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Title page

Title: Lung development

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Abstract

Epidemiological studies have demonstrated an association between maternal vitamin D deficiency and an increased risk of chronic lung disease in offspring. While vitamin D, and UV induced non-vitamin D pathways, have the capacity to modulate immune function, this relationship may also be explained by an effect on lung development which is an independent predictor of lung function and the risk of lung disease later in life. To date there are not sufficient data to support a role for non-vitamin D pathways in this association, however, *in vivo* and *in vitro* data suggest there is a causal relationship between vitamin D and lung development. Despite this, equivocal results in recent high profile clinical trials have dampened enthusiasm for vitamin D as an important public health intervention for improving lung development. In this narrative review we summarise our current understanding of the link between UV exposure, vitamin D and lung development.

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Background

Exposure to ultraviolet (UV) radiation exposure from the sun has impacts on human health and disease. While UV radiation is well-known for its direct deleterious effects on the skin, it is also the major source of vitamin D synthesis [1] which is important in calcium homeostasis [2]. However, recent studies have also suggested a link between vitamin D and the development of non-communicable chronic lung diseases. In particular, epidemiological studies have consistently shown a relationship between low maternal vitamin D levels and the risk of developing asthma in children [3-5]. Vitamin D, and UV radiation through non-vitamin D pathways, are well known for their potential to modulate immune function which is important in asthma pathogenesis [6]. However, this association may also be explained by the potential importance of this pathway in modulating lung growth, which is an independent predictor of susceptibility to the development of chronic lung disease [7].

Several recent studies have highlighted the potential role for vitamin D in modulating lung development, which may explain this link, however the recent equivocal results in high profile randomised controlled trails examining the impact of maternal vitamin D supplementation on postnatal lung health suggest that this relationship is not straight-forward [8-10]. In this narrative review we will summarise our current understanding of the vitamin D, and the non-vitamin D effects of UV exposure, on lung development.

Overview of lung development

The lungs bud from the primitive foregut *in utero* at 3 weeks gestation [11]. Organogenesis of the lung is characterised by the embryonic, pseudoglandular, canalicular, saccular, and alveolar stages of development (summarized in Figure 1). Lung development is a complex and dynamic process such that any insults that occur during this process have the potential to

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impact on normal lung development resulting in increased susceptibility to lung disease and long-term deficits in lung function [7].

During the embryonic stage (3-6 weeks gestation), the human fetal lung makes its first appearance as a ventral diverticulum that arises from the caudal end of the laryngotracheal groove of the foregut [11]. By 4 weeks of gestation, the end of the diverticulum divides to form two primary bronchi buds, which then develop lobar buds that corresponding to the mature lung lobes (three on the right and two on the left). The primitive lung bud is lined by endoderm-derived epithelium, which eventually differentiates into both the airway and alveolar epithelium[12]. The pulmonary arteries bud off from the aortic arches and grow down into the mesenchyme, surrounding the lung tubules where they form a vascular plexus [13].

During the pseudoglandular phase (5-17 weeks gestation) the primitive conducting airways begin to form as epithelial tubes push into the mesenchyme [14]. The primitive airway epithelium starts to differentiate to form neuroendocrine, ciliated and goblet cells, while mesenchymal cells begin to form cartilage and smooth muscle cells [15]. During this phase, mesenchymal-epithelial interactions play a critical role in the regulation of growth and the airway branching pattern. The mesenchyme is directly responsible for budding of the epithelial tube, and as the mesenchymal mass increases, epithelial differentiation is shifted from bronchial (ciliated and goblet cells) to alveolar (primarily Type II pneumocytes) [16]. The accompanying arterial branches are laid down during this phase [17]. Towards the end of the pseudoglandular stage, the major conducting airways to the terminal bronchioles are developed down to 16 generations [18].

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During the canalicular stage (16-27 weeks gestation) the compact acinar clusters grow by further peripheral branching, lengthening of the branches, and widening of the distal airspaces with a concomitant decrease in interstitial mesenchyme. The epithelium has differentiated into Type I and II pneumocytes by this stage, and surfactant synthesis begins [19]. As the peripheral airways grow and mature the capillary network develops [13], and airway branching is completed by ~24 weeks gestation [20]. During the saccular phase (26 weeks to birth), interstitial tissue projects into the distal airspaces and divides them into saccules [11], and true alveoli appear [19]. Along with the expansion of respiratory airways, blood vessels grow in length and diameter and new bronchial arteries and veins are formed [21].

Alveolarization begins around 29 weeks gestation and continues postnatally [22]. Studies have shown postnatal alveolar number increases dramatically within the first 2-3 years of life [23]. While there is some debate about when this process finishes, recent evidence suggests that human lung growth continues through late adolescence [24], and the alveoli may continue to increase in size and number into early adulthood [25]. During the alveolar stage, alveoli are formed through a septation process that greatly increases the gas-exchange surface area [13]. From birth until maturity, there is a 20-fold increase in gas-exchange surface area and 30-fold increase in lung volume [23].

Lung development and susceptibility to lung disease

Due to the complexity of normal lung development [11], any environmental insults that occur during fetal and early post-natal growth can have profound and lifelong impacts on lung structure and function [26]. This is because deficits in lung development persist throughout life such that infants born with low lung function have a persistent deficit in lung function,

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relative to their peers, throughout life [27, 28]. Importantly, these early deficits in lung function are associated with an increased susceptibility to chronic lung disease [29-31].

While the mechanisms linking *in utero* and early post-natal environmental exposures with altered lung development will depend on the type of exposure, alterations in somatic growth are associated with a range of developmental insults. Interestingly, somatic growth has been associated with poor post-natal lung function in a number of cohort studies whereby, after adjusting for other maternal factors, there is an association between birth weight (as a crude marker of somatic growth) and lung function in adulthood [32, 33]. In addition, young adults born at very low birth weight have reduced airflow [34, 35]. A recent meta-analysis of data from 25,000 children found that younger gestational age, being smaller for gestational age and having lower infant weight gain were independent risk factors for the development of asthma [7]. Thus, maternal exposures that impact on somatic growth also have the potential to have a long-term influence on lung health.

Season, latitude and lung development

Ambient UV levels depend on season, latitude, climate, and atmospheric pollution [36]. Given that vitamin D production is strongly and consistently associated with the duration of the photoperiod, which in turn is influenced by latitude and season [37, 38], an impact of early life vitamin D levels or UV exposure, should be evident in associations between season of birth or latitude and post-natal lung function. However, to our knowledge, there are no studies examining the relationship between latitude at birth and objective measures of post-natal lung function. Likewise, there are few studies that have examined the association between season of birth and lung function. One study on mortality in extremely pre-term infants, who are at a high risk for death due to respiratory insufficiency, showed improved

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survival if they were exposed to higher levels of UVB at 17 and 22 weeks gestation [39]. Another study that examined the link between a range of early life factors, including season of birth, using data from two European population cohorts found that individuals born during winter had a greater decline in lung function in mid-late adulthood [40]. However, due to the lack of experimental models that have attempted to isolate the effect of UV on lung development it is not clear whether these associations are due to UV exposure or vitamin D synthesis.

While data linking season of birth and post-natal lung function are limited, a number of studies have examined the relationship between season of birth and birth weight which, as detailed earlier, may be linked to lung development. However, the relationship that has been described is inconsistent. For example, an Australian study examined within-year fluctuations in birth weight and found peak birth weight occurred in Spring and Autumn [41]. In contrast, a Northern Ireland study found that individuals born during late spring and summer were lighter than those born in winter [42] while a more recent study in UK found that children born in summer had higher mean birth weight, later pubertal development, and taller adult height [43]. So, while season, and perhaps UV/vitamin D, seems to influence birth weight the nature of the relationship is unclear. This may reflect the complex processes that may be driving this association including other environmental factors (e.g. infection) that may differ geographically and be related to season and/or lung development. Further complicating these relationships is the fact that season of birth and birth weight are examples of a single snapshot in time that ignores the fluctuating UV exposure, and hence vitamin D levels, that would occur during the entire gestation period.

Maternal vitamin D and post-natal lung function

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A number of longitudinal epidemiologic studies have reported associations between maternal vitamin D status during pregnancy (which reflects fetal vitamin D status [44]), post-natal lung function, as a marker of lung development, and the susceptibility to respiratory disease. A cohort study in the United States found an association between a higher maternal vitamin D intake during pregnancy and a reduced risk of recurrent wheeze in children at 3 years of age [3]. This observation was supported by a birth cohort study from the United Kingdom [4]. A more recent study, using objective measures of vitamin D status (serum 25(OH)D) and lung function, found an association between maternal vitamin D deficiency at 16-20 week's gestation, impaired lung function and an increased risk of asthma at 6 years of age [45]. However, other similar studies have shown no association between maternal vitamin D status and early post-natal lung outcomes [46-48]. Given the range of experimental evidence supporting a role for maternal vitamin D in fetal lung development (outlined below), it is possible that this variability is due to the method of assessing vitamin D status and the gestational time point used for assessing maternal vitamin D status. Of course, these studies could also be confounded by an as yet undetermined role for maternal UV exposure in lung development via non-vitamin D related pathways.

Evidence supporting a causal role for vitamin D in lung development comes from a number of sources. For example, a principal component analysis of vitamin D sensitive genes derived from PubMED and Gene Ontology surveys found common vitamin D related genes in both the human and mouse lung transcriptome; particularly in the later stages of lung development [49]. Many of these genes were also upregulated in cells from asthmatics providing a link between vitamin D, lung development and the susceptibility to chronic lung disease. This link is supported by a number of *in vivo* rodent studies.

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An early study found that rachitic rats had decreased lung compliance and altered lung structure characterised by disturbed alveolar formation and increased connective tissue [50]. A subsequent study in mice, that had sufficient calcium levels, showed that early life vitamin D deficiency cause impaired lung function at 2 weeks of age (early alveolar stage of lung development; see Figure 1) including increased airway resistance, increased tissue stiffness and a smaller lung volume (Figure 2) suggesting that this effect is mediated by vitamin D rather than impaired calcium homeostasis [51]. Similarly, 3 weeks old rats born to vitamin D deficient mothers have increased airway resistance [52] while vitamin D receptor (VDR) knockout mice, which have severe physiological phenotype characterised by hair loss and impaired bone development, show evidence of early-onset emphysema and lung function decline as a result of matrix metalloproteinase (MMP) upregulation in the lung [53].

One of the characteristic features of asthma is airway hyperresponsiveness (AHR); an increased propensity for the airway constrict in response to bronchoconstricting stimuli [54]. While the mechanisms of AHR are complex it is consistently associated with altered airway structure; including the increased airway smooth muscle (ASM) mass that is typical of the asthmatic airway [55]. Thus, it is interesting to note that *in utero* vitamin D deficiency in mice increases ASM in adulthood with the addition of post-natal deficiency also causing AHR [56] (Figure 2). This link between vitamin D and ASM has also been demonstrated in severe steroid resistant asthmatic children, where lower vitamin D levels were associated with increased ASM mass and worse lung function [57]. Taken together, these data suggest that there is a causal relationship between *in utero* vitamin D deficiency, altered airway structure and impaired post-natal lung function. The mechanisms underlying this link will be discussed in more detail below.

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Mechanisms linking maternal vitamin D and lung development

A recent proteomic analysis of the effects of vitamin D deficiency on fetal and neonatal mouse lung during the key stages of lung development suggested that maternal vitamin D deficiency affected lung development by reducing surfactant production and enhancing collagen synthesis [58], which are both likely to increase lung stiffness [51]. Interestingly, differential expression in the lung proteome seemed to be restricted to the saccular/alveolar stages of lung development, which are almost exclusively post-natal in the mouse (Figure 1), and is consistent with a previous lung transcriptome study[49].

The role of vitamin D in epithelial cell structure and function during the later stages of lung development is supported by *in vitro* studies showing that alveolar Type I and Type II cells isolated from fetal rat lungs during late gestation express vitamin D receptors (VDRs) [59]. Application of vitamin D to primary cell cultures from fetal, neonatal and adult rat alveolar epithelial cells stimulates DNA synthesis [60] while the active form of vitamin D, $1,25(OH)_2D$ stimulates surfactant production in Type II cells [61] [62]. Vitamin D may also play a critical in controlling epithelial-mesenchymal interactions during normal lung development [59] and contribute to lung maturation by modulating septal thinning during alveolarisation [63] (Figure 2).

Vitamin D, in a number of settings, has been shown to alter expression of the transforming growth factor beta (TGF-β) pathway. This is important because TGF-β_1 expression influences surfactant production [64], regulates epithelial-mesenchymal interactions [65] and is critical in overall lung development [66]. For example, vitamin D reduces expression of TGF-β_1 induced extracellular matrix proteins in lung epithelial cells and blunts epithelial to mesenchymal transition [67]. Likewise, $1,25(OH)_2D$ inhibits TGF-β induced cell migration in

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human bronchial epithelial cells [68] and attenuates cytokine induced remodelling in human fetal ASM cells by blunting TGF-β induced collagen III deposition [69]. This latter observation highlights the proliferative influence of vitamin D on cells [70] that have structurally important roles in determining post-natal lung function and susceptibility to lung disease later in life. Interestingly, vitamin D deficiency seems to reduce TGF-β expression *in vivo* [6], suggesting that the role of TGF in this association is complex.

In addition to these effects on epithelial and mesenchymal cells, there is recent evidence to suggest that vitamin D may also have a role in angiogenesis. In particular, in *in utero* models of endotoxin exposure, prenatal vitamin D treatment can prevent vascular dysfunction [71] and improve endothelial cell growth and tube formation [72]. Interestingly, post-natal vitamin D supplementation also seems to be beneficial in these models [73]. While the role of vitamin D in lung developmental angiogenesis in the healthy lung remains to be determined, any alteration in endothelial function is likely to have a significant impact on lung development and, potentially, post-natal lung structure and function.

Vitamin D supplementation trials and lung development

In response to the epidemiological and experimental associations between maternal vitamin D and lung outcomes there have been a number of recent randomised controlled trials to assess the effect of maternal vitamin D supplementation on lung health in offspring. On balance, the outcomes of these trials suggest no beneficial effect of vitamin D supplementation on indirect measures of lung development [8-10] (Figure 2).

A small London based study of 180 women at 27 weeks' gestation found no effect of small (800 IU) daily doses of ergocalciferol or a single bolus does of 200,000 IU of cholecalciferol

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on the risk of wheeze or lung function at 3 years of age in offspring [8]. Interestingly, both interventions only had a limited effect on cord blood levels of vitamin D suggesting that the interventions may not have been effective at improving vitamin D status in the participants. Two more recent studies, published as companion, also showed equivocal effects of vitamin D supplementation on post-natal lung outcomes [9, 10].

The Copenhagen Prospective Studies on Asthma in Childhood study [10] and (US) Vitamin D Antenatal Asthma Reduction Trial (VDAART) [9] had similar designs with all women receiving 400 IU of vitamin D daily and the intervention group receiving additional daily doses of vitamin D. The main differences between the studies being the higher intervention dose in the VDAART trial (4000 IU vs 2400 IU) and earlier gestational start point (10-18 weeks' gestation vs 24 weeks). Both had similar primary outcomes although they did not measure lung function in the children which is not surprising given their age (3 years). For the primary outcomes related to wheeze/asthma there was no effect of the intervention in both trials [9, 10].

Based on these observations one could conclude that there is no effect of maternal vitamin D supplementation on lung development. However, a few issues need to be addressed. Firstly, both studies used "community" samples, which included mothers with a range of baseline vitamin D levels. While there was a significant increase in serum 25(OH)D levels in response to the intervention in both studies the mean baseline levels in the Copenhagen study were relatively high (31 ng/mL = 77.4 nmol/L) [10]. While the levels in the VDAART study were lower (~ 23 ng/mL = 57.4), suggesting a higher level of deficiency (cutoff ~ 50 nmol/L), the placebo group (who received 400 IU of vitamin D daily) had an increase in vitamin D levels (26.8 ng/mL = 66.9 nmol/L) which may have been sufficient to protect the offspring from the

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detrimental effects of maternal vitamin D deficiency [9]. The authors of the VDAART trial also suggest that their study may have been underpowered and the authors of the Copenhagen study acknowledge the variability in the effect size estimates; both of which may be due to the issues outlined above. Thus, whether maternal vitamin D supplementation in mothers who are vitamin D deficient can improve lung development and reduce the risk of chronic lung disease in their offspring remains an open question.

Given the issues outlined above, particularly the absence of lung function measures in the children, follow up studies are likely to be enlightening. Given that a recent Cochrane review suggests that there is moderate evidence for increased growth in offspring following maternal supplementation with vitamin D [74], and the link between being small for gestational age and lung growth, measuring somatic growth in the children in these studies may also provide further insight. The importance of this is highlighted by the fact that vitamin D deficient mice also have similar early life deficits in somatic growth [51].

Conclusion

In summary, whether vitamin D supplementation in pregnant women is necessary to improve lung development and reduce the risk of chronic lung disease is unclear. Throughout this review we have largely ignored the potential effects of UV via non-vitamin D pathways. This is due to the fact that there are almost no data on these pathways in terms of their role in modulating lung development. There are some epidemiological studies that have implied an effect of UV exposure on early growth and development, however, the presumptive mediator in all of these cases has been vitamin D which is supported by the plethora of studies demonstrating a biologically plausible link between vitamin D and lung maturation. These studies have shown that *in utero* vitamin D deficiency can alter post-natal lung growth

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resulting in increased lung stiffness and an increase in ASM mass. Mechanistic studies suggest that the lung is most sensitive during the saccular/aveolar stage of development through altered surfactant production, increased collagen synthesis, epithelial mesenchymal transition and ASM proliferation; all of which may be modulated by the TGF-β pathway. Future studies should focus on the effects of vitamin D supplementation during the later stages of lung development, particularly in mothers who are vitamin D deficient at this point in gestation. Taken together there appears to be consistent evidence to support a role for vitamin D in modulating lung development and there are sufficient issues with recent clinical trials to suggest that further investigation in this field is required.

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Figure legends

Figure 1. Schematic representation of the major stages of human and mouse lung development. While the lung development process in humans and mice is qualitatively similar, there are important differences; in particular, alveolarisation is exclusively post-natal in the mouse. These discrepancies need to be taken into account when considering *in vivo* experimental data and their relevance to human lung development.

Figure 2. Diagram summarising the relationship between vitamin D and lung development. *In vivo* descriptive studies have shown that early life vitamin D deficiency impairs lung growth resulting in increased lung stiffness (and reduced lung volume) and *in utero* vitamin D deficiency increases airway smooth muscle (ASM) mass in adulthood. Mechanistic studies, from both *in vitro* and *in vivo* models, suggest that these observations are due to vitamin D's impact on epithelial-mesenchymal interactions, cell migration, cell proliferation, surfactant production and collagen deposition. However, vitamin D supplementation trials have shown no effect on post-natal outcomes that are indicative of lung development (wheeze and lung function at 3 years of age).

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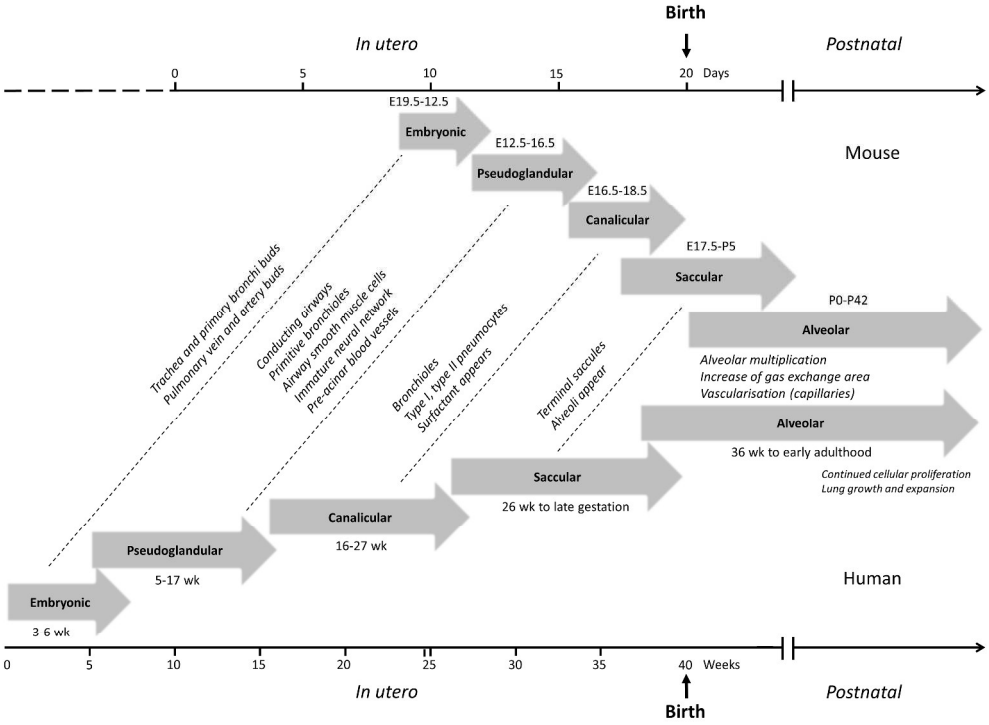

Schematic representation of the major stages of human and mouse lung development. While the lung development process in humans and mice is qualitatively similar, there are important differences; in particular, alveolarisation is exclusively post-natal in the mouse. These discrepancies need to be taken into account when considering *in vivo* experimental data and their relevance to human lung development.

Figure 1
355x266mm (300 x 300 DPI)

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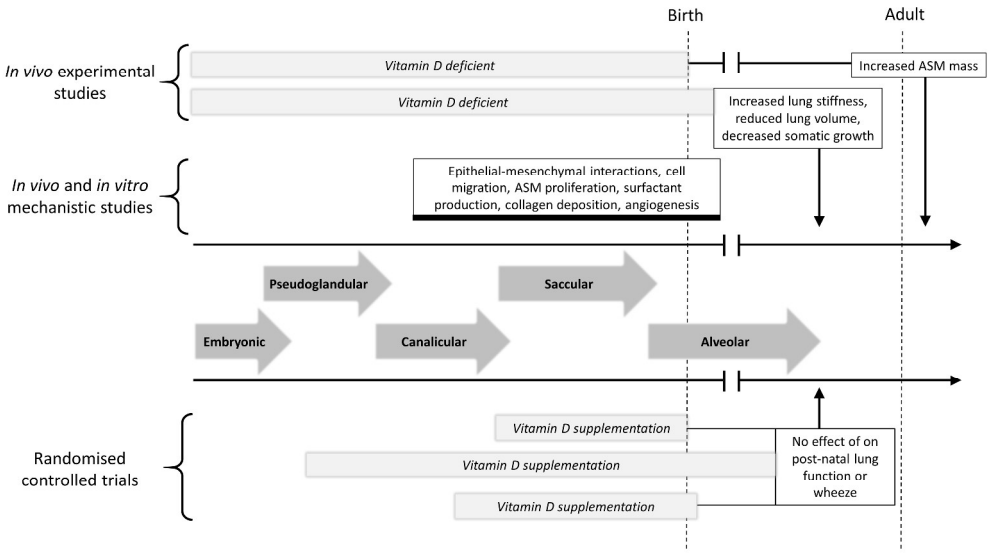

Diagram summarising the relationship between vitamin D and lung development. *In vivo* descriptive studies have shown that early life vitamin D deficiency impairs lung growth resulting in increased lung stiffness (and reduced lung volume) and *in utero* vitamin D deficiency increases airway smooth muscle (ASM) mass in adulthood. Mechanistic studies, from both *in vitro* and *in vivo* models, suggest that these observations are due to vitamin D's impact on epithelial-mesenchymal interactions, cell migration, cell proliferation, surfactant production and collagen deposition. However, vitamin D supplementation trials have shown no effect on post-natal outcomes that are indicative of lung development (wheeze and lung function at 3 years of age).

Figure 2
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Recent equivocal results in high profile randomised controlled trials suggest that the impact of vitamin D deficiency on lung development is complex. In this narrative review we summarise our current understanding of the link between UV exposure, vitamin D and lung development.