codons 416 and 420 of exon 11 of the *DBP* gene give rise to the three major electrophoretic variants of DBP, termed group-specific component 1 fast (Gc1F), Gc1 slow (Gc1S) and Gc2. These variants differ in their functional characteristics: the Gc1F and Gc1S variants have been reported to have greater affinity for 25-hydroxyvitamin D (25(OH)D) than the Gc2 variant [43], potentially leading to more efficient delivery of 25(OH)D to the target tissues, while the Gc2 variant is associated with decreased circulating concentrations of 25(OH)D, 1,25(OH)₂D and DBP [44, 45]. Case control studies conducted in India, Russia and Kuwait have not reported any association between DBP genotype and susceptibility to TB [46-48], but a more recent study reported an association between the Gc2 allele of vitamin D binding protein and susceptibility to active TB among Gujarati Asians living in London. This association was preserved if serum 25(OH)D concentration was <20 nmol/L, but not if serum 25(OH)D was ≥ 20 nmol/L, suggesting that profound vitamin D deficiency and Gc2 genotype may interact to increase susceptibility to TB [39].

PROSPECTIVE OBSERVATIONAL STUDIES

In contrast to the numbers of published cross-sectional studies, relatively few cohort studies investigating associations between vitamin D status/VDR genotype and TB have been conducted. Two studies have examined the influence of VDR genotype on response to antimicrobial therapy: Roth and colleagues reported that the FF genotype of the *FokI* VDR polymorphism and the Tt genotype of the *TaqI* VDR polymorphism associated with faster sputum culture conversion in a cohort of pulmonary TB patients in Peru [49], while Babb and colleagues reported no difference in time to sputum culture conversion according to *TaqI* or

FokI VDR genotype among South African TB patients [50]. Recently, a cohort study conducted in Pakistan [51] reported that profound vitamin D deficiency among healthy household TB contacts at baseline associated with increased risk of development of active TB over the subsequent 4 years: 7/30 contacts with baseline plasma 25(OH)D < 17.5 nmol/L developed active TB during follow-up, compared with 1/32 with plasma 25(OH)D 17.5 – 33.5 nmol/L and 0/30 with plasma 25(OH)D > 33.5 nmol/L. This association retained significance after adjustment for age and sex, although other potential confounders were not taken into account in the analysis. The observation that increased risk of TB reactivation was almost exclusively confined to individuals with profound vitamin D deficiency is interesting, particularly when taken together with reports from case control studies that profound vitamin D deficiency is most strongly associated with susceptibility to TB [32]: the implication is that, if vitamin D deficiency does indeed predispose to active TB, then relatively modest elevations of serum 25(OH)D might be effective for prevention of active disease.

INTERVENTION STUDIES

Despite the compelling results from the laboratory and observational studies reviewed above, no randomised controlled trials of vitamin D supplementation for the prevention of active TB have been published to date. The absence of such studies reflects the very considerable methodological and logistic challenges of conducting them. Because the annual risk of reactivation of latent TB is low in immunocompetent individuals (<1% even in individuals with a strongly positive and newly-converted tuberculin skin test [2]), very large sample sizes and prolonged follow-up will be needed to detect all but the largest effects of vitamin D supplementation on TB incidence in such populations. Our own group has attempted to circumvent this problem by investigating the effect of vitamin D supplementation on a surrogate outcome measure of antimycobacterial response – the BCG-lux assay, which measures the ability of whole blood to restrict bioluminescence of a reporter mycobacterium [52]. We found that a single dose of 2.5 mg vitamin D enhanced the ability of TB contacts' whole blood to restrict mycobacterial bioluminescence at 24 hours post-inoculation [53], providing further evidence that trials of vitamin D supplementation for prevention of TB are justified.

In contrast to prevention studies, randomised controlled trials to determine whether adjunctive vitamin D enhances response to antimicrobial therapy can be powered with more modest numbers, because the majority of TB patients respond rapidly to antimicrobial therapy. Seven such studies have been published to date (summarised in Table 1). Three of these trials had biochemical primary outcomes: two reported no hypercalcaemia in tuberculosis patients receiving either 125 mcg vitamin D daily [54] or a single oral dose of 2.5 mg vitamin D [55], and one reported hypercalcaemia occurring in 19/30 tuberculosis patients receiving daily doses of 10 – 95 mcg vitamin D daily [56]. However, this third study, by Narang and colleagues, also reported that a daily dose of 60 mcg vitamin D elevated mean serum calcium in healthy controls – a finding which contrasts with other

studies which demonstrate that identical [57] or considerably higher [58] doses of vitamin D do not induce hypercalcaemia in healthy people. It is possible, therefore, that the actual doses of vitamin D administered in Narang's study were considerably higher than reported. The remaining four clinical trials listed in the table had clinical primary outcomes. Morcos and colleagues investigated the effects of 25 mcg vitamin D daily in a 24 children in Egypt receiving antimicrobial therapy for tuberculosis, and showed no effect on body weight or resolution of symptoms [59]. Nursyam and colleagues subsequently conducted a trial of a daily dose of 250 mcg vitamin D in 67 pulmonary TB patients in Indonesia [60]. In this study, adjunctive vitamin D enhanced sputum smear conversion at 6 weeks after initiation of antimicrobial therapy (34/34 vs 25/33 smear-converted in intervention vs control arm at 6 weeks, $p=0.002$); no effect of the intervention was seen at 8 weeks. The vitamin D status of participants was not assessed at either baseline or follow-up in this study, and details of safety monitoring, including monitoring of serum calcium concentrations, were not reported. In the largest treatment trial to date, Wejse and colleagues randomised 365 adult TB patients in Guinea-Bissau to receive three doses of 2.5 mg vitamin D3 or placebo at initiation of antimicrobial therapy, and again at 5 and 8 months [61]. The intervention had no effect on the primary outcome measure (a specially designed TB score) or on serum 25(OH)D concentration. Mean serum 25(OH)D concentrations at baseline were 78 nmol/L vs 79 nmol/L in intervention vs control groups. Most recently, our own group investigated the effect of a 2-weekly dose of 2.5 mg vitamin D on time to sputum culture conversion in 146 patients with smear-positive pulmonary TB in the UK [62]. A 79 nmol/L increase in serum 25(OH)D was seen among participants in the intervention arm of the study, which was associated with a non-statistically significant trend towards faster sputum culture conversion ($p=0.14$). A pre-planned sub-group analysis revealed that adjunctive vitamin D significantly hastened sputum culture conversion by more than 17 days in participants with the *tt* genotype of the *TaqI* vitamin D receptor polymorphism (hazard ratio 8.09, 95% CI 1.36–48.01; $p=0.02$).

CONCLUSIONS

Much remains to be done to evaluate whether vitamin D might have a role in the prevention or treatment of TB. A key research priority is to establish randomised controlled trials of vitamin D supplementation for the prevention of tuberculosis in individuals with latent MTB infection. Although some question the need for such studies to be conducted on the grounds that the methodological challenges they pose are too great [63], we remain convinced that these trials are necessary, fundable and feasible. Equivalent trials have been conducted to establish the role of chemoprophylaxis for tuberculosis prevention [64], and investigation of the role of vitamin D supplementation in this regard should be no less of a research priority, given the safety and low cost of this intervention. Investigations of the potential role of vitamin D as an adjunct to antimicrobial therapy are more advanced, but results from clinical trials published to date have shown little if any benefit in drug-sensitive disease. This is not the end of the road for this line of enquiry however. First, five other similar trials are

Table 1. Summary of Randomised Controlled Trials Investigating Effects of Adjunctive Vitamin D in Patients with Tuberculosis

First Author, Year, Reference	Sample Size, Setting	Vitamin D Dose Administered	Effect on Serum 25-Hydroxyvitamin D Concentration	Primary Outcome
Gwinup 1981 [54]	23 adults, USA	125 mcg daily	Not reported	Serum calcium: no change
Narang 1984 [56]	30 adults, India	10-95 mcg daily	Not reported	Serum calcium: hypercalcaemia in 63%
Morcos 1998 [59]	24 children, Egypt	25 mcg daily	Not reported	Body weight/symptoms: no change
Nursyam 2006 [60]	67 adults, Indonesia	250 mcg daily	Not reported	Smear conversion: increased rate at 6 wks
Martineau 2009 [55]	25 adults, UK	1 x 2.5 mg D2 @ 0 months	22 nmol/L increase in active arm	Serum 25(OH)D: small increase at 8 wks
Wejse 2009 [61]	365 adults, Guinea Bissau	3 x 2.5 mg D3 @ 0/5/8 months	25 nmol/L increase both arms	TB score: no effect
Martineau 2011 [62]	146 adults, UK	4 x 2.5 mg D3 @ 0/2/4/6 weeks	79 nmol/L increase in active arm	Culture conversion: no effect in study population as a whole, but effect seen in subgroup with tt genotype of the <i>TaqI</i> VDR polymorphism

ongoing [63]; a meta-analysis of the results of these studies may reveal a benefit which existing studies have not been powered to demonstrate. Second, the doses of vitamin D administered in trials conducted to date are considerably lower than those reported to be effective historically [27]; the effects of pharmacological dosing regimens are worthy of investigation. Finally, on-going investigations from our recently completed trial [62] reveal that administration of adjunctive vitamin D is associated with favourable immunomodulatory activity; this observation raises the possibility that individuals with multi-drug resistant TB, in whom antimicrobial therapy is less effective, might derive a clinical benefit from enhancement of their antimycobacterial immune response using adjunctive vitamin D therapy.

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Vitamin D and Lung Cancer

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Abstract: Lung cancer is one of the most common cancers but treatment has limited success and survival rates remain low. The active metabolite of vitamin D, 1,25-dihydroxyvitamin D, has anti-proliferative and pro-apoptotic actions in lung carcinoma cell lines *in vitro*. Vitamin D deficiency is associated with poor prognosis in lung cancer, and lung cancer survival varies according to vitamin D receptor genotype. Randomized controlled trials should be performed to determine whether vitamin D supplementation might have a role as an adjunct to conventional treatments for lung cancer.

Keywords: Vitamin D, lung cancer, incidence, mortality.

INTRODUCTION

Lung cancer is the leading cause of cancer death among both men and women [1], responsible for 1.3 million deaths per year worldwide [2]. The two major forms are non-small-cell lung cancer (NSCLC), representing 85% of all lung cancers, and small-cell lung cancer (SCC), accounting for 15%. Treatments include surgery, chemotherapy, radiotherapy or a combination of the above. Overall 5-year survival remains low at 14% [3].

The most common cause of lung cancer is long-term exposure to tobacco smoke, but a growing body of literature suggests that vitamin D deficiency may also be independently associated with increased lung cancer incidence and decreased lung cancer survival in some groups. Moreover, some lung cancer cell lines have been reported to express both vitamin D receptor (VDR) and the enzyme capable of synthesising the active metabolite of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)₂D), which exerts anti-proliferative and pro-apoptotic effects *in vitro*. In this article, I will describe the *in vitro* actions of vitamin D metabolites in lung cancer cell lines, review the epidemiological literature relating to the association between vitamin D deficiency and lung cancer incidence and mortality and highlight the need for randomised controlled trials of vitamin D supplementation in patients with lung cancer.

MECHANISMS OF ACTION OF VITAMIN D IN CANCER CELLS

Vitamin D, obtained through cutaneous synthesis on exposure to ultraviolet B (UVB) light or diet, is transported in the circulation bound to vitamin D binding protein, and is hydroxylated in the liver by the mitochondrial cytochrome P450 enzyme 25-hydroxylase (CYP27A1) to form 25-hydroxyvitamin D (25(OH)D), the major circulating

metabolite of vitamin D. The production of 25(OH)D is not tightly regulated and correlates with substrate availability rather than with physiological requirement [4, 5]. Determination of circulating 25(OH)D concentrations thus provides a useful indicator of vitamin D status that is widely applied in clinical practice [6]. 25(OH)D is metabolised by another mitochondrial cytochrome P450 enzyme, 1 alpha-hydroxylase (CYP27B1) into the hormonally active form 1,25(OH)₂D; CYP27B1 is expressed in the kidney and in extra-renal tissues including NSCLC cells [7]. Synthesis of 1,25(OH)₂D is tightly regulated, both by 1,25(OH)₂D itself and by parathyroid hormone, fetal growth factor 23 and serum concentrations of calcium and phosphate [4], which influence expression of the major vitamin D catabolic enzyme, 24-hydroxylase (CYP24). The gene encoding this enzyme has been identified as a candidate oncogene in a number of cancer cell lines, and its expression is increased in NSCLC cells [8].

1,25(OH)₂D exerts its biological effects by binding VDR within the nuclei of cells in target organs. VDR is expressed in at least thirty-six tissues of the body [4], and its presence has been detected in approximately 65% of primary non-small cell lung cancers [8]. VDR regulates the expression of as many as 500 genes of the $\approx 20,488$ genes in the human genome [9]; many of these are involved in the control of apoptosis, cell division and adhesion, raising the possibility that 1,25(OH)₂D may influence the growth of malignant cells [10].

In vitro, 1,25(OH)₂D has been reported to possess anti-proliferative, anti-angiogenic and pro-apoptotic activity in a wide variety of cancer cell lines including leukaemia cells and malignant cells from skin, breast, colon and prostate [11-23]. 1,25(OH)₂D has also been reported to inhibit proliferation of two SCLC cell lines (NCI-H82 and NCI-H209) [24] and one out of five squamous cell carcinoma cell lines investigated (EBC-1) [25]; in the latter study, exertion of anti-proliferative action by 1,25(OH)₂D was found to be dependent on expression of VDR. *In vivo*, both 1,25(OH)₂D and a low-calcaemic analogue, 22-oxa-1 α ,25-dihydroxyvitamin D₃, reduced the number of metastases and tumour burden in mice injected with a lung carcinoma cell line [26].

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VITAMIN D AND LUNG CANCER INCIDENCE

The overwhelming contribution of smoking as a cause of lung cancer dominates amongst the causes, and this may obscure associations between vitamin D status and susceptibility to lung cancer, particularly because smokers have been reported to have lower serum 25(OH)D concentrations than non-smokers [27]. Nevertheless, there is some evidence that greater UVB exposure or supplemental vitamin D may be independently associated with reduced incidence of the disease. Grant has recently summarised the evidence for increased solar UVB exposure and higher vitamin D status as factors for the prevention of cancer using Hill's criteria for causality [28]. Vitamin D status/UVB exposure satisfied the causality criteria most convincingly for breast and colorectal cancer, he found evidence that they also fulfilled the causality criteria for 14 other cancers – one of them being cancer of the lung.

In an ecological study, Mohr *et al.* examined incidence rates of lung cancer in 111 countries according to the UVB irradiance while controlling for cigarette smoking, cloud cover and anthropogenic aerosols. Lower UVB irradiance was associated with higher incidence rates of lung cancer. Latitude was positively related to incidence rates in both men ($r^2=0.55$, $p<0.01$) and women ($r^2=0.36$, $p<0.01$) [29]. In a prospective study, Kilkkinen *et al.* followed up 6937 patients in Finland over 24 years and 122 lung cancers were identified. After adjustment for potential confounders, serum 25(OH)D concentration was inversely associated with lung cancer incidence for women (RR, 0.16; 95% CI, 0.04-0.59; $P < 0.001$) and younger participants (RR, 0.34; 95% CI, 0.13-0.90; $P = 0.04$) but not for men (RR, 1.03; 95% CI, 0.59-1.82; $P = 0.81$) or older individuals (RR, 0.92; 95% CI, 0.50-1.70; $P = 0.79$) [30].

Clinical trials of vitamin D supplementation specifically for the prevention of lung cancer have not been performed, but secondary analysis of trials of vitamin D supplementation related to bone health may yield pertinent data. In one such randomised controlled trial, 1179 post menopausal women were randomised to receive 1100 IU vitamin D + calcium vs calcium alone vs placebo. A 77% reduction of all cancer risk, with 35% of the cancer risk reduction attributed to the vitamin D, was reported [31]. In this trial there was one lung cancer in the vitamin D + calcium group after four years, but three in the placebo group. The reason why such a large treatment success was observed after the study's relatively short duration of four years might be because vitamin D is thought to be effective in preventing progress of early cancers, consistent with the 15 - 20 year latency period before clinical detection [32]. Two previous negative studies analysed data from the women's health initiative and found no effect of vitamin D supplementation on incidence of cancer of the colon [33] or the breast [34]; however these studies have been criticised for the low dosing regimen (400 IU vitamin D daily) used in the intervention arm, and for having had poor compliance [35, 36].

VITAMIN D AND LUNG CANCER MORTALITY

The hypothesis that solar UVB radiation and cancer mortality were inversely related was first proposed by

Apperly in 1941 [37]. Grant, who carried out an ecological study in the US, was the first to identify an inverse association between UVB irradiance/vitamin D status and lung cancer mortality rates [32]. Another ecological study has since reported an inverse association between UVB irradiance and lung cancer mortality rates in male, but not female, black Americans [38]. Zhou *et al.* investigated the effects of seasonal influences and vitamin D intake on survival of early stage NSCLC and found that both higher UVB exposure (patients who had surgery in the summer) and higher vitamin D intake (diet and supplement, assessed by a validated food frequency questionnaire) was associated with improved lung cancer survival [39]. They observed a 26% improved survival in those with serum 25(OH)D concentration >50 nmol/L vs those with serum 25(OH)D concentration <25 nmol/L. Chen *et al.* recently reported an inverse relation between ambient UVB and mortality rates for all cancers in a large study conducted in China, with lung cancer showing the strongest inverse relation, even if adjusted for smoking [40]. However, cross-sectional analysis of a very large data set from the US National Health and Nutrition Examination Survey III did not demonstrate an association between serum 25(OH)D concentration and lung cancer mortality [41]. A large prospective study has estimated that a 25 nmol/L difference in predicted 25(OH)D concentration was associated with a 20% reduction in lung cancer mortality risk in men, although this trend did not attain statistical significance [42].

A number of studies have investigated whether VDR genotype is associated with lung cancer survival. Zhou *et al.* conducted a cohort study of 373 patients with early stage NSCLC has demonstrated an association between the G/A+A/A genotype group of the Cdx-2 VDR polymorphisms and improved survival among patients with squamous cell carcinoma (SCC) but not among those with adenocarcinoma; 5-year survival rates were 41% for the G/G and 55% for the G/A+A/A genotypes. Concerning the joint effects of the three relevant VDR polymorphisms, Cdx-2 G>A, FokI C>T, and BsmI C>T, SCC patients with two or more “protective” alleles had better overall survival, with adjusted hazard ratios of 0.20 (95% CI, 0.09-0.48), 0.40 (95% CI, 0.19-0.87) and 0.43 (95% CI, 0.19-0.97) for subjects with two, three and four or more “protective” alleles, respectively, when compared with subjects with zero or one “protective” allele [43]. The same research group has also reported that the C/C genotype of the FokI VDR polymorphism was associated with better survival in a cohort of 294 patients with advanced stage NSCLC (21.4 months for C/C versus 12.1 months for C/T and 15.6 months for T/T). The G-T-C (Cdx-2-FokI-BsmI) haplotype was associated with worse survival compared with the most common haplotype of G-C-T (adjusted hazard ratio, 1.61; 95% CI, 1.21 to 2.14; $P = .001$) [44]. To date, no randomised controlled trials of vitamin D supplementation have been performed in patients with lung cancer. However, Cannell and Hollis have proposed that patients with cancer require significantly higher doses of vitamin D and should maintain serum 25(OH)D concentrations >137 nmol/L [45]. It is reasonable to hypothesise that vitamin D might produce maximal clinical benefit in the early stages of tumour development, when malignant cells are more likely to retain both the VDR and the enzymes needed to activate vitamin D

[9]. Two observations support this hypothesis: firstly the finding of an association between improved survival for cancers diagnosed in the summer (when serum 25(OH)D concentrations are higher) vs the winter [39], and secondly, the higher metabolic clearance of 25(OH)D once the cancer has developed [45]. If supplementation in early cancer is considered, frequent monitoring of serum 25(OH)D and calcium concentrations should guide dosing [45, 46].

CONCLUSIONS

Randomised controlled trials are needed to test the hypothesis that increases in circulating 25(OH)D concentration are effective in reducing cancer risk before any public health recommendations can be made for vitamin D supplementation on a population level. Considering the widespread vitamin D insufficiency and deficiency which has in particular been observed in cancer and lung cancer patients, it might be prudent to consider treating vitamin D deficiency and insufficiency in those diagnosed with cancer. Further information from trials is needed to determine whether vitamin D supplementation might prolong survival in lung cancer. However, sufficient observational, ecological and epidemiological evidence is now available to argue the case to conduct clinical trials of vitamin D supplementation in this patient group.

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Immunomodulatory Actions of Vitamin D Metabolites and their Potential Relevance to Human Lung Disease

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Abstract: The non-classical role for vitamin D in maintaining immune homeostasis has been recognised for 30 years. A definitive link between vitamin D status and the immune response has now been established by a multitude of association studies which link both vitamin D deficiency and genetic polymorphisms in vitamin D-related genes to susceptibility to respiratory diseases including tuberculosis, upper respiratory tract infection, chronic obstructive pulmonary disease (COPD), asthma and lung cancer. This review considers the mechanisms by which immune cells and lung epithelial cells respond to infection or injury by inducing intracellular metabolism of vitamin D. The effects of vitamin D metabolites on induction of phagocyte antimicrobial responses, modulation of DC maturation and T cell priming, skewing of the cytokine milieu towards a type 2 inflammatory response and promotion of regulatory T (Treg) cell development will also be described.

Keywords: Immunomodulation, 1,25-dihydroxyvitamin D, cytokine, macrophage, T-cell, dendritic cell, T-reg, autoimmunity, infection.

INTRODUCTION

Over the past three decades our understanding of the role vitamin D plays in human physiology has changed dramatically. Once considered primarily as a regulator of calcium and bone homeostasis, vitamin D is now recognised to have a diverse range of physiological functions. One reason for its pleiotropic actions may be the fact that vitamin D differs from other vitamins in that its primary active metabolite, 1 α ,25-dihydroxyvitamin D (1,25(OH)₂D), is a steroid hormone. Moreover, unlike many other vitamins which act as antioxidants or enzyme co-factors, vitamin D metabolites complex with their receptor to form a ligand-dependant transcription factor complex that regulates the expression of more than 900 genes [1].

The classic model of vitamin D metabolism involves its initial 25-hydroxylation in the liver to produce 25-hydroxyvitamin D (25(OH)D), the major circulating vitamin D metabolite and accepted measure of vitamin D status. Once 25(OH)D reaches the kidney it is 1 α -hydroxylated by the CYP27B1 enzyme to form 1,25(OH)₂D. This metabolite acts on the kidney, the gastro-intestinal tract and bone to regulate serum calcium concentration as well as its own catabolism, *via* induction of the 24-hydroxylase enzyme CYP24A1 (Fig. 1). In 1981 it was discovered that 1,25(OH)₂D can also be produced in extra-renal tissues, including macrophages [2]. Subsequent *in vitro* studies have demonstrated that during certain infections, microbial binding to pattern recognition receptors (PRRs) on leucocytes and epithelial cells can upregulate expression of CYP27B1 in these cells to induce local synthesis of 1,25(OH)₂D [3, 4]. These observations suggest that during an immune response cells require high levels of 1,25(OH)₂D for

optimum functionality. It is now known that many cell types upregulate CYP27B1 to induce synthesis of 1,25(OH)₂D in response to a range of stimuli. Consequently, vitamin D is now recognised to regulate numerous aspects of cellular physiology, including cell proliferation, differentiation, activation and death.

Vitamin D metabolites modulate both innate and adaptive immune responses (summarised in Fig. 2). The wide-ranging clinical implications of this immunomodulatory activity are reflected by the steady increase in the number of diseases being associated with vitamin D deficiency and the large number of clinical trials of vitamin D for the prevention and treatment of respiratory infections, cancer, diabetes mellitus, cardiovascular disease and multiple sclerosis [5-12]. Moreover, with the worldwide prevalence of vitamin D deficiency on the increase [13, 14], two internationally recognised bodies have released new supplementation guidelines [15, 16] within the last year.

The aim of this review is to discuss the emerging immunomodulatory roles of 1,25(OH)₂D and its precursors, catabolites and analogues in controlling the development and function of monocytes (MN), macrophages, dendritic cells (DC), T cells, B cells, mast cells and airway epithelial cells. Particular attention will be paid to the mechanisms by which vitamin D metabolites modulate the immune response to respiratory pathogens.

VITAMIN D STATUS AND DISEASE ASSOCIATION

Vitamin D exists in two forms: vitamin D₃ (cholecalciferol) and vitamin D₂ (ergocalciferol) (Fig. 1). Vitamin D₃ is the predominant form in humans and is primarily obtained through skin exposure to UVB irradiation. This cleaves 7-dehydrocholesterol, stored in the epidermis, into pre-vitamin D₃ which spontaneously isomerises to form pro-vitamin D₃. Vitamin D₃ is also obtained from dietary sources such as oily fish and their derived products like cod liver oil, while vitamin D₂ is

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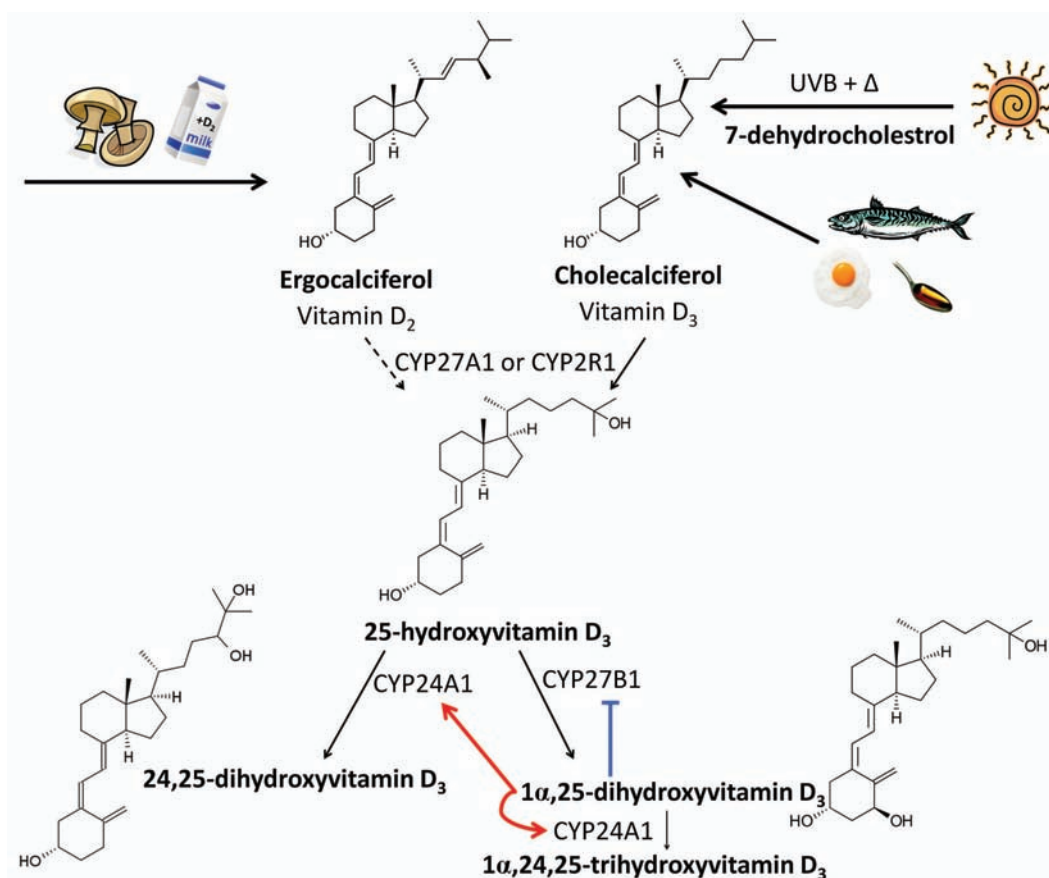


Fig. (1). Vitamin D metabolism pathway. The primary form of vitamin D in humans, D₃, is obtained by ultra violet B irradiation and photolytic conversion of 7-dehydrocholesterol stored in the epidermis or directly from animal products such as oily fish and egg yolk. Vitamin D₂ is obtained nutritionally from fungus or dairy products supplemented with D₂. Cytochrome p450 (CYP) enzymes are responsible for vitamin D metabolism, CYP27A1 and CYP2R1 are the primary 25-hydroxylases found in the liver. 25-hydroxyvitamin D (25(OH)D) is the major circulatory form of vitamin D and is 1 alpha-hydroxylated to produce the primary active metabolite 1α,25-dihydroxyvitamin D (1,25(OH)₂D) by CYP27B1, either in the kidney or in a tissue-specific manner. 1,25(OH)₂D controls its own levels by inhibiting CYP27B1 and inducing its own catabolising enzyme CYP24A1, which can also 24-hydroxylate 25(OH)D into 24,25-dihydroxyvitamin D.

obtained from plant sources, primarily fungus and yeast, and from some nutritional supplements. Some countries also fortify dairy products, margarine and other foodstuffs with vitamin D to prevent profound vitamin D deficiency arising as a consequence of limited sunlight exposure and poor dietary vitamin D intake.

The sum of 25(OH)D₂ and 25(OH)D₃ concentrations in serum is used as a measure of vitamin D status; the half-life of 25(OH)D is 15-20 days [17, 18] compared to that of 1,25(OH)₂D, which is 4-8 hours [19]. A serum concentration of total 25(OH)D <25 nmol/L was the traditional clinical cut-off for deficiency due to its association with susceptibility to rickets. However, recent studies suggest that circulating 25(OH)D concentrations lower than 75-80 nmol/L may be insufficient for optimal physiological function. Recent studies and meta-analyses have identified significant associations between lower serum 25(OH)D concentrations and impaired lung function [20], decreased survival from lung cancer [21, 22], and increased rates of severe asthma exacerbation [23], tuberculosis (TB) [24], acute respiratory tract infections [25] and chronic obstructive pulmonary disease COPD [26]. Consequently, new supplementation guidelines released by the Institute of

Medicine (IOM) in November 2010 defined serum 25(OH)D concentrations <50 nmol/L as deficient and increased the recommended daily intake (RDI) from 400 IU to 600 IU [15]. However, many scientists within the field believe that concentrations of 50-75 nmol/L are insufficient for optimal nonskeletal health benefits of vitamin D [27]. Therefore, in opposition to the IOM report, the Endocrine Society released its own guidelines in July 2011 recommending an RDI of at least 600 IU is needed to prevent deficiency but 1500-2000 IU/day is needed to elevate serum 25(OH)D above 75 nmol/L [16].

Both vitamin D₂ and vitamin D₃ may be administered to correct vitamin D deficiency. However, controversy exists over whether vitamin D₂ elicits the same physiological responses as vitamin D₃. Some investigators have reported that 25(OH)D₂ has a shorter half-life than 25(OH)D₃ and have questioned whether vitamin D₂ is an appropriate supplement to correct vitamin D deficiency [28]. However, others have reported that vitamin D₂ and vitamin D₃ can induce equivalent increases in serum 25(OH)D concentration [29]. Studies comparing the effect of 25(OH)D₂ and 25(OH)D₃ on immune cell function are needed to determine

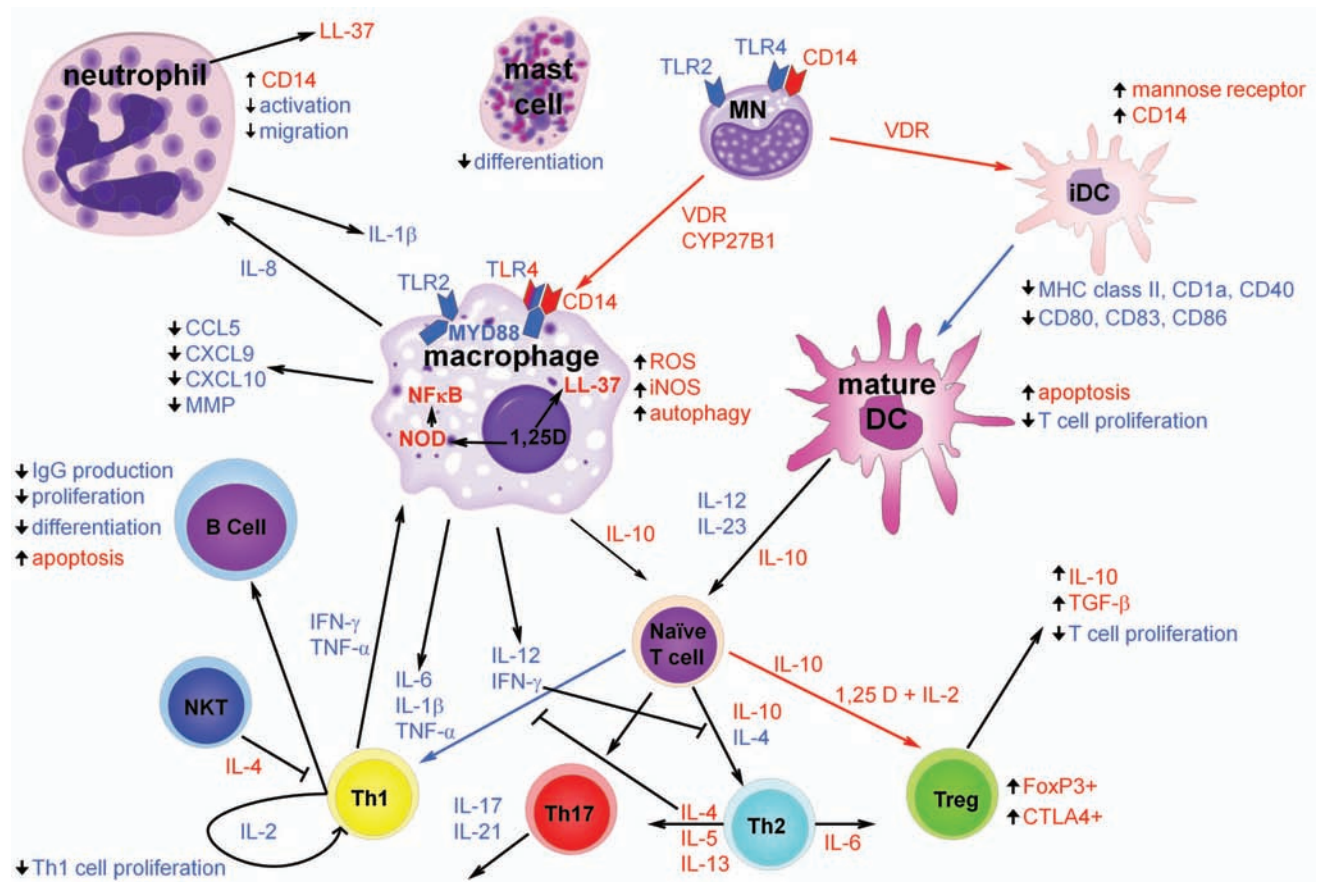


Fig. (2). Vitamin D has a wide range of actions on all immune cells. Vitamin D can induce (red) monocyte (MN) differentiation into macrophages and immature dendritic cells (iDC). Vitamin D inhibits (blue) iDC maturation towards mature DC. Vitamin D also inhibits type 1 cytokine production from mature DC and enhances the anti-inflammatory cytokines IL-10 which primes naïve T cells to differentiate into Th2 cells, producing type 2 cytokines and along with IL-2 vitamin D (1,25D) can induce regulatory T (Treg) cell development. Vitamin D also inhibits TLR2 and MYD88 (the toll-like receptor (TLR) adapter molecule), but upregulates CD14 and in some cases TLR4. Vitamin D also upregulates the antimicrobial peptide cathelicidin (LL-37) which has direct antimicrobial properties and induces autophagy. The intracellular pattern recognition receptor NOD is also upregulated by vitamin D and this induces NFκB. However, vitamin D also inhibits pro-inflammatory cytokine production by macrophages and Th1 cells and the interferon-gamma (IFN-γ) inducible chemokines CXCL9, CXCL10 and CCL5 and matrix metalloproteinases (MMP). Less information is known regarding its effects on B cells, mast cells and neutrophils, but vitamin D has a general inhibitory effect on either their differentiation, activation, proliferation or antibody (IgG) production. In general, vitamin D enhances direct anti-microbial properties of antigen presenting cells, it inhibits pro-inflammatory cytokine and promotes a type 2 inflammatory response.

whether or not these metabolites possess equivalent immunomodulatory activity.

Environmental and dietary intakes of vitamin D are not the only determinant of vitamin D status. Circulating levels of 25(OH)D also depend on levels of vitamin D binding protein (DBP), and on the activity of CYP450 enzymes which regulate vitamin D metabolism and catabolism. Moreover, genetic variation in the genes encoding these proteins also influences serum 25(OH)D concentrations [30]. DBP (also known as group-specific component, GC) contains 3 major haplotypes, due to two polymorphisms: GC-1F, GC-1S (D416E amino acid change) and GC-2 (T420K amino acid change). The GC-1F gene product has the highest affinity for 25(OH)D, while the GC-1S gene product has 2-fold lower affinity and the GC-2 gene product has 4-fold lower affinity [31]. Consequently, individuals possessing the GC-2 allele have lower circulating levels of

25(OH)D than non-carriers [32, 33]. Gujarati Asians who carry the GC-2 allele and who are vitamin D deficient have greater susceptibility to active TB, raising the possibility that low 25(OH)D levels and impaired ability to traffic 25(OH)D to the site of disease may have a combined detrimental effect on antimycobacterial immunity [34]. Conversely, GC-1F has been identified as a risk factor for COPD in a Chinese Han population, and carriage of the GC-2 allele has been suggested to have a protective effect on pathogenesis of COPD [35]. CYP24A1 and CYP27B1 polymorphisms are associated with circulating 25(OH)D levels [36] and CYP24B1 polymorphisms are associated with increased risk of asthma [33].

An effective response to vitamin D is dependent on a functional vitamin D receptor (VDR). There are a number of single nucleotide polymorphisms (SNPs) in the VDR which affect its function and abundance, including a start codon

SNP identified by *FokI* restriction enzyme digestion and multiple SNPs in the 3' untranslated region (UTR) identified by digestion with *ApaI*, *BsmI* and *TaqI* restriction enzymes. *TaqI* *TT* and *FokI* *FF* genotypes have been associated with high VDR mRNA levels, and the *TT* genotype of the *TaqI* polymorphism has been associated with high VDR protein levels [37]. Six CpG islands (associated with DNA methylation) have recently been identified in VDR; the promoter region encompassing the above described SNPs falls within the CpG island with the highest variability in methylation status [38]. Furthermore, the *TaqI* genotype showed an interaction with regional methylation status, ethnicity and disease susceptibility, indicating that epigenetic factors may influence relationships between VDR genotype and susceptibility to disease. In the context of respiratory disease, VDR polymorphisms have been associated with susceptibility to asthma [39], lung cancer [40], acute lower respiratory tract infection [41], COPD [42, 43] and TB [38, 44-46]. VDR polymorphisms have also been associated with enhanced response to antimicrobial therapy for active TB; however, the two studies to present such data had conflicting observations. One found that patients with *FokI* *ff* and *TaqI* *Tt* genotypes had faster time to sputum culture conversion [47], while the other found that patients with *ApaI* *AA* and *TaqI* *TT* genotypes had faster smear conversion [48]. These studies used different measures of treatment outcome and were performed in small populations without measuring baseline serum 25(OH)D concentrations. Recently a clinical trial has reported that high dose adjunctive vitamin D therapy accelerates sputum culture conversion in TB patients with the *tt* genotype of the *TaqI* VDR polymorphism, but not in those with *TT* or *Tt* genotypes [11]. More studies in larger population groups from various ethnogeographic backgrounds are needed to confirm these results.

TISSUE-SPECIFIC VITAMIN D UPTAKE AND METABOLISM

The greatest leap forward in elucidating non-classical roles of vitamin D occurred in 1981 with the discovery of extra-renal 1 α -hydroxylation of 25(OH)D [2]. Shortly afterwards it was reported that 1,25(OH)₂D₃ induces differentiation of monocytes into macrophage-like cells [49] and that both these cell types express VDR [50]. In 1983, it was shown that macrophages isolated from sarcoid granulomas could convert 25(OH)D₃ into 1,25(OH)₂D₃; they lacked 24-hydroxylase activity however [51, 52]. These observations also provided an explanation for the high serum 1,25(OH)₂D levels and low serum 25(OH)D levels that are sometimes seen in chronic granulomatous diseases such as sarcoidosis, TB and lymphoma [53]. Interferon-gamma (IFN- γ) was also found to enhance macrophage levels of 1,25(OH)₂D₃ by antagonising the ability of 1,25(OH)₂D₃ to induce CYP24A1 and suppress CYP27B1 [54, 55]. In 2006 it was discovered that stimulation of the macrophage PRR Toll-like receptor 2 (TLR2) with *M. tuberculosis* (MTB)-derived TLR2/1 ligand induces intracellular expression of CYP27B1 and VDR to enhance action of endogenously synthesised 1,25(OH)₂D₃ [4]. Treatment of TLR2/1 stimulated macrophages with 25(OH)D₃ also led to upregulation of CYP24A1, suggesting the presence of its inducer, 1,25(OH)₂D₃, and thus intracellular conversion of 25(OH)D₃ to 1,25(OH)₂D₃. Intracellular 1,25(OH)₂D₃

production has subsequently been confirmed in monocyte-derived macrophages (MDM) and immature DC (iDC) using radio-labelled 25(OH)D₃ [56].

Since the discovery that MN and MDM express a repertoire of vitamin D-regulated genes, further studies have made similar observations in a range of other cell types. Activated T and B cells, DC and lung epithelial cells all express VDR, CYP27B1 and CYP24A1, indicating that they possess the machinery to utilise and generate 1,25(OH)₂D₃ from 25(OH)D₃ [57-60]. Mast cells have also been shown to express VDR and respond to 1,25(OH)₂D₃, but not 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃) [61]. Furthermore, MDM have been shown to have high expression of the 25-hydroxylase enzyme CYP27A1, whereas MN and DC have only low expression. Accordingly, MDM were also found to be able to convert vitamin D₃ to 1,25(OH)₂D₃ with greater efficiency than DC [62]. It could be argued that lymphocytes activated *in vitro* do not represent the same population of cells which infiltrate the lung during pathological processes. However, the fact that they express both VDR and the enzymatic machinery capable of producing intracellular 1,25(OH)₂D₃ indicates that they have the capacity to utilise circulating 25(OH)D when stimulated to do so and to respond to locally produced 1,25(OH)₂D₃. *In vivo* work is needed to characterise the response of these cells to vitamin D supplementation in patients with respiratory disease.

In order to produce intracellular 1,25(OH)₂D₃ cells also need a mechanism to internalise its precursor compounds. Megalin and cublin, two proteins involved in active transport of the 25(OH)D-DBP complex in the renal proximal tubule have also been shown to be expressed by non-renal cells, including lung epithelium [63]. Interestingly, macrophages do not express these proteins but can still obtain and utilise 25(OH)D₃. *In vitro*, human MN cultured in serum from individuals carrying the DBP GC-2 allele have an enhanced transcriptional response to 25(OH)D₃ treatment [64]. It has been suggested that this is due to less 25(OH)D₃ being bound to DBP, making more free 25(OH)D₃ available for cellular uptake *via* diffusion. Similar experiments using serum from DBP knockout (KO) mice during *in vitro* culture of mouse osteoblastic cells showed that co-culture with DBP KO serum resulted in greater sensitivity to 1,25(OH)₂D₃ treatment [65]. Moreover, DBP KO mice had higher tissue concentrations of 1,25(OH)₂D₃ than wild-type mice despite having lower circulating concentrations of 25(OH)D₃. Thus, it appears that the concentration of 'free' circulating 25(OH)D may be a more important determinant of cellular response to vitamin D than the total circulating 25(OH)D concentration. This genotypic effect may therefore be an important point to take into consideration when prescribing vitamin D therapy.

Apart from transporting 25(OH)D, DBP also plays a role in scavenging extracellular globular(G)-actin [66], activation of macrophages [67] and enhancement of the chemotactic activity of C5a for neutrophils and monocytes [68, 69]. G-actin is released by necrotic cells and is thus a major by-product of inflammation; DBP binding facilitates clearance of G-actin, preventing harmful effects of filamentous (F)-actin formation during inflammation [70]. Furthermore, F-actin can bind the antimicrobial peptide cathelicidin, inhibiting its function. Dissociation of these F-actin bundles

restores cathelicidin's antimicrobial activity [71]. DBP variants with different binding affinities may therefore affect the role of DBP in resolution of inflammation [72] and indirectly control antimicrobial peptide activity.

The peptide encoded by the GC-2 allele is also the only DBP variant which cannot be converted to the potent macrophage activation factor GC-MAF, as it lacks a threonine residue that is targeted for deglycosylation by T- and B-cell glycosidases during formation of the sialic acid-free variant of DBP [73]. Thus, the lower binding capacity for 25(OH)D₃ and the inability to form GC-MAF makes carriage of the GC-2 variant potentially detrimental for a vitamin D deficient individual. This observation is consistent with the finding that vitamin D deficient carriers of the GC-2 allele are more susceptible to TB [34]. Polymorphisms which affect the binding efficiency of DBP may therefore associate with variation in responses to vitamin D deficiency.

VDR-LIGAND INTERACTION AND RECEPTOR SIGNALLING

The VDR can function at both the cell membrane and as a nuclear receptor. Formation of an active receptor in the nucleus requires 1:1 heterodimerisation with the vitamin A receptor, retinoid X receptor (RXR). Binding of 1,25(OH)₂D to the nuclear receptor complex results in nuclear translocation and a conformational change which exposes the VDR DNA binding site and thus activates the transcription factor complex. This complex binds repeats of PuG(G/T)TCA, known as vitamin D response elements (VDREs), in the promoter of more than 900 genes to regulate their expression [1].

1,25(OH)₂D₃ is the most potent ligand for this receptor complex as it has the strongest affinity for the VDR-RXR ligand binding domain of all natural vitamin D metabolites (K_d-value 0.1nM - 50x stronger than for 25(OH)D₃) [74, 75]. 1,25(OH)₂D₃ is therefore recognised as the primary active metabolite of vitamin D₃. However, circulating 25(OH)D₃ and its 24-hydroxylated catabolite, 24,25(OH)₂D₃, can also activate VDR ligand-dependant transcription [76, 77], although higher concentrations are required to achieve the same effect. Moreover, the natural 26-hydroxylated forms of 25(OH)D₃ and 1,25(OH)₂D₃ (25,26-dihydroxyvitamin D₃ and 1,25,26-trihydroxyvitamin D₃, respectively) induce similar restriction of *M. tuberculosis* growth in MDM as 1,25(OH)₂D₃ when they are used at the supra-physiological concentration of 10 µmol/L [78]. However, 1,25(OH)₂D₃ can achieve the same degree of restriction as 25(OH)D₃ at much lower concentrations (1 nmol/L), while 1,24,25(OH)₃D₃ can achieve this at 100 nmol/L [79]. Numerous synthetic analogues of vitamin D that can transactivate VDR without inducing hypercalcemia (a detrimental effect of excessive vitamin D supplementation) have also been produced [80]. 22-oxa-1,25-dihydroxyvitamin D₃ is one such analogue that has been reported to have the same potential as 1,25(OH)₂D₃ to inhibit functional differentiation of DC, cytotoxic T cells and helper T cells [81].

Vitamin D metabolites are not the sole activators of VDR-RXR. Retinoic acid (RA) in combination with 1,25(OH)₂D₃ (both at 1 µmol/L) has been shown to down-regulate expression of coronin-1 (also known as Tryptophan

Aspartate containing Coat protein, TACO), an endogenous macrophage actin-binding protein that inhibits phagosome maturation during infection [82]. Pre-treatment with RA/1,25(OH)₂D₃ also inhibited macrophage phagocytosis of MTB [83]. Taken together, this suggests that maintaining physiological levels of both vitamin D and vitamin A may be important for correct functioning of the VDR-RXR complex.

Immunomodulatory actions of 1,25(OH)₂D₃ may also be mediated by activation of membrane VDR and phosphatidylinositol 3-kinase (PI3K) signal induction. PI3K signalling has been linked to induction of monocyte-macrophage differentiation [84], generation of reactive oxygen species (ROS) [85], induction of nitric oxide (NO) [86] and phagosome/lysosome fusion during MTB-MDM infection [87]. However, experiments using selective agonists and antagonists of membrane and nuclear VDR indicate that 1,25(OH)₂D₃-induced restriction of mycobacterial growth during *in vitro* peripheral blood mononuclear cell (PBMC) culture was predominantly due to nuclear VDR signalling [88]. Moreover, the capacity for all immune cells to generate intracellular 1,25(OH)₂D suggests that the main action of vitamin D during an immune response is *via* nuclear VDR. This is further supported by recent work showing that T cell receptor signalling in naïve T cells induces nuclear VDR expression and VDR-mediated transcription of phospholipase C-γ1 (PLC-γ1), which controls the Ras-mitogen-activated protein kinase (MAPK) cascade during T cell activation [89].

INDUCTION OF ANTI-MICROBIAL RESPONSES BY VITAMIN D

The ability to mount an immune response to microbial infection is reliant on the expression of PRR by myeloid cells. 1,25(OH)₂D₃ stimulates the expression of CD14 (which forms a functional heterodimer with TLR4) in MN and MDM, but it inhibits TLR4 and TLR2 expression during 1,25(OH)₂D₃-induced MDM differentiation [90]. In the HL-60 macrophage cell line, however, 1,25(OH)₂D₃ does not inhibit TLR4 expression [91]. These results indicate that the effect of 1,25(OH)₂D₃ on TLR4 expression depends on macrophage phenotype, raising the possibility that, *in vivo*, macrophages in different tissues may have different responses to 1,25(OH)₂D₃. In the context of lung disease, more work therefore needs to be performed using alveolar macrophages (AM) as well as MDM.

Along with experiments showing that 25(OH)D₃ treatment of TLR2/1-stimulated MDM induces 1,25(OH)₂D₃ production, Liu and colleagues have also reported that 25(OH)D₃ treatment induces expression of the gene which encodes the antimicrobial peptide cathelicidin (*hCAP18*) which is proteolytically cleaved to produce the active antimicrobial peptide LL-37 [92]. Co-treatment with itraconazole, a non-specific CYP450 enzyme antagonist, inhibited cathelicidin expression in these experiments, suggesting that 25(OH)D₃ metabolism was required for *hCAP18* induction. It was subsequently reported that *in vitro* treatment of MTB-infected PBMC with 1,25(OH)₂D₃ induces *hCAP18* mRNA expression and that LL-37 restricts growth of MTB in liquid broth culture [88]. Further studies have reported that epithelial cells in the upper and lower respiratory tract also secrete cathelicidin and that viruses can also induce cathelicidin expression when sufficient 25(OH)D is available [93, 94]. Furthermore, neutrophils, T cells, B cell, NK cells, DC and mast

cells also express *hCAP18* [95, 96], suggesting that $1,25(\text{OH})_2\text{D}_3$ could induce cathelicidin expression in diverse cell populations that infiltrate the lung in response to a range of bacterial and viral infections. The finding that MTB induces greater cathelicidin expression in AM isolated from healthy controls, than in MDM differentiated *in vitro*, suggests that models using MDM may be underestimating the *in vivo* cathelicidin-mediated immune response [97]. However, interestingly, granuloma tissue from TB patients show very weak LL-37 staining, whereas tissue sections from patients with acute pneumonia show intense LL-37 staining, particularly in activated macrophages [97]. The lack of LL-37 in TB patients may be due to the common occurrence of vitamin D deficiency in these patients and highlights one of the potential benefits of vitamin D supplementation in these patients. Although there has been a number of recent vitamin D supplementation trials in TB patients [10-11, 98], obtaining clinical samples from the lower airway of these patients is methodologically problematic. Furthermore, the presence of three VDREs in the cathelicidin gene promoter (consequently making it one of the most highly induced genes by vitamin D) appears to be a recent evolutionary addition to the innate immune response, which only exists in humans and non-human primates [99]. Thus, non-primate animal models are not an appropriate model in which to study the effect of vitamin D on LL-37 distribution *in vivo*, when the aim is to translate findings to humans.

The bactericidal activity of cathelicidin was first shown to be mediated by its ability to bind and disrupt bacterial cell wall phosphatidylglycerol monolayers [100]. However, recent work has shown that the cathelicidin peptide also induces 2 genes (*Beclin-1* and *Atg5*) involved in autophagy *via* activation of p38 MAPK and extracellular signal-regulated kinase (ERK) 1/2 signalling, but not *via* activation of nuclear factor (NF)- κB . Autophagy is an intracellular process inducing autophagosome/lysosome fusion and subsequent lysosome-mediated degradation of phagosome contents [101]. This finding is key to tuberculosis pathogenesis as MTB is known to subvert the immune response by inhibiting phagosome/lysosome fusion [102]. Thus, cathelicidin appears to have direct and indirect antimicrobial properties.

Since it was discovered that TLR2/1 stimulation induced VDR expression in MN, it has also been shown that $1,25(\text{OH})_2\text{D}_3$ induces expression of another PRR, the intracellular nucleotide-binding oligomerization domain containing 2 (NOD2) receptor in MN and intestinal epithelial cells [103]. NOD2 signalling activates the NF κB transcription factor, which together with the VDR transcription factor complex induces expression of the antimicrobial peptide beta-defensin 2 (hBD-2 or DEFB4) by binding the one VDRE and two NF κB sites in its promoter. By contrast, the cathelicidin promoter has 3 VDREs but no NF κB sites [104]. The observation that cathelicidin activates MAPK and ERK signalling, but not NF κB , during $1,25(\text{OH})_2\text{D}_3$ -induced autophagy [101] indicates that $1,25(\text{OH})_2\text{D}_3$ regulates the immune response *via* two separate mechanisms: one *via* NF κB signalling and one *via* cathelicidin-induced MAPK signalling (Fig. 2).

VITAMIN D INHIBITS A TYPE 1 AND PROMOTES A TYPE 2 INFLAMMATORY RESPONSE

Paradoxically, despite its ability to induce NF κB *via* NOD2 activation [103], $1,25(\text{OH})_2\text{D}_3$ inhibits NF κB -induced

type-1 pro-inflammatory cytokines TNF α , IFN- γ , IL-12p40, IL-6, IL-8, IL-1 β and IL-23 and induces the anti-inflammatory cytokine IL-10 in MN, MDM and DC [88, 105-107]. Pre-treatment of human MN with $1,25(\text{OH})_2\text{D}_3$ induces maturation and augments TNF α production following stimulation with lipopolysaccharides (LPS, a TLR4 ligand found in the cell wall component of gram negative pulmonary pathogens such as *Hemophilus influenzae*, *Klebsiella pneumoniae*, *Legionella pneumophila* and *Pseudomonas aeruginosa*) [108]. However, combined pre-treatment of mouse bone marrow-derived macrophages (BMDM) with $1,25(\text{OH})_2\text{D}_3$ and IFN- γ decreased the bactericidal activity of BMDM by inhibiting the phagocyte oxidase-mediated oxidative burst and suppressing IFN- γ induction of chemokines CCL5 (RANTES), CXCL10 (IP10) and CXCL9 (MIG) and PRRs TLR2 and Fc γ receptor 1 and 2 [109]. Thus, it is possible that $1,25(\text{OH})_2\text{D}_3$ controls a negative feedback loop, limiting the inflammatory immune response at the same time as inducing a direct anti-microbial response.

T cells also have the capacity to generate endogenous $1,25(\text{OH})_2\text{D}_3$, and this has been shown to have direct effects on different T cell populations. $1,25(\text{OH})_2\text{D}_3$ treatment of human CD8 $^+$ and CD4 $^+$ PBMC promotes a type 2 phenotype, reducing the number of IL-2 secreting T cells and completely blocking IL-12-induced IFN- γ production by CD4 $^+$ and CD8 $^+$ subsets [110] and IL-17 and IL-21 production by CD4 $^+$ CD25 $^+$ T cells [111]. Conversely, $1,25(\text{OH})_2\text{D}_3$ has also been shown to increase IL-4 and IL-13 positivity in CD4 $^+$ and CD8 $^+$ subsets, along with IL-6 positive CD4 $^+$ T cells. In combination with IL-4, $1,25(\text{OH})_2\text{D}_3$ enhances IL-6 induction in CD4 $^+$ and CD8 $^+$ cells [110]. Furthermore, in the presence of IL-2, $1,25(\text{OH})_2\text{D}_3$ induces expression of FoxP3, the marker of adaptive regulatory T (Treg) cells that suppress T cell proliferation. This *in vitro* observation is validated by *in vivo* work showing that serum $25(\text{OH})\text{D}$ levels are positively correlated with Treg function [112]. Moreover, IL-10 producing Tregs have been shown to be protective in the mouse asthma model [113], suggesting that vitamin D treatment of asthma patients may limit its severity. In contrast to its actions in activated CD4 $^+$ and CD8 $^+$ T cells, $1,25(\text{OH})_2\text{D}_3$ has been reported to suppress IL-4 production by naïve CD62 ligand $^+$ CD4 $^+$ T cells during their *in vitro* polarization. This effect was greatest if $1,25(\text{OH})_2\text{D}_3$ was given before initiation of differentiation and it was significantly diminished if it was added after polarization [114]. Taken together, the studies above provide further evidence that the immunomodulatory effects of $1,25(\text{OH})_2\text{D}_3$ are dependent on the activity and environment of its target cells at the time of exposure. The relevance of these *in vitro* findings to the clinical situation may also be limited, and while there are some *in vivo* findings in animal models, more findings from clinical trials are needed to characterise the effect of vitamin D supplementation on human T cell responses.

VITAMIN D REGULATES DC MATURATION AND DC-MEDIATED T-CELL ACTIVATION

DC play a fundamental role bridging innate and adaptive immunity, due their ability to present antigen and activate T helper cells. DC are hypothesized to be responsible both for

extra-pulmonary dissemination of MTB during chronic infection [115] and for delayed lymph-node trafficking of MTB, resulting in the delayed induction of the adaptive immune response following infection [116]. $1,25(\text{OH})_2\text{D}_3$ inhibits the maturation of immature DC, blocking CD14 down-regulation and inhibiting cell surface expression of MHC-class II molecules, CD40, CD80, CD83 and CD86. Functionally, this results in inhibition of chemokine secretion, inhibition of Th1 and Th17 cytokine production and induction of mature DC apoptosis [105, 106, 117]. Consequently, $1,25(\text{OH})_2\text{D}_3$ -treated DC have a reduced capacity to activate Th1 T cell proliferation and to induce CD4+ cells to secrete IFN- γ [106, 118]. Moreover, $1,25(\text{OH})_2\text{D}_3$ switches DC phenotype towards production of type 2 cytokines, with upregulation of IL-10 and promotion of differentiation of naïve T cells into Tregs, independently of its effect on DC maturation [119]. This effect of $1,25(\text{OH})_2\text{D}_3$ on DC-mediated Treg induction has been reported in a mouse model of asthma: pre-treatment of DC with $1,25(\text{OH})_2\text{D}_3$ and dexamethasone induced IL-10 production, generated DC which stimulated CD4+ naïve T cells to differentiate into Tregs, and ultimately resulted in reduced airway hyper-responsiveness [120].

VITAMIN D AND DESTRUCTION OF PULMONARY EXTRACELLULAR MATRIX

Matrix metalloproteinases (MMP) are a family of enzymes capable of degrading all components of pulmonary extracellular matrix. They are generally upregulated in activated cells where their primary role is to facilitate tissue remodelling and repair. MMP also regulate the innate immune response by controlling cytokine and chemokine processing, apoptosis and antimicrobial peptide cleavage and activation [121]. However, excess MMP activity can lead to excess matrix degradation and ultimately lung tissue destruction in chronic inflammatory conditions. MN, MDM and PBMCs infected with MTB induce expression and secretion of MMP-1, MMP-7 and MMP-10 [122, 123]. Increased expression of *MMP-1*, *MMP-7* and *MMP-9* has been demonstrated in cells isolated from the lungs of TB patients, with MMP-1 and MMP-7 co-localising to macrophages around the central area of necrosis in tuberculous granulomata [123, 124]. Circulating concentrations of MMP-9 have also been shown to correlate with the severity of pulmonary TB [125]; in a zebra fish model of TB, disruption of MMP-9 function attenuates granuloma formation and bacterial growth [126]. TNF α up-regulates MMP-9 expression and activity in alveolar macrophages from COPD patients, and elevated MMP-9 levels have been detected in the sputum of COPD patients [127, 128]. Airway smooth muscle cells from asthma patients have increased MMP-9 expression [39], raising the possibility that chronic asthma-associated lung destruction is also linked to MMP-9.

$1,25(\text{OH})_2\text{D}_3$ has also been reported to inhibit the expression, secretion and activity of a number of MMP during *in vitro* MTB infection [122, 129]. There is limited *in vivo* data, however, to support a role for vitamin D in limiting lung destruction through inhibiting MMP. However, inhibition of MMP activity has the potential to limit AMP activity. Thus, the net effect of vitamin D supplementation

on MMP function, if any, needs to be investigated in clinical trials.

CONCLUSION

There is increasing evidence that $1,25(\text{OH})_2\text{D}_3$ and other vitamin D metabolites regulate innate and adaptive immune responses to pulmonary pathogens, and elicit direct antimicrobial activity in cells which populate the lung. Vitamin D metabolites also inhibit pro-inflammatory cytokine production, enhance Treg proliferation, induce type 2 cytokine production and inhibit MMP expression and secretion. Taken together, these observations indicate that vitamin D has the potential to boost innate antimicrobial responses and limit adaptive inflammatory responses to prevent the excessive tissue destruction which often accompanies chronic lung disease. *In vitro* observations now need to be taken forward into clinical trials to characterise the immunomodulatory actions of vitamin D supplementation in patients with infectious and immune-mediated lung disease.

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