Title: Vitamin D deficiency causes deficits in lung function and alters lung structure

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Funding source: National Health and Medical Research Council of Australia (Grant)

Running title: Vitamin D deficiency and lung function

Descriptor: 1.8 Airway responsiveness: physiology (Integrative physiology and pathology)

Word count: 2988

Scientific knowledge on the subject: The prevalence of vitamin D deficiency is increasing and has been associated with obstructive lung disease. There is an association between vitamin D deficiency and lung function which may explain this link, however causal evidence is lacking.
What this study adds to the field: This is the first study to provide direct evidence for a causal link between vitamin D deficiency, deficits in lung function and altered lung structure. These functional and structural abnormalities provide a mechanism explaining the link between vitamin D deficiency and obstructive lung disease.

Author contributions:

GZ – was involved in the conceptualisation of the study, conducted all of the lung function experiments, analysed the results and wrote the first draft of the manuscript.

LB – conducted and analysed all of the stereological measurements in the study.

JE and AJ – were involved in the conceptualisation of the study, provided intellectual input into the stereological measurements and had input into the manuscript.

SG and PH – were involved in the conceptualization of the study, were involved in analysis and interpretation of the results and design of the mouse colonies and made substantial contributions to the manuscript.
Abstract

Rationale: The prevalence of vitamin D deficiency is increasing and has been linked to obstructive lung diseases including asthma and COPD. Recent studies suggest that vitamin D deficiency is associated with reduced lung function. The relationship between vitamin D deficiency and lung function is confounded by the association between physical activity levels and vitamin D status. Thus, causal data confirming a relationship between vitamin D and lung function are lacking.

Objective: To determine if vitamin D deficiency alters lung structure and function.

Methods: A physiologically relevant BALB/c mouse model of vitamin D deficiency was developed by dietary manipulation. Offspring from deficient and replete colonies of mice were studied for somatic growth, lung function and lung structure at 2 weeks of age.

Measurements: Lung volume and function were measured by plethysmography and the forced oscillation technique respectively. Lung structure was assessed histologically.

Main results: Vitamin D deficiency did not alter somatic growth but decreased lung volume. There were corresponding deficits in lung function which could not be entirely explained by lung volume. The volume dependence of lung mechanics was altered by deficiency suggesting altered tissue structure, however the primary histological difference between groups was lung size rather than an alteration in architecture.

Conclusions: Vitamin D deficiency causes deficits in lung function which are primarily explained by differences in lung volume. This study is the first to provide direct mechanistic evidence for linking vitamin D deficiency and lung development which may explain the association between obstructive lung disease and vitamin D status.

Words: 250
Introduction

There has been a dramatic increase in the prevalence of vitamin D deficiency around the world (1, 2). Vitamin D deficiency is associated with a number of diseases; in particular the bone disorder rickets as a result of the role of vitamin D in calcium homeostasis (3). However, the active form of vitamin D ($1\alpha25(OH)2D$) is also critical in immune regulation (4) and deficiency of this vitamin has been linked to both autoimmune disease (5) and cardiovascular disease (6). Additionally, the vitamin D axis has been implicated in the pathogenesis of chronic respiratory diseases including asthma (7, 8) and COPD (9-11).

Epidemiological studies have shown an association between 1) low maternal vitamin D intake and wheeze in children (12-14), 2) decreased serum levels of vitamin D and increased asthma severity (15) and steroid use (16) in asthmatic children and, 3) reduced glucocorticoid responses in adult asthmatics with low serum vitamin D (17). A similar association exists between COPD severity and low levels of serum vitamin D (10). However, it has been demonstrated that low serum vitamin D levels are associated with physical inactivity (18-20). Thus, given the known association between increased asthma (21) and COPD (22) severity and low physical activity levels, a causal link between vitamin D and these respiratory diseases has been difficult to establish.

Given the immunomodulatory properties of vitamin D (23) previous studies have primarily focused on immune mechanisms of lung disease. However, vitamin D may also play a role in...
lung development which could explain the association between vitamin D deficiency and lung
disease in the absence of alterations in immune regulation. For example, data from the third U.S.
NHANES survey showed a strong relationship between serum vitamin D and baseline lung
function (FEV$_1$ and FVC) (24). This association between vitamin D levels and lung function is
also seen in COPD (10). Similarly, vitamin D increases surfactant synthesis (25), inhibits airway
smooth muscle proliferation (26) and has a critical role in epithelial-mesenchymal interactions
during lung growth (25). However, there has been no study to directly determine whether vitamin
D deficiency alone results in altered lung function in vivo. Additionally, the effect of vitamin D
deficiency in utero on fetal growth is controversial and appears to be dependent on maternal
calcium status (27). There is a well known relationship between body size and lung function, so
any effect of vitamin D on somatic growth will ultimately influence lung function in the absence
of a direct effect on the lung. The nature of the cross-sectional population based studies that have
shown an apparent relationship between vitamin D deficiency and lung function means that a
causal relationship between vitamin D deficiency alone, without additional confounders, and
altered lung growth resulting in altered lung function is yet to be established.

To date there is only limited mechanistic evidence for a direct role for vitamin D in the
progression of obstructive respiratory disease which can be partially explained by the limited
utility of experimental mouse models of altered vitamin D regulation. This is due to the extreme
phenotype of both the 1α-hydroxylase (28) and vitamin D receptor (29) knockout mouse models
which both develop severe hypocalcaemia (and the associated bone malformations), and
hyperparathyroidism. In order to overcome this problem we have developed a physiologically
relevant mouse model of vitamin D deficiency with serum levels of vitamin D matching those seen in deficient human populations.

The aim of this study was to determine if vitamin D deficiency results in altered lung function and/or structure as a potential explanation for the association between vitamin D and chronic respiratory disease. Specifically we aimed to determine if vitamin D deficiency 1) has an influence on somatic growth, 2) results in delayed lung growth as indicated by a decrease in lung volume after controlling for changes in somatic growth, 3) alters the mechanical properties of the lung tissue as indicated by the volume dependence of lung mechanics, and 4) results in alterations in lung morphology.
Methods

Model

3 week old female BALB/c mice (ARC, Murdoch, Western Australia) were provided with vitamin D deficient or replete (2195 IU.kg\(^{-1}\)) diets (Specialty Feeds, Glen Forrest, Western Australia) for at least 5 weeks prior to mating. In all cases, female mice on the vitamin D deficient diets were confirmed as being deficient (by assay of serum vitamin D levels) prior to mating at 8 weeks of age. Deficient diets were supplemented with calcium 25g.kg\(^{-1}\) (vs 15 g.kg\(^{-1}\)) to avoid hypocalcaemia and caloric content of the diets was adjusted to ensure that all mice had similar calorie intake (deficient, 15.3 MJ.kg\(^{-1}\); replete, 15.8 MJ.kg\(^{-1}\)). Mice were housed in rooms with a 12:12 hr ambient UV-B free light:dark cycle. Food and water were provided ad libitum.

Female mice were mated with vitamin D replete males and offspring of both sexes were studied at 2 weeks of age for somatic growth, lung volume, lung function and lung structure. All studies were carried out according to animal health and welfare guidelines and were approved by the Institutional Animal Ethics Committee.

Mechanical ventilation

Mice were anaesthetized by i.p injection with ketamine (20 mg.mL\(^{-1}\); Troy Laboratories, NSW, Australia) and xylazine (1 mg.mL\(^{-1}\); Troy Laboratories) at a dose of 0.01 mL.g\(^{-1}\). Two-thirds of the dose was given prior to tracheostomy and cannulation. The remaining anaesthetic was given and mice were placed in a plethysmograph and mechanically ventilated (HSE-Harvard MiniVent, Harvard Apparatus, USA) at 400 breaths.min\(^{-1}\) with a tidal volume of 10 mL.kg\(^{-1}\) and 2 cmH\(_2\)O PEEP.
Lung volume

Thoracic gas volume (TGV) was measured as described previously (30). The trachea was occluded at elastic-equilibrium lung volume (EELV) and inspiratory efforts were induced by intramuscular electrical stimulation. TGV was calculated by applying Boyle’s law to the tracheal and box pressure signals (30).

Lung mechanics

Lung mechanics were assessed using a modified low-frequency forced oscillation technique (31). Briefly, a speaker generated an oscillatory signal containing 9 frequencies ranging from 4 to 38 Hz. The signal was delivered to the tracheal cannula via a wave tube of known impedance. A model with constant phase tissue impedance was fit to the respiratory impedance spectrum ($Z_{rs}$) allowing calculation of the Newtonian resistance ($R_{aw}$; which approximates airway resistance in mice), airway inertance ($I_{aw}$; which is negligible after correcting for the tracheal cannula), tissue damping ($G$) and tissue elastance ($H$). Hysteresivity ($\eta$) was calculated by $G/H$ (32). This system allowed assessment of the volume dependence of lung mechanics (31).

Lung structure

Lung structure was assessed according to ATS/ERS guidelines (33). Following euthanasia the tracheal cannula was instilled with 2.5% glutaraldehyde at 10 cmH$_2$O. This fixation pressure was chosen to fall within the range of volumes that lung function was measured at EELV (34). Lungs were randomly oriented (35) and embedded in paraffin. Starting at a random point sections (5 µm) were taken at regular (500 µm) intervals throughout the lung and stained with H&E. Lung volume ($V_L$) was calculated using the Cavalieri method (36) and point counts were used to obtain total tissue volume ($V_t$), volume of the alveolar septa ($V_s$) and air in the major airways ($V_a$).
alveolar ducts ($V_{ad}$) and alveoli ($V_{alv}$). Alveolar surface area ($S_a$) was calculated using a linear grid and $S_a$ and $V_s$ were used to estimate the mean (arithmetic) septal thickness ($T_s$) (33). The depth to diameter ratio of the alveoli was also calculated by direct measurement (37) as an index of alveolar septation. Alveolar number ($N_a$) was calculated using a physical dissector (38).

Statistics

Between group comparisons were made using t-tests. Additional analyses involving correction for continuous variables (e.g. body size and lung volume) were conducted using ANCOVA. Data were analysed in Stata (v11, StataCorp) and reported as mean(SD).
Results

Model characteristics

Vitamin D deficiency had no effect on litter size [deficient 4.9(2.8) vs replete 3.9(2.1); p = 0.39]. N = 34 replete (female, n = 13; male, n = 21) and n = 46 deficient (female, n = 25; male, n = 22) offspring were studied for somatic growth and lung function. Serum vitamin D levels in the deficient mice [12.8(2.3) nmol.L\(^{-1}\)] were significantly lower than those in the replete mice [81.5(27.9) nmol.L\(^{-1}\)] and below that of the consensus cutoff value for deficiency in humans of 50 nmol.L\(^{-1}\) (3). There was no difference in serum calcium (Ca\(^{2+}\)) levels between the two groups [deficient 8.90(3.87) mg.dL\(^{-1}\) vs replete 9.05(2.45) mg.dL\(^{-1}\); p = 0.94] and no evidence for a difference in the percentage bone mineral content per body weight between the groups as measured by dual energy x-ray absorptiometry (DEXA; GE Lunar Prodigy, GE Lunar Corporation, U.S.A) [deficient 9.1(0.7)% vs replete 9.1(1.2)%, p = 0.97].

Somatic growth

There was no evidence for a difference in body weight between mice born to vitamin D deficient or replete mothers (female, p = 0.34; male, p = 0.40) (Figure 1). There was some evidence to suggest that male vitamin D mice were significantly shorter (p = 0.04) than their replete counterparts, however this was not the case in females (p = 0.42) (Figure 1) and the magnitude of the difference in the males was small (approx. 2 mm or 3.5%).

Thoracic gas volume
Both male (p < 0.001) and female (p = 0.001) vitamin D deficient mice had significantly smaller TGV than replete controls (Figure 2). These differences were still apparent after correcting for body length (male, p < 0.001; female, p = 0.001) (Figure 2).

Baseline lung mechanics

Airway resistance and tissue mechanics (tissue damping and tissue elastance) were significantly higher in both male (R_\text{aw}, p < 0.001; G, p < 0.001; H, p < 0.001) and female (R_\text{aw}, p < 0.001; G, p = 0.004; H, p = 0.03) vitamin D deficient mice compared to their respective replete controls (Figure 3 and Figure 4; data not shown for G). Whereas there was no difference in hysteresivity (a fundamental property of the lung tissue describing the ratio of energy dissipation to energy storage) between deficient and replete mice for either sex (male, p = 0.53; female, p = 0.29; data not shown). For males these differences were still evident in airway resistance (p = 0.001) and tissue elastance (p = 0.04), but not tissue damping (p = 0.10), after correcting for lung volume. In contrast only airway resistance was significantly higher in females after correcting for lung volume (p = 0.001) while this was not the case for tissue damping (p = 0.15) or tissue elastance (p = 0.40) suggesting that differences in tissue mechanics at baseline in female vitamin D deficient mice could be explained by differences in lung volume (Figure 3 and Figure 4; data not shown for G).

Volume dependent lung mechanics

Due to the differences in lung volume at baseline, the PV curve in male and female vitamin D deficient mice was shifted downward. There was a corresponding difference in the lung volume reached at 20 cmH₂O transrespiratory pressure (P_{\text{rs}}) in male (p < 0.001) and female (p < 0.001) mice. However, this difference appeared to be proportional to lung volume at baseline in both
sexes (male, p = 0.07; female, p = 0.44) (Figure 5). At 20 cmH₂O airway resistance (male, p = 0.003; female, p = 0.01), tissue damping (male, p = 0.002; female, p = 0.002) and tissue elastance (male, p < 0.001; female, p < 0.001) were all higher in the vitamin D deficient mice compared to replete mice (Figure 6; data not shown for G). For airway resistance this could be explained by the difference in lung volume at 20 cmH₂O as all values for airway resistance appeared to fall upon a master curve describing the relationships with lung volume, whereas this was not the case for tissue damping or tissue elastance where the relationship between these parameters and lung volume in vitamin D deficient mice clearly deviated substantially from that observed in the vitamin D replete mice.

Lung structure

Post-fixation and embedding lung volume was significantly smaller in vitamin D deficient mice (female, n = 8, male, n = 6) compared to replete mice (female, n = 8, male, n = 7) (p = 0.05) with no effect of sex (p = 0.92) (Figure 7). There was no difference in the volume of the major airways (Vₐ) between the groups (p = 0.64). However, the volume of air in the alveolar ducts (Vₐd) was significantly lower in the vitamin D deficient mice (p = 0.02) with no effect of sex (p = 0.25) (Figure 7). There was some evidence to suggest that the volume of tissue in the alveolar septa (p = 0.08) was lower in vitamin D deficient mice compared to the replete mice however there was no difference in either surface area (p = 0.27) or septal thickness (p = 0.55) between groups (data not shown). The total tissue volume (Vₜ) was significantly lower (p = 0.01) in the vitamin D deficient mice (female, vit D+ 0.074[0.009] mL vs vit D- 0.062[0.010] mL; male, vit D+ 0.070[0.007] mL vs vit D- 0.059[0.016] mL) with no effect of sex (p = 0.44). In contrast, there was no difference in the depth to diameter ratio of the alveoli (p = 0.92) between the groups (female, vit D+ 0.890[0.013] vs vit D- 0.871[0.029]; male, vit D+ 0.877[0.013] vs vit D-
The number of alveoli in vitamin D deficient female mice was lower than that in replete females ($p = 0.06$) whereas this was not the case in male mice ($p = 0.97$) (Figure 8). Despite this, there was no difference in the arithmetic mean volume of the alveoli between groups of either sex (female, $p = 0.18$; male, $p = 0.20$) (data not shown).
Discussion

This study clearly demonstrated that vitamin D deficiency causes decrements in lung function. These differences could not be attributed to alterations in somatic growth and appeared to be over and above the influence of differences in lung volume. These deficits in lung function were reflected histologically and related primarily to differences in overall lung size.

For the first time direct mechanistic evidence has been provided supporting a relationship between vitamin D and lung growth *in vivo* whereby vitamin D deficiency resulted in a significant deficit in lung volume. It is well known that there is a strong association between body size and lung volume (39). Importantly, while there was a small but statistically significant difference in body length between male vitamin D deficient and replete mice, vitamin D did not appear to have a large impact on somatic growth. Correspondingly, differences we observed in lung volume and mechanics could not be explained by differences in body length in these mice. This observation suggests that vitamin D deficiency has a direct effect on lung growth in the absence of a major effect on somatic growth.

The deficit in lung volume in the vitamin D deficient mice was substantial (approx. 18% in females and 28% in males). It is important to recognize that this measure of lung volume was made at elastic equilibrium lung volume (EELV) which represents the result of opposing forces generated by the elastic recoil of the lung and the outward force generated by the chest wall at zero transrespiratory pressure. Thus, differences in TGV could be influenced by changes in lung structure and/or differences in the stiffness of the chest wall. This context is important given that vitamin D deficiency alters skeletal muscle growth and function (40). However, it has been
shown previously that the chest wall impedance of the mouse is minimal (41) suggesting that the skeletal muscle component of the chest wall is unlikely to have made a significant contribution to the measured TGV in this instance. Additionally, while it is possible that the structural integrity of the ribs may have been altered by vitamin D deficiency which may influence TGV, whole body DEXA scans suggested that bone mineral content was not altered by exposure to the vitamin D deficient diets. This argues against the possibility of altered rib structure contributing to decreases in lung volume and highlights the importance of our calcium supplementation regime which allowed us to identify the effect of vitamin D deficiency alone. Importantly, this deficit in TGV was maintained over the range of the PV curve thus demonstrating a clear effect of vitamin D deficiency, in the absence of hypocalcaemia, on total lung volume.

Differences in lung size, in their own right, may be sufficient to explain altered lung function and respiratory disease prevalence. In humans there is a strong association between low birth weight (as a marker of lung size), lung function later in life (42) and risk of hospitalization due to respiratory illness (43). This link can be explained intuitively by the smaller airways associated with small lungs resulting in higher resistance to airflow and a decreased capacity to clear pathogens. Not surprisingly, given the large differences in lung volume between the groups of mice, there were substantial differences in lung mechanics. However, these differences in lung volume were not sufficient to explain the differences in lung mechanics we measured. In particular, for a given lung volume, $R_{aw}$ was substantially higher in the deficient mice. G and H could be partially explained by differences in lung volume at EELV, however this was not the case when the lung was inflated. Specifically, in the anaesthetised state we allowed TGV to be self established at EELV which, for the reasons discussed earlier, may be significantly influenced by chest wall structure. However, by inflating the lungs and tracking lung mechanics we were
able to show substantial, physiologically relevant, differences in the volume dependence of lung mechanics in the vitamin D deficient mice. These differences were particularly evident in the rate of change of parenchymal mechanics with increasing $P_{rs}$. These data clearly suggest an effect of vitamin D deficiency on the mechanical properties of the lung tissue, however the nature of this structural difference was not clear from our data.

The primary difference in lung structure between groups, that was consistent between sexes, was a smaller lung volume and size. Differences in the volume of the major airways were not observed, however we did measure differences in the volume of the alveolar ducts. $R_{aw}$ in the model of the frequency dependence of $Z_{rs}$ that was used in this study represents the frequency independent Newtonian resistance to flow. In mice, due to the relatively low contribution of the chest wall (41, 44), this primarily reflects resistance of the airways where air moves by bulk flow. The anatomy of the mouse lung, whereby large airways rapidly give way to alveolar ducts (44), is such that the differences we observed between groups in the volume of the alveolar ducts can explain the differences we observed in $R_{aw}$. At EELV the parenchymal lung mechanics in females was explained entirely by differences in lung volume between groups whereas this was not the case in male mice. The only structural parameter that showed a different response between males and females was the number of alveoli. The decrease in the number of alveoli in deficient female mice compared to replete females may explain why the parenchymal mechanics could be explained by differences in lung volume between the groups. The fact that lung volume did not explain differences in parenchymal mechanics at EELV in male mice suggests a difference in the underlying structure of the lung parenchyma although the nature of this was not clear from the structural parameters we assessed.
Due to the nature of this study we were not able to determine whether the differences in the lung size and function we observed in the offspring were the result of their own deficient status or as a consequence of developmental deficits that occurred *in utero* due to the mother’s deficiency. It is important to note that extreme nutritional manipulation resulting in caloric restriction is a potent model of intra-uterine growth restriction. Animal models have been used to demonstrate that *in utero* caloric restriction results in decreased body weight and a lower lung volume to body weight ratio (45). Notwithstanding the issues associated with standardising lung size by body weight, these data suggest that caloric restriction alters lung growth. In the present study the calorie content of both diets was similar. Additionally, there was little evidence for an effect of exposure to vitamin D deficient diets on somatic growth suggesting that the mice were not grossly undernourished *in utero*. Thus, the effects of maternal vitamin D status versus the role of the vitamin D status of the individual after birth on lung development in this study could not be distinguished.

For the first time we have demonstrated a direct role for vitamin D in causing decreased lung function in the absence of known confounders; thus confirming the assertion by epidemiological studies that there is a relationship between vitamin D deficiency and lung function. Specifically, vitamin D deficiency resulted in physiologically significant decreases in lung volume without a major influence on somatic growth. There were corresponding deficits in lung function in the deficient mice which could not be entirely explained by differences in lung volume. There was some evidence to suggest that the structure of the lung was altered due to differences in the volume dependence of lung mechanics at high transrespiratory pressure, however the nature of this structural difference was not apparent from our data. These differences in lung volume were also apparent histologically. The observed differences in lung volume and lung mechanics, which
were substantial and physiologically relevant, raise serious concerns regarding the increased prevalence of vitamin D deficiency in the community and the potential impact this may have on general lung health and in particular susceptibility to obstructive lung disease.


Figure Legends

Figure 1. Box plots (median, interquartile range and range) of weight (wt) and snout vent length for female (A, B) and male (C, D) vitamin replete (white) and deficient (grey) mice at 2 weeks of age.

Figure 2. Box plots (median, interquartile range and range) of thoracic gas volume (TGV) and scatter plots of TGV against snout vent (SV) length with regression lines from ANCOVA for female (A, B) and male (C, D) vitamin D replete (white symbols, dashed lines) and deficient (dark symbols, solid lines) mice at 2 weeks of age.

Figure 3. Box plots (median, interquartile range and range) of airway resistance ($R_{aw}$) and scatter plots of $R_{aw}$ against thoracic gas volume (TGV) length with regression lines from ANCOVA for female (A, B) and male (C, D) vitamin D replete (white symbols, dashed lines) and deficient (dark symbols, solid lines) mice at 2 weeks of age.

Figure 4. Box plots (median, interquartile range and range) of tissue elastance ($H$) and scatter plots of $H$ against thoracic gas volume (TGV) length with regression lines from ANCOVA for female (A, B) and male (C, D) vitamin D replete (white symbols, dashed lines) and deficient (dark symbols, solid lines) mice at 2 weeks of age.

Figure 5. Pressure-volume curves for female (A) and male (B) vitamin D replete (white symbols) and deficient (black symbols) mice at 2 weeks of age. Shown are the group mean curves for each group.
Figure 6. Plots of the volume dependence of airway resistance ($R_{aw}$) and tissue elastance ($H$) against thoracic gas volume (TGV) during slow inflation-deflation manoeuvres up to 20 cmH$_2$O transrespiratory pressure in female (A, C) and male (B, D) vitamin D replete (white symbols) and deficient (black symbols) mice at 2 weeks of age. Shown are the mean curves for each group.

Figure 7. Lung volume ($V_L$; A), volume of air in the major airways ($V_a$; B), volume of alveolar septa ($V_s$, C) and volume of air in the alveolar ducts ($V_{ad}$, D) measured by stereology from fixed lungs of 2 week old female and male (cross hatched) vitamin D replete (white bars) and deficient (grey bars) mice. Data are mean(SD).

Figure 8. Number of alveoli measured by stereology from fixed lungs of 2 week old female and male (cross hatched) vitamin D replete (white bars) and deficient (grey bars) mice. Data are mean(SD).
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Figure 4. Box plots (median, interquartile range and range) of tissue elastance (H) and scatter plots of H against thoracic gas volume (TGV) length with regression lines from ANCOVA for female (A, B) and male (C, D) vitamin D replete (white symbols, dashed lines) and deficient (dark symbols, solid lines) mice at 2 weeks of age.
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