

# The Effects of *In Utero* Vitamin D Deficiency on Airway Smooth Muscle Mass and Lung Function

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## Abstract

We have previously demonstrated increased airway smooth muscle (ASM) mass and airway hyperresponsiveness in whole-life vitamin D-deficient female mice. In this study, we aimed to uncover the molecular mechanisms contributing to altered lung structure and function. RNA was extracted from lung tissue of whole-life vitamin D-deficient and -replete female mice, and gene expression patterns were profiled by RNA sequencing. The data showed that genes involved in embryonic organ development, pattern formation, branching morphogenesis, Wntless/Int signaling, and inflammation were differentially expressed in vitamin D-deficient mice. Network analysis suggested that differentially expressed genes were connected by the hubs matrix metalloproteinase 9; NF- $\kappa$  light polypeptide gene enhancer in B cells inhibitor,  $\alpha$ ; epidermal growth factor receptor; and E1A binding protein p300. Given our findings that developmental pathways may be altered, we investigated if the timing of vitamin D exposure (*in utero* vs. postnatal) had an impact on lung health outcomes. Gene expression was measured in *in utero* or postnatal vitamin D-deficient mice, as well as whole-life vitamin D-deficient and -replete mice at 8 weeks of age. Baseline lung function, airway hyperresponsiveness, and airway inflammation were measured and

lungs fixed for lung structure assessment using stereological methods and quantification of ASM mass. *In utero* vitamin D deficiency was sufficient to increase ASM mass and baseline airway resistance and alter lung structure. There were increased neutrophils but decreased lymphocytes in bronchoalveolar lavage. Expression of inflammatory molecules S100A9 and S100A8 was mainly increased in postnatal vitamin D-deficient mice. These observations suggest that *in utero* vitamin D deficiency can alter lung structure and function and increase inflammation, contributing to symptoms in chronic diseases, such as asthma.

**Keywords:** airway remodeling; inflammation; RNA sequencing; mouse model; vitamin D

## Clinical Relevance

This research demonstrates that maternal vitamin D deficiency has an effect on long-term lung health outcomes in offspring. Vitamin D deficiency *in utero* alters the expression of genes involved in lung development and inflammation.

Vitamin D deficiency is associated with chronic lung diseases, such as asthma and chronic obstructive pulmonary disease (1, 2). Asthma and chronic obstructive

pulmonary disease are both characterized by airway hyperresponsiveness (AHR) and airway remodeling (3), including an increase in airway smooth muscle (ASM)

mass (4). We have recently demonstrated increased ASM mass in mice exposed to whole-life vitamin D deficiency (5). The increases in ASM were associated with gene

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expression levels of transforming growth factor- $\beta$  signaling molecules in vitamin D-deficient embryos at Embryonic Day 17.5, indicating that vitamin D deficiency may impinge on developmental pathways in early life. These observations support previous studies showing that low serum vitamin D levels are associated with increased ASM mass in children with severe asthma (6). Similarly, *in vitro* studies show that 1,25-hydroxyvitamin D ( $1,25(\text{OH})_2\text{D}_3$ ), the active metabolite of vitamin D, inhibits the proliferation of ASM cells (7), providing evidence that vitamin D has the capacity to modulate airway remodeling.

Experimental animal studies suggest that vitamin D affects lung development. The maturation of fetal rat type II alveolar epithelial cells, which are important for the synthesis of surfactant, and represent a key event in fetal lung development, is induced by  $1,25(\text{OH})_2\text{D}_3$  (8). Alveolar epithelial-mesenchymal interactions that are important for alveolar development are also influenced by  $1,25(\text{OH})_2\text{D}_3$  (9). Furthermore, genetic studies show that a high number of vitamin D-regulated genes are represented in the developing human and murine lung transcriptome (10). One-third of these vitamin D-regulated genes were differentially

expressed in children with asthma compared with sibling control subjects without asthma, suggesting that vitamin D deficiency during lung development may lead to asthma later in life.

A recent study from our group, investigating the effects of maternal vitamin D status on postnatal lung function outcomes, found impaired lung function and increased asthma risk in children exposed to maternal vitamin D deficiency (11). This was consistent with our earlier work showing a causal association between early life vitamin D deficiency and lung structure and function in a mouse model (12). Based on our recent observation that

**Table 1.** Functional Metagroups Associated with Up-Regulated Genes in Whole Lung of Vitamin D-Deficient Mice

| Metagroup | Genes                                            | Adjusted <i>P</i> Value | Terms                                                                                                                                                                                                                                                                                                                             |
|-----------|--------------------------------------------------|-------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1         | Gata3, Hhex, Foxa2, Wnt7a, Ccdc85b, Dcdc2a, Ier3 | $2.16 \times 10^{-10}$  | *GO:0048568: embryonic organ development (BP); GO:0048646: anatomical structure formation involved in morphogenesis (BP); GO:0060065: uterus development (BP); GO:0045599: negative regulation of fat cell differentiation (BP); GO:0001764: neuron migration (BP); GO:0050728: negative regulation of inflammatory response (BP) |
| 2         | Foxa2, Hhex, Wnt7a                               | $6.98 \times 10^{-9}$   | GO:0009952: anterior/posterior pattern formation (BP); GO:0007492: endoderm development (BP); GO:0090009: primitive streak formation (BP); GO:0009887: organ morphogenesis (BP); GO:0042127: regulation of cell proliferation (BP); GO:0016055: Wnt receptor signaling pathway (BP)                                               |
| 3         | Hoxb5, Wnt7a, Foxa2, Hoxb2, Hhex                 | $3.22 \times 10^{-7}$   | GO:0009952: anterior/posterior pattern formation (BP); GO:0009953: dorsal/ventral pattern formation (BP); GO:0048704: embryonic skeletal system morphogenesis (BP)                                                                                                                                                                |
| 4         | Wnt7a, Foxa2, Hoxb2                              | $5.81 \times 10^{-7}$   | GO:0009952: anterior/posterior pattern formation (BP); GO:0009953: dorsal/ventral pattern formation (BP); GO:0048705: skeletal system morphogenesis (BP)                                                                                                                                                                          |
| 5         | Lyl1, Hhex, Cx3cr1, Pclo                         | $1.12 \times 10^{-6}$   | GO:0030183: B cell differentiation (BP); GO:0016525: negative regulation of angiogenesis (BP); GO:0008283: cell proliferation (BP); GO:0007049: cell cycle (BP)                                                                                                                                                                   |
| 6         | Cxcr2, Csf3r, Cx3cr1                             | $1.14 \times 10^{-6}$   | GO:0019221: cytokine-mediated signaling pathway (BP); GO:0030593: neutrophil chemotaxis (BP); GO:0007186: G protein-coupled receptor protein signaling pathway (BP)                                                                                                                                                               |
| 7         | Wnt7a, Snai1, Nrarp                              | $1.52 \times 10^{-6}$   | GO:0060021: palate development (BP); GO:0090263: positive regulation of Wnt receptor signaling pathway through $\beta$ -catenin (BP)                                                                                                                                                                                              |
| 8         | Wnt7a, Atf5, Hhex, Gadd45a                       | $4.08 \times 10^{-5}$   | GO:0042127: regulation of cell proliferation (BP); GO:0050768: negative regulation of neurogenesis (BP); GO:0051726: regulation of cell cycle (BP)                                                                                                                                                                                |
| 9         | Mmp8, Mmp9, Atp1a3, Cx3cr1                       | $4.59 \times 10^{-6}$   | GO:0008152: metabolic process (BP); GO:0006508: proteolysis (BP); GO:0030574: collagen catabolic process (BP); GO:0042493: response to drug (BP); GO:0045766: positive regulation of angiogenesis (BP)                                                                                                                            |
| 10        | Foxa2, Atp1a3, F13a1                             | 0.001                   | GO:0008344: adult locomotory behavior (BP); GO:0007596: blood coagulation (BP)                                                                                                                                                                                                                                                    |

Definition of abbreviation: BP, biological process.

\*GO: repository of gene and gene product ontological attributes across species.

whole-life vitamin D deficiency increases ASM mass, we sought to investigate pathways that might be altered by vitamin D deficiency in the adult lung using RNA sequencing (RNA-Seq). We focused on female mice, because vitamin D deficiency-induced physiological changes are only present in female mice (5). Moreover, the maternal effects of vitamin D deficiency on postnatal lung function are strongest in girls (11). Given the existing evidence supporting a link between early-life vitamin D deficiency and lung health, we also investigated the relative impact of *in utero* and/or postnatal vitamin D deficiency on ASM, lung mechanics, AHR, inflammation, and gene expression patterns to determine

when lung structure and function are the most sensitive to the effects of vitamin D deficiency.

## Materials and Methods

### Mouse Model

A mouse model of vitamin D deficiency was established by dietary manipulation, as described previously (5). To compare the effects of *in utero* and postnatal vitamin D deficiency, pups were cross-fostered to produce *in utero* vitamin D-deficient (VitD<sup>-/+</sup>) or postnatally vitamin D-deficient (VitD<sup>+/-</sup>) mice, as well as whole-life vitamin D-deficient (VitD<sup>-/-</sup>) or whole-life vitamin D-replete (VitD<sup>+/+</sup>)

mice. All studies were performed on female mice when they were 8 weeks of age. All procedures were approved by the Telethon Kids Institute Animal Ethics Committee and conformed to National Health and Medical Research Council of Australia guidelines. Further details are provided in the online supplement.

### RNA-Seq Protocol and Data Analysis

Total RNA was extracted from lung tissue of whole-life vitamin D-deficient and -replete mice using TRIzol (Life Technologies, Mulgrave, Victoria, Australia) followed by RNeasy (Qiagen, Chadstone, Victoria, Australia). Library preparation (TruSeq RNA Sample Prep

**Table 2.** Functional Metagroups Associated with Down-Regulated Genes in Whole Lung of Vitamin D-Deficient Mice

| Metagroup | Genes                                                                                                                            | Adjusted <i>P</i> Value | Terms                                                                                                                                                                                                                                                                                |
|-----------|----------------------------------------------------------------------------------------------------------------------------------|-------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1         | Gbp5, Gbp8, Gbp6, Gbp7, Gbp3, Gbp10, Gbp2, Gbp1, ligp1, Tgtp1, Igtp, Abca1                                                       | $5.27 \times 10^{-14}$  | GO:0071346: cellular response to interferon- $\gamma$ (BP); GO:0050830: defense response to Gram-positive bacterium (BP); GO:0042832: defense response to protozoan (BP); GO:0035458;GO:0051856: adhesion to symbiont (BP); GO:0071222: cellular response to lipopolysaccharide (BP) |
| 2         | Fat1, Pkd1, Lrp6, Tnxb, Slit2, Egfr, Clec7a, Mgp, Sema5a, Dst, Klf7, Apc, Robo2                                                  | $2.73 \times 10^{-7}$   | GO:0016337: cell-cell adhesion (BP); GO:0048754: branching morphogenesis of a tube (BP); GO:0007409: axonogenesis (BP); GO:0007411: axon guidance (BP)                                                                                                                               |
| 3         | Serpina3h, Plcb1, Serpina3i, Serpina3 g, Gatm, Serpina3f                                                                         | $3.75 \times 10^{-7}$   | GO:0043434: response to peptide hormone stimulus (BP);GO:0034097: response to cytokine stimulus (BP)                                                                                                                                                                                 |
| 4         | Uhmk1, Lrrk2, Cdkl5, Sik2, Ern1, C230081A13Rik, Aak1, Egfr, Dst, Pkd1, Apc, Tnks                                                 | $6.18 \times 10^{-7}$   | GO:0046777: protein amino acid autophosphorylation (BP); GO:0007050: cell cycle arrest (BP); GO:0018105: peptidyl-serine phosphorylation (BP)                                                                                                                                        |
| 5         | Lrrk2, Peli2, Ep300, Bmpr2, Fam129a, Plcb1, Rgs1, Tbc1d9, Adap2, Agfg1, Rab3c, Rhobtb1, Rapgef5, Rab43, Bcar3, Nipbl, Pkd1, Mib1 | $1.42 \times 10^{-6}$   | GO:0001934: positive regulation of protein amino acid phosphorylation (BP); GO:0043547: positive regulation of GTPase activity (BP); GO:0007264: small GTPase mediated signal transduction (BP); GO:0007507: heart development (BP)                                                  |
| 6         | Synrg, Mrc1, Sorl1, Lrp6, Steap2, Siglec1, Aak1, Ly75                                                                            | $8.06 \times 10^{-6}$   | GO:0006897: endocytosis (BP)                                                                                                                                                                                                                                                         |
| 7         | Crebbp, Ep300, Lrp6, Arid5b, Fzd4                                                                                                | $1.21 \times 10^{-5}$   | GO:0051091: positive regulation of transcription factor activity (BP)                                                                                                                                                                                                                |
| 8         | Mertk, Fzd4, C230081A13Rik, Axl, Sorl1, Shroom2, Apc                                                                             | $7.72 \times 10^{-5}$   | GO:0034446: substrate adhesion-dependent cell spreading (BP); GO:0016477: cell migration (BP)                                                                                                                                                                                        |
| 9         | Nipbl, Plcb1, Arid5b, Alms1, Creb5, Shroom4, Shroom2                                                                             | $8.49 \times 10^{-5}$   | GO:0045444: fat cell differentiation (BP); GO:0007420: brain development (BP)                                                                                                                                                                                                        |
| 10        | Hipk2, Lrp6, Bmpr2, Zbtb16, Apc, Foxo3, Nfkb1a, Egfr, Fzd4, Strn, Tnks                                                           | $2.05 \times 10^{-4}$   | GO:0009952: anterior/posterior pattern formation (BP); GO:0042127: regulation of cell proliferation (BP); GO:0016055: Wnt receptor signaling pathway (BP)                                                                                                                            |
| 11        | ligp1, Tlr8, Axl, Clec7a, Nlrc5, Trim56, Sema7a, Chi3l3, Olr1                                                                    | $3.40 \times 10^{-4}$   | GO:0045087: innate immune response (BP); GO:0006954: inflammatory response (BP)                                                                                                                                                                                                      |
| 12        | Cacna1c, Atp2b4, Itpr2, Efcab4b, Pkd1, Slc24a4                                                                                   | $4.88 \times 10^{-4}$   | GO:0006816: calcium ion transport (BP)                                                                                                                                                                                                                                               |
| 13        | Plxn2, Lphn3, Fzd4, Egfr, Cbl, Crebbp, Mki67, Glul                                                                               | $1.53 \times 10^{-3}$   | GO:0007166: cell surface receptor-linked signaling pathway (BP);GO:0008283: cell proliferation (BP)                                                                                                                                                                                  |

Definition of abbreviations: BP, biological process; Wnt, Wingless/Int.

Kit v2; Illumina, Scoresby, Victoria, Australia) and sequencing (50 bp single-end using an Illumina HiSeq2000) was performed by the Australian Genome Research Facility Ltd. (Parkville, Victoria, Australia). The raw sequencing results are available at the National Center for Biotechnology Information Short Read Archive under accession number SRP046087 ([www.ncbi.nlm.nih.gov/sra](http://www.ncbi.nlm.nih.gov/sra)). Further details regarding the RNA-Seq data analysis are provided in the online supplement.

#### RT Quantitative PCR

A subset of differentially expressed genes identified in the RNA-Seq experiment was validated using RT quantitative PCR (RT-qPCR). Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR primer assay sequences were obtained from Primerbank (13), and the primer list is included in the online supplement. QuantiTect SYBR Green (Qiagen) was used for qPCR on the ABI7900HT instrument (Life Technologies). Relative standard

curves were prepared from serially diluted PCR products (14).

#### Lung Function and Methacholine Challenge

Baseline lung function was assessed using the low-frequency forced oscillation technique (15). After lung function measurements, mice were transferred to a flexiVent system (SCIREQ, Montreal, PQ, Canada) for assessment of responsiveness to methacholine ( $\beta$ -methacholine chloride; Sigma-Aldrich, St. Louis, MO) as described

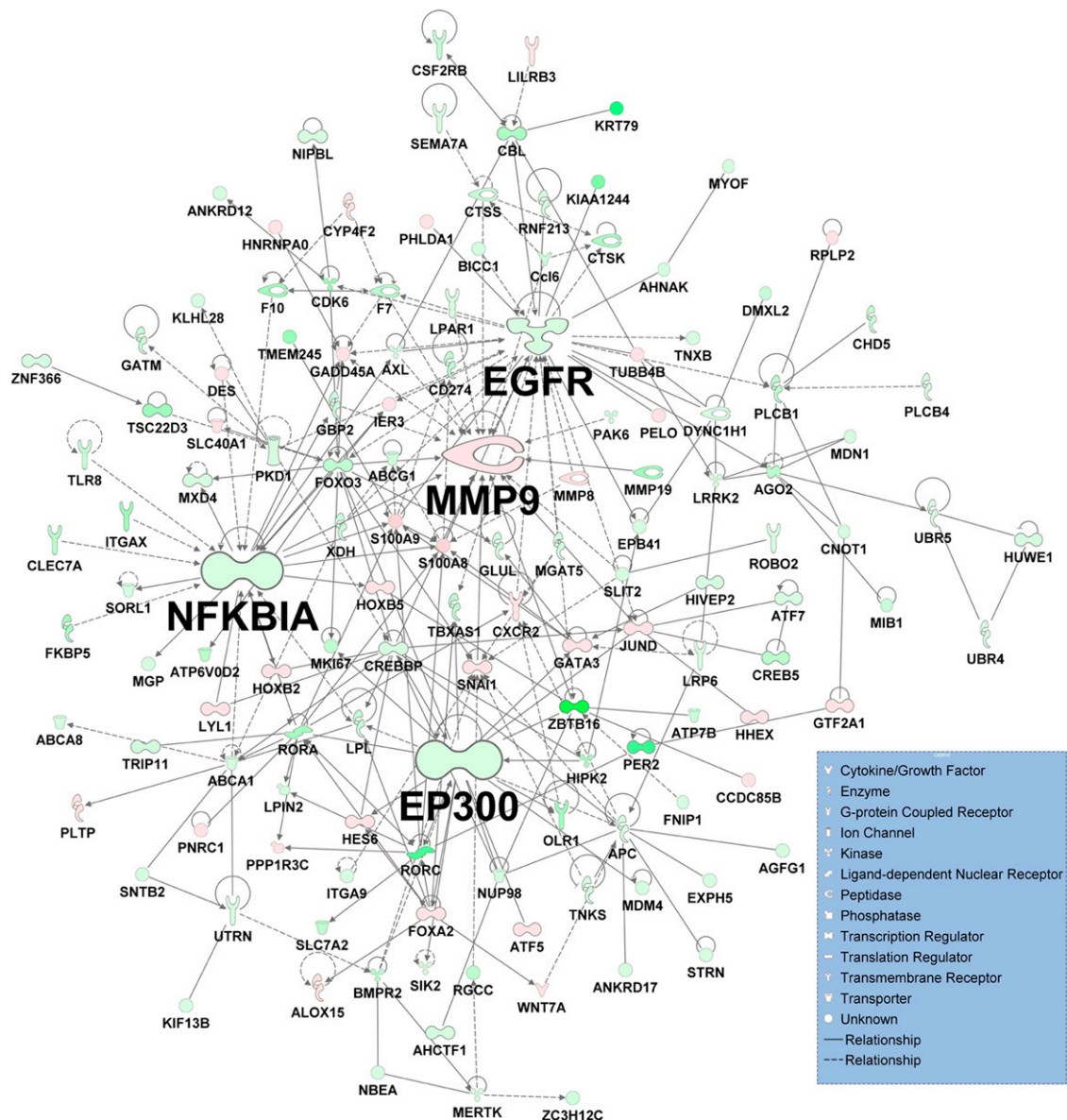


**Figure 1.** Gene network analysis of differentially expressed genes in vitamin D-deficient female mice. Major hub genes are enlarged. Up-regulated genes are shown in pink and down-regulated genes in green.

previously (5). Further details are provided in the online supplement.

### Differential Cell Counts

Bronchoalveolar lavage (BAL) was collected from mice by washing the lungs three times with 500  $\mu$ l of sterile saline. Total cells collected in the BAL were counted using a hemocytometer. The remaining cells were cytopun and stained with Leischman's stain for differential cell counts.

### Histology and Immunohistochemistry

Lung structure was assessed in independent groups of mice following American Thoracic Society/European Respiratory Society guidelines (16). The right lung was used for lung stereology as described previously (12). Transverse sections from the left lung were stained with Masson's trichrome for measurement of ASM mass (5). Immunohistochemical staining was performed using primary antibodies for S100A9, S100A8, phosphorylated I $\kappa$ B $\alpha$ , phosphorylated epidermal growth factor receptor (EGFR), and matrix metalloproteinase (MMP) 9. Further details are provided in the online supplement.

### Statistical Analyses

Between-group comparisons were made using *t* tests or two-way ANOVAs with Holm-Sidak *post hoc* tests. Pearson's correlation tests were performed to assess correlations between AHR and inflammatory parameters. Statistical analyses were performed using SigmaPlot software (Systat Software, San Jose, CA) and Prism (GraphPad Software, La Jolla, CA). Data are shown as mean ( $\pm$ SD). *P* values of less than 0.05 were regarded as statistically significant.

## Results

Gene expression patterns were profiled by RNA-Seq in lung tissue samples collected from whole-life vitamin D-deficient and -replete mice. The data were analyzed using the *edgeR* statistical method, and this analysis revealed that 383 genes were differentially expressed at a false discovery rate less than 0.05 (see Table E2 in the online supplement).

GeneTermLinker (<http://gtlinker.cnb.csic.es>) was then used to classify these genes into functionally coherent metagroups of genes and terms (17). The data showed that the up-regulated genes were characterized by

10 functional metagroups, which included embryonic organ development and morphogenesis, pattern formation, and Wingless/Int (Wnt) receptor signaling (Table 1). The down-regulated genes were associated with 13 metagroups, most notably the cellular response to IFN- $\gamma$ . Wnt receptor signaling and branching morphogenesis were also enriched in the down-regulated genes (Table 2).

### Network Analysis Identified Hub Genes that May Regulate Differentially Expressed Genes in the Lungs of Vitamin D-Deficient Mice

To provide some insight into the regulation of the differentially expressed genes

identified previously here, we used the Ingenuity Systems Knowledge base of functional molecular interaction data derived from previous studies to build a network of functionally interacting genes (18) (Figure 1). This analysis demonstrated that EGFR was the most dominant hub in the network, with 31 links to other genes, followed by NF- $\kappa$  light polypeptide gene enhancer in B cells inhibitor,  $\alpha$  (NFKBIA) with 23 links. Two other dominant hubs identified were MMP9 and E1A binding protein p300 (EP300), both with 18 links each. NFKBIA, an inhibitor of NF- $\kappa$ B-mediated inflammation, was down-regulated with vitamin D deficiency. EGFR,

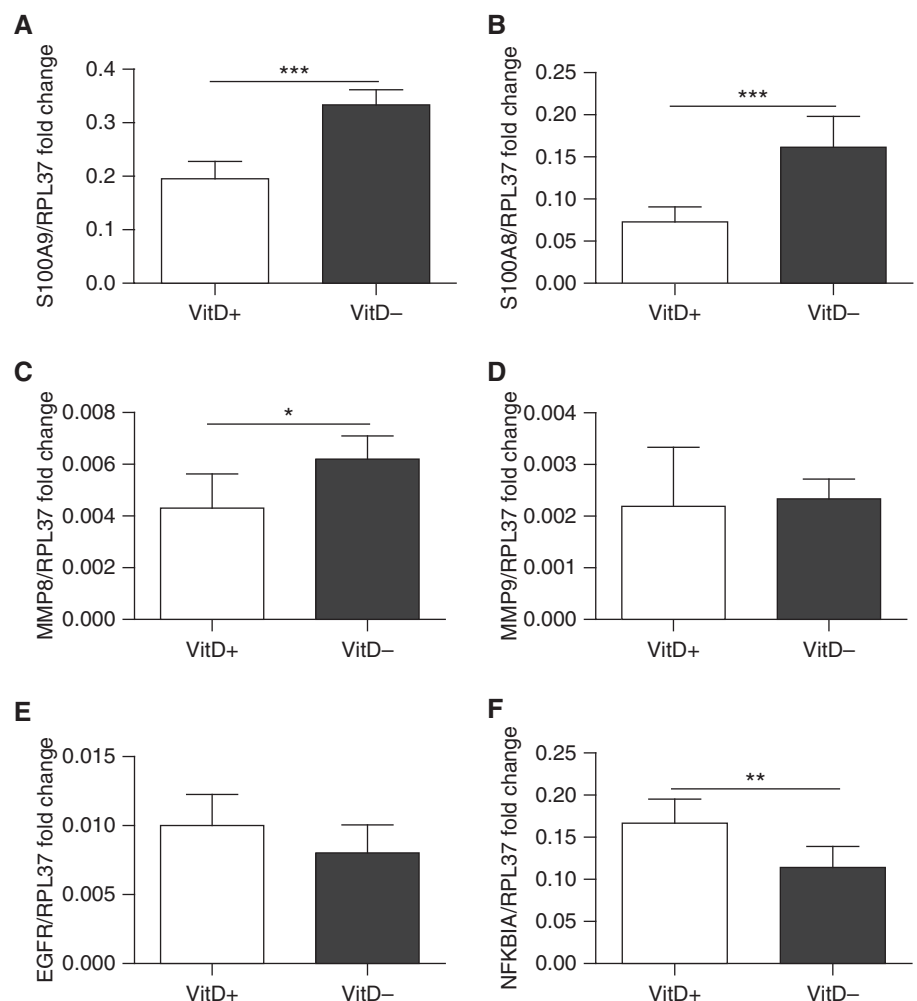


**Figure 2.** Gene expression of S100A9 (A), S100A8 (B), matrix metalloproteinase (MMP) 8 (C), MMP9 (D), epidermal growth factor receptor (EGFR) (E), and NF- $\kappa$  light polypeptide gene enhancer in B cells inhibitor,  $\alpha$  (NFKBIA) (F) in lungs from whole-life vitamin D-replete (VitD<sup>+</sup>), and whole-life vitamin D-deficient (VitD<sup>-</sup>) mice used for the RNA sequencing experiment. Data are expressed as fold changes obtained by normalizing candidate genes to the ribosomal protein L37 (RPL37) reference gene. \*\*\**P* < 0.001; \*\**P* < 0.01; \**P* < 0.05. Data are presented as mean ( $\pm$ SD); *n* = 6/group.

signaling from which regulates cell differentiation, proliferation, and inflammation, was also down-regulated. MMP9, which is involved in the breakdown of extracellular matrix and degrades type IV and V collagens, was up-regulated with vitamin D deficiency, whereas EP300, which codes for a transcriptional coactivator that has a central role in Wnt signaling, was down-regulated.

### Validation of Gene Expression Patterns by RT-qPCR

A subset of the differentially expressed genes that are relevant to the biology of airway remodeling and respiratory disease was selected for RT-qPCR validation. These genes included S100A8, S100A9, MMP8, as well as the hubs—EGFR, NFKBIA, MMP9, and EP300. Genes were initially validated in the same samples that were used for the RNA-Seq experiment.

In the samples from the RNA-Seq experiment, S100A9 and S100A8 RNA expression were significantly up-regulated in vitamin D-deficient mice compared with -replete mice (Figures 2A and 2B;  $P < 0.001$ ). MMP8 was also significantly up-regulated in vitamin D-deficient mice (Figure 2C;  $P = 0.013$ ), whereas there were no significant differences between groups for MMP9 (Figure 2D;  $P = 0.764$ ), EGFR (Figure 2E;  $P = 0.136$ ), and EP300

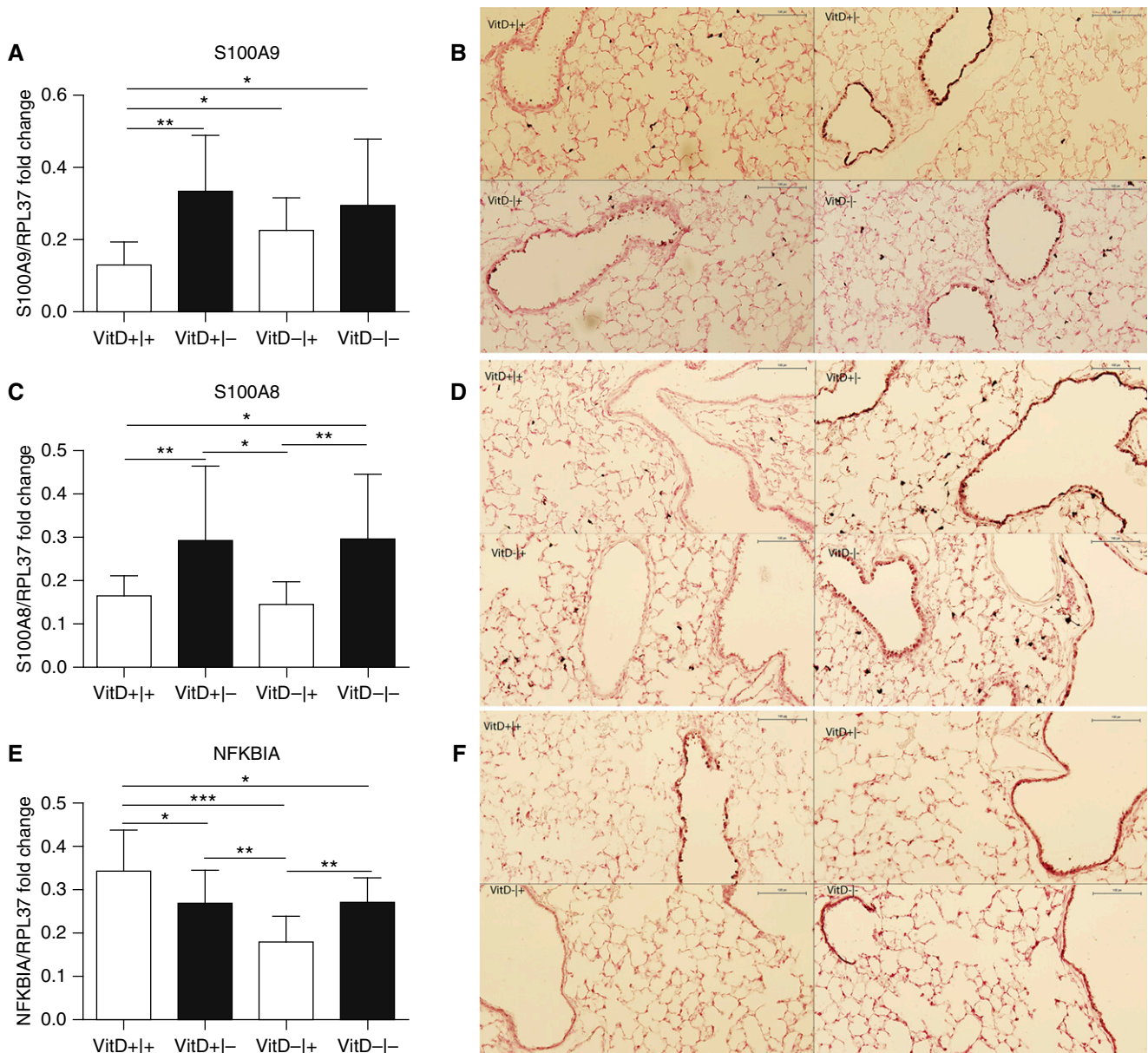


**Figure 3.** Gene and protein expression of S100A9 (A), S100A8 (C), and NFKBIA (E) in lungs from whole-life vitamin D-replete (VitD<sup>+/+</sup>), postnatal vitamin D-deficient (VitD<sup>+/-</sup>), vitamin D-deficient *in utero* (VitD<sup>-/+</sup>), and whole-life vitamin D-deficient (VitD<sup>-/-</sup>) mice. Gene expression data are expressed as fold changes obtained by normalizing candidate genes to the RPL37 reference gene. Representative images of lung sections immunohistochemically stained for the protein products of S100A9 (S100A9; B), S100A8 (MRP8; D), and NFKBIA (phosphorylated I $\kappa$ B $\alpha$ ; F) are shown. Scale bars, 100  $\mu$ m. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ . Data are presented as mean ( $\pm$ SD);  $n = 10$ /group.

( $P = 0.583$ ) (data not shown). NFKBIA was down-regulated in vitamin D-deficient mice (Figure 2F;  $P = 0.007$ ).

### Gene Expression and Lung Function Patterns Depend on the Timing of Vitamin D Deficiency

Given our findings that vitamin D deficiency may alter developmental pathways within the lung, we designed a study to investigate if the timing of vitamin D deficiency (i.e., *in utero* and/or postnatal exposure) had an impact on gene expression, lung structure, and function. We measured RNA expression in lungs of *in utero* vitamin D-deficient (VitD<sup>-/+</sup>) or postnatally vitamin D-deficient (VitD<sup>+/-</sup>) mice, and included an independent group of whole-life vitamin D-deficient (VitD<sup>-/-</sup>) or whole-life vitamin D-replete (VitD<sup>+/+</sup>) mice, as controls.

S100A9 was significantly up-regulated in VitD<sup>+/-</sup>, VitD<sup>-/+</sup>, and VitD<sup>-/-</sup> mice compared with VitD<sup>+/+</sup> mice (Figure 3A;  $P < 0.05$ ), whereas S100A8 was significantly up-regulated in mice that were postnatally deficient (VitD<sup>+/-</sup> and VitD<sup>-/-</sup>; Figure 3C;  $P < 0.001$ ). NFKBIA was also significantly down-regulated in the VitD<sup>+/-</sup>, VitD<sup>-/+</sup>, and VitD<sup>-/-</sup> groups (Figure 3E;  $P = 0.001$ ). MMP8 was not differentially expressed between groups, although there was a trend for increased expression in postnatal vitamin D-deficient mice ( $P = 0.067$ ). MMP9 was significantly down-regulated in the VitD<sup>+/-</sup> and VitD<sup>-/+</sup> groups ( $P < 0.001$ ), whereas EGFR was only down-regulated in the VitD<sup>-/+</sup> group ( $P = 0.015$ ). There were no differences in RNA expression for EP300 between all groups (data for MMP8, MMP9, EGFR, and EP300 are shown in Figure E1 in the online supplement). In addition, MUC5AC RNA expression was measured, but there was no difference in expression between groups (data not shown). There were also no differences in expression of the reference gene, RPL37 (data not shown).

Immunohistochemical staining was then performed on lung sections to determine protein expression. Immunohistochemical staining revealed more intense staining of S100A9 and S100A8 (myeloid-related protein [MRP] 8) in lung sections from VitD<sup>+/-</sup> and VitD<sup>-/-</sup> mice (Figures 3B and 3D), whereas phosphorylated I $\kappa$ B $\alpha$  staining was evident in lung sections from VitD<sup>+/+</sup>, VitD<sup>+/-</sup>, and VitD<sup>-/-</sup> mice (Figure 3F), reflecting

our findings in RNA expression.

Immunohistochemical staining for phosphorylated EGFR and MMP9 is shown in Figure E1.

### Baseline Lung Function

At baseline, *in utero* vitamin D-deficient (VitD<sup>-/+</sup> and VitD<sup>-/-</sup>) mice had increased airway resistance compared with *in utero*

vitamin D-replete (VitD<sup>+/+</sup> and VitD<sup>+/-</sup>) mice (Figure 4A;  $P = 0.01$ ). There were no differences between groups in tissue damping (Figure 4B) or tissue elastance (Figure 4C).

### Responsiveness to Methacholine

Responsiveness to methacholine was presented as a percentage increase from saline at the maximal dose (100 mg/ml) due

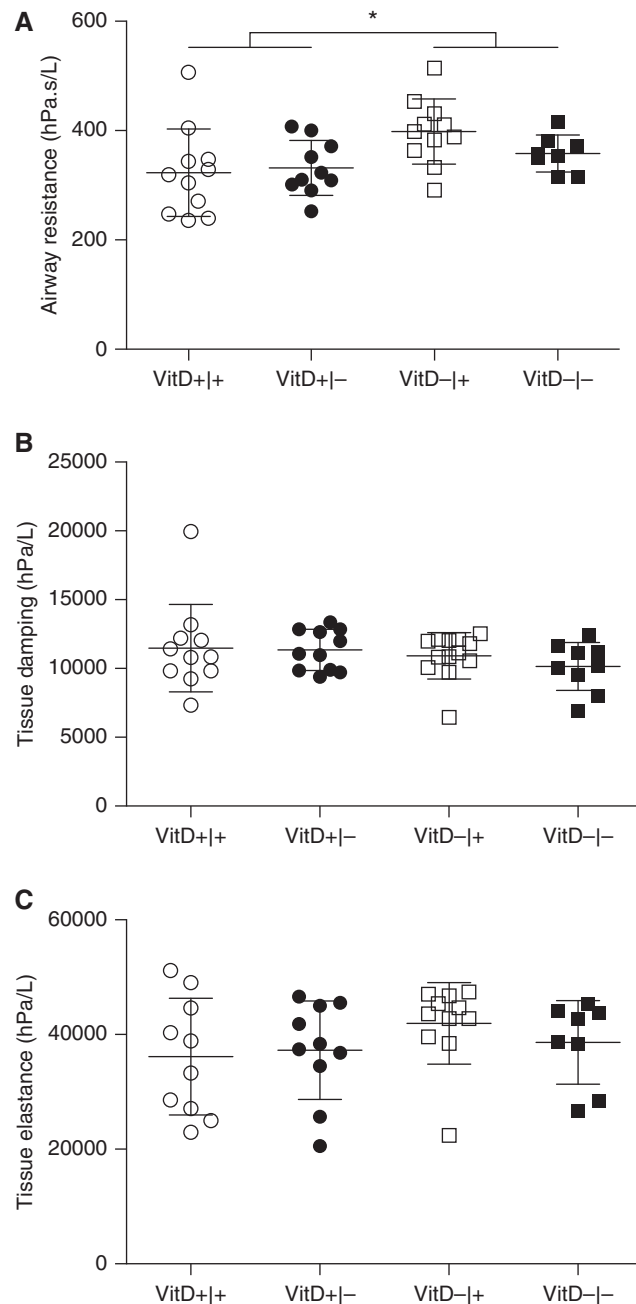


**Figure 4.** Baseline airway resistance (A), tissue damping (B), and tissue elastance (C) in whole-life vitamin D-replete (VitD<sup>+/+</sup>), postnatal vitamin D-deficient (VitD<sup>+/-</sup>), vitamin D-deficient *in utero* (VitD<sup>-/+</sup>), and whole-life vitamin D-deficient (VitD<sup>-/-</sup>) mice. \* $P < 0.05$ . Data are presented as mean ( $\pm$ SD);  $n = 8$ –11/group.

to differences in baseline lung function.  $\text{VitD}^{-/-}$  mice had a greater increase in airway resistance in response to the highest dose of methacholine compared with the other groups (Figure 5A;  $P = 0.036$ ). There were no differences for tissue damping (Figure 5B) or tissue elastance (Figure 5C) between groups.

### ASM Mass

*In utero* vitamin D-deficient ( $\text{VitD}^{-/+}$  and  $\text{VitD}^{-/-}$ ) mice had increased ASM compared with *in utero* vitamin D-replete ( $\text{VitD}^{+/+}$  and  $\text{VitD}^{+/-}$ ) mice (Figure 6E;  $P = 0.029$ ).

### Lung Stereology

Postfixation and embedding lung volume was significantly smaller in mice that were vitamin D-deficient *in utero* ( $\text{VitD}^{-/+}$  and  $\text{VitD}^{-/-}$ ) compared with mice that were vitamin D-replete *in utero* ( $\text{VitD}^{+/+}$  and  $\text{VitD}^{+/-}$ ) ( $P = 0.019$ ). The volume of the parenchyma ( $P = 0.002$ ) and volume of air in the major airways ( $P = 0.03$ ) were also significantly reduced in *in utero* vitamin D-deficient ( $\text{VitD}^{-/+}$  and  $\text{VitD}^{-/-}$ ) mice. There was no difference in the volume of alveolar septae (data not shown) and surface area of the alveoli between groups. The data are presented in Figure E2.

### Inflammatory Cell Counts

#### Correlate with AHR

Differential cell counts of the BAL revealed that, in mice that were vitamin D deficient *in utero* ( $\text{VitD}^{-/+}$  and  $\text{VitD}^{-/-}$ ), neutrophil numbers were increased (Figure 7B;  $P = 0.027$ ), whereas lymphocyte numbers were decreased (Figure 7C;  $P = 0.014$ ), but there were no differences in macrophage cell counts between groups (Figure 7A;  $P = 0.554$ ).

AHR, presented as the percentage increase from saline of airway resistance, significantly correlated with neutrophil cell counts ( $P = 0.007$ ). Neutrophil cell counts also correlated with MMP8 ( $P < 0.001$ ) and MMP9 ( $P = 0.02$ ) expression. The data are presented in Figure E3.

## Discussion

In this study, we set out to uncover the mechanisms contributing to increased ASM and AHR in 8-week-old adult vitamin D-deficient female mice by examining changes in gene expression. RNA-Seq

analysis of gene expression patterns in lung tissue found that whole-life vitamin D deficiency resulted in differential expression of genes involved in embryonic organ development, branching morphogenesis, and Wnt signaling. These data provide a plausible mechanism to explain how maternal vitamin D deficiency may impair lung development *in utero*. We

subsequently studied the impact of *in utero* versus postnatal vitamin D deficiency on gene expression, as well as ASM, lung mechanics, and AHR. *In utero* vitamin D deficiency, irrespective of postnatal vitamin D status, was sufficient to cause increased ASM mass and baseline airway resistance, as well as altered lung structure. Increased neutrophilic inflammation and a reduced

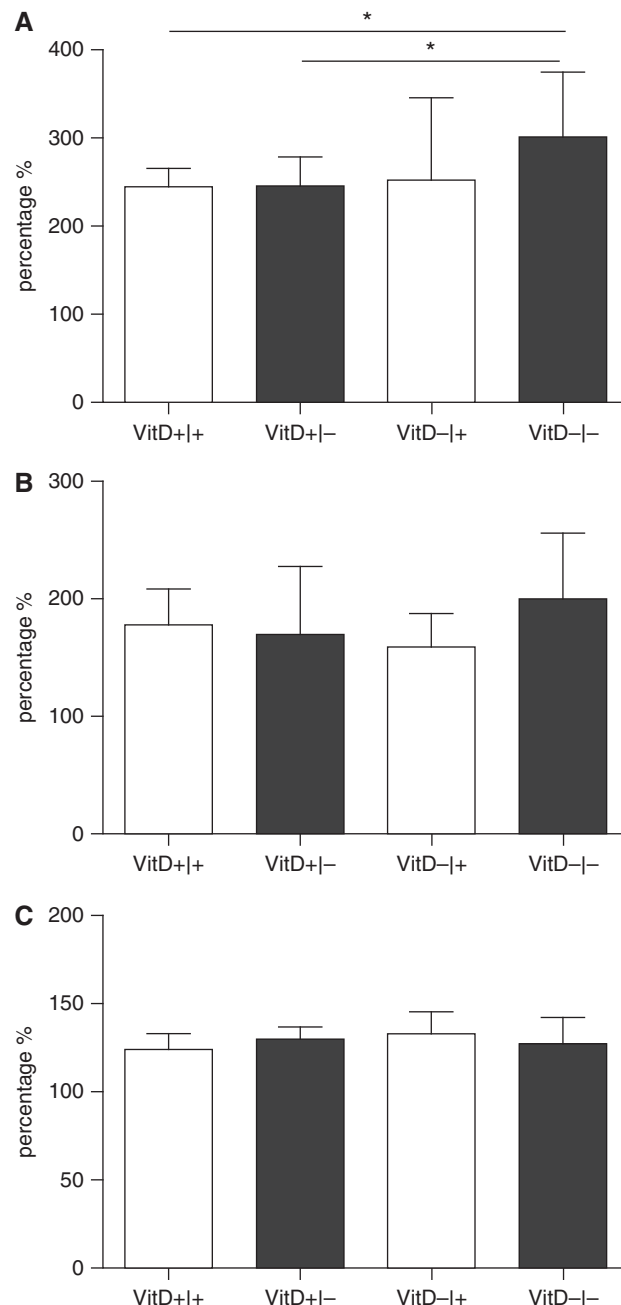


**Figure 5.** The percentage increase from saline of airway resistance (A), tissue damping (B), and tissue elastance (C) in response to 100 mg/ml of methacholine in whole-life vitamin D-replete ( $\text{VitD}^{+/+}$ ), postnatal vitamin D-deficient ( $\text{VitD}^{+/-}$ ), vitamin D-deficient *in utero* ( $\text{VitD}^{-/+}$ ), and whole-life vitamin D-deficient ( $\text{VitD}^{-/-}$ ) mice. \* $P < 0.05$ . Data are presented as mean ( $\pm$ SD);  $n = 9$ –10/group.

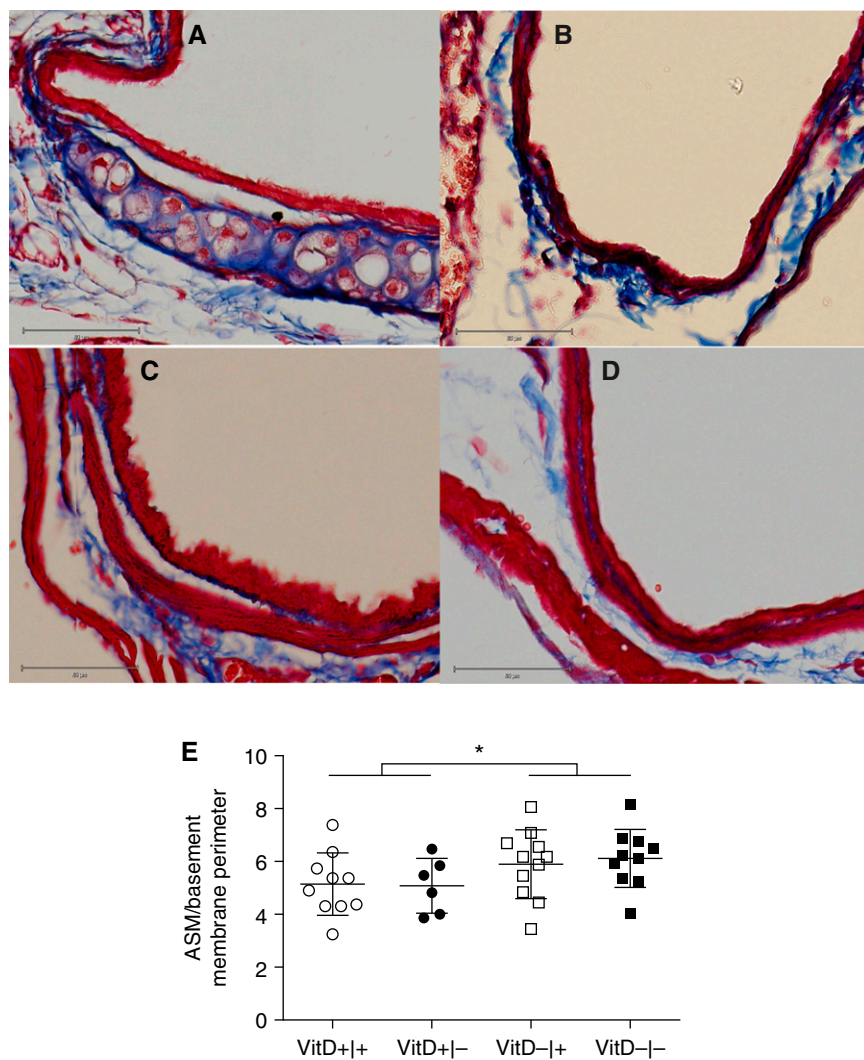


**Figure 6.** Representative images for airway sections stained with Masson's trichrome in whole-life vitamin D-replete (VitD<sup>+/+</sup>; A), postnatal vitamin D-deficient (VitD<sup>+/-</sup>; B), vitamin D-deficient *in utero* (VitD<sup>-/+</sup>; C), and whole-life vitamin D-deficient (VitD<sup>-/-</sup>; D) mice. Scale bars, 60  $\mu$ m. Airway smooth muscle (ASM) mass corrected for the basement membrane perimeter was then quantified in lung sections from VitD<sup>+/+</sup>, VitD<sup>+/-</sup>, VitD<sup>-/+</sup>, and VitD<sup>-/-</sup> mice. \* $P < 0.05$ . Data are presented as mean ( $\pm$ SD), as represented by horizontal lines;  $n = 7$ –12/group.

number of lymphocytes were also observed. This suggests that *in utero* vitamin D deficiency has the potential to have long-lasting impacts on the lung. In addition to these effects on lung development, vitamin D deficiency up-regulated the expression of inflammatory molecules, S100A9 and S100A8. Collectively, these observations suggest that the lung is highly susceptible to vitamin D deficiency during lung development, and that low vitamin D levels could lead to increased inflammation after an insult, which may contribute to symptoms in chronic inflammatory diseases, such as asthma.

Vitamin D deficiency altered the expression levels of genes with roles in embryonic organ development, branching morphogenesis, and Wnt receptor signaling pathways. Canonical Wnt signaling, of which EP300, Wnt7a, and the frizzled (Fzd) 4 receptor are members, has been shown to be active during lung development in both the murine and human lung (19). Furthermore, genes that regulate Wnt signaling during airway branching morphogenesis have been associated with impaired lung function in two cohorts of childhood asthma (20). A recent review by Larriba and colleagues (21) found that 1,25

(OH)D inhibits canonical Wnt signaling, thus supporting our findings that vitamin D deficiency *in utero* can alter Wnt signaling pathways. Wnt signaling is also associated with the development of airway remodeling (22). An aberration in Wnt signaling may lead to impaired lung development and altered lung structure, as we have observed in our model, and this impairment may track into later life, resulting in increased ASM mass and airway resistance.

We have previously shown that whole-life vitamin D deficiency increases ASM leading to increased responsiveness to methacholine (5). In the present study, *in utero* deficiency alone was sufficient to increase ASM mass and baseline airway resistance. The link between airway structure and function is not surprising, given that increased ASM mass may lead to a thicker airway wall and smaller airway lumen, which is likely to increase resistance to flow and contribute to the increase in baseline airway resistance that we observed. Human data support a link between vitamin D and ASM; Gupta and colleagues (6) demonstrated that serum 25(OH)D levels were negatively correlated with volume fraction of ASM in endobronchial biopsies from children with severe asthma with a median age of 11 years, suggesting that vitamin D deficiency may be associated with increased ASM from an early age.

It is noteworthy that ASM thickness correlates with AHR in adult patients with asthma (23). Interestingly, we only found an increased response to methacholine in mice that were whole-life vitamin D deficient, despite the fact that *in utero* deficiency was sufficient to increase ASM mass. This indicates that, although maternal vitamin D deficiency caused airway remodeling and subsequent increased baseline airway resistance, having sufficient vitamin D postnatally may protect against AHR. S100A8 and S100A9 RNA expression was up-regulated with postnatal vitamin D deficiency, and immunohistochemical staining showed that it was highly expressed in the airway epithelium of postnatal vitamin D-deficient mice. These S100 proteins are damage-associated molecular patterns, which are proinflammatory mediators that are overexpressed in inflammatory and autoimmune diseases (24). S100A8 and S100A9 dimerize to form a S100A8/A9 heterodimeric complex and are released by neutrophils and activated macrophages

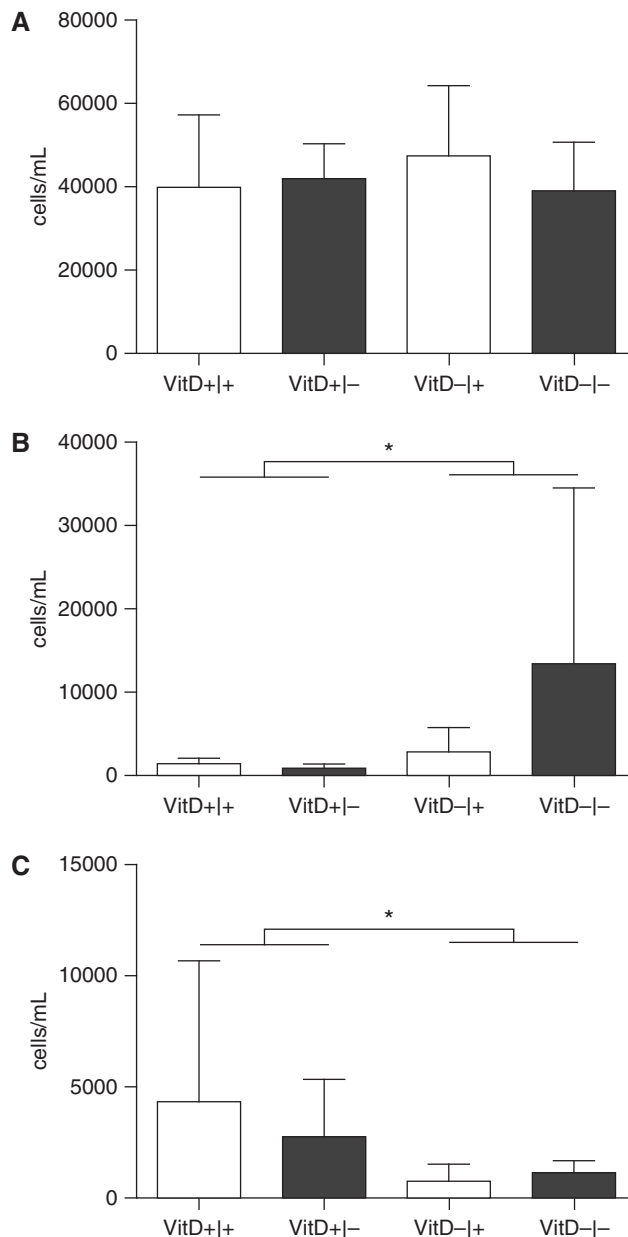


**Figure 7.** Differential cell counts showing the number of macrophages (A), neutrophils (B), and lymphocytes (C) collected from bronchoalveolar lavage of whole-life vitamin D-replete (VitD<sup>+/+</sup>), postnatal vitamin D-deficient (VitD<sup>+/-</sup>), vitamin D-deficient *in utero* (VitD<sup>-/+</sup>), and whole-life vitamin D-deficient (VitD<sup>-/-</sup>) mice. \**P* < 0.05. Data are presented as mean (±SD); *n* = 7–12/group.

during inflammatory responses (24). BAL was performed in mice after a methacholine challenge and, although this may affect inflammatory cell counts, interestingly, we demonstrated that neutrophils were increased in the airways of mice that were vitamin D deficient *in utero*, and neutrophil cell count correlated with AHR. Thus, neutrophilic inflammation and its contribution to the release of S100 molecules may explain, in part, the

induction of the AHR phenotype in this model.

Network analysis suggested that NFKBIA, EGFR, MMP9, and EP300 may be major players in the regulation of gene expression patterns during vitamin D deficiency. We chose to verify the expression of NFKBIA, EGFR, MMP9, and EP300, together with the differentially regulated genes, S100A8, S100A9, and MMP8, as these genes are associated with respiratory disease.

Of these six genes, expression of MMP9, EGFR, and EP300 was not significantly different between the groups in the samples used for the RNA-Seq study. It should be noted that the log fold change of these genes, as determined by RNA-Seq analysis, were small to begin with. Moreover, RNA-Seq considers reads along the full length of the mRNA transcript, whereas RT-qPCR measures a small region of the transcript consisting of around 200 bps. Notwithstanding this, it is pertinent to note that hubs need not be differentially expressed to play important roles in biological processes, and hubs have been reported to exhibit low expression changes in asthma (25). We decided to expand this study and test these genes in an independent group of whole-life vitamin D-deficient and -replete mice, together with the additional *in utero* or postnatal vitamin D-deficient groups. Differential RNA expression of MMP8 was not significant in these analyses, although a trend was observed with a *P* value of 0.067, suggesting that there was some variation between the independent groups of mice. A potential contributor to variation is the fostering of pups at birth to different dams in the whole-life vitamin D-deficient and -replete groups of mice. Fostering was performed as they were controls for the *in utero* and postnatal vitamin D-deficient groups. RNA expression of MMP9 and EGFR were, however, both significantly down-regulated in the *in utero* vitamin D-deficient group, but not the whole-life vitamin D-deficient group and, interestingly, followed a similar trend in our RT-qPCR results. Previous studies have shown that the expression of MMP9 is dependent on EGFR activation (26, 27). Although it is unknown as to why expression would be lower in the *in utero* vitamin D-deficient group and not in whole-life vitamin D deficiency, MMP9 has been found to be highly expressed in asthmatic ASM and down-regulated with vitamin D treatment (28). EGFR expression in human ovarian cancer cells is also down-regulated by vitamin D (29). Thus, it is possible that the “supplementation” of vitamin D postnatally reduced MMP9 and EGFR expression. Furthermore, MMP9 and MMP8 RNA expression correlated with neutrophil cell count; thus, reduced inflammation with higher vitamin D levels may be another contributing factor to reduced expression of these genes.

NFKBIA was the second most dominant hub in the network, and encodes I $\kappa$ B $\alpha$ , an inhibitor of NF- $\kappa$ B activity (30). We found that phosphorylated I $\kappa$ B $\alpha$  was expressed in the airway epithelium. A network analysis of microarray datasets for children with respiratory syncytial virus bronchiolitis, asthma, and bronchopulmonary dysplasia has also demonstrated that NFKBIA is a significant hub in networked cellular transcriptional responses (30), demonstrating its importance in respiratory disease in early life. Previous studies have shown that degradation of NFKBIA increases the transcription of both S100A8 and S100A9 (31). S100A8 and S100A9 mediate the expression of MUC5AC by airway epithelial cells via activation of NF- $\kappa$ B pathways (32). Although we did not observe altered expression of MUC5AC, in our model, NFKBIA expression was down-regulated with vitamin D deficiency, and this may lead to increased NF- $\kappa$ B activity, and subsequent up-regulation of S100A8/A9 and inflammation. Notably, Zhang and colleagues (33) recently demonstrated that maternal vitamin D deficiency during pregnancy in rats decreased the expression of I $\kappa$ B $\alpha$  in offspring and subsequently increased inflammation after birth. It is also noteworthy that vitamin D treatment of airway epithelial cells increases expression of I $\kappa$ B $\alpha$  in a dose-dependent manner, leading to decreased viral induction of NF- $\kappa$ B-driven inflammatory mediators (34), further suggesting that NFKBIA is

a regulator of inflammation in response to vitamin D deficiency.

This study has limitations, which should be acknowledged. First, whole lung tissue was used for gene expression studies; thus, we are unable to pinpoint the cellular source(s) of the inflammatory mediators. Also, because whole lung is a highly complex and heterogeneous tissue, it is likely that follow-up studies on highly purified cell populations would identify additional differentially expressed genes. However, at the outset of the study, it was not clear which cell types were likely to be the most relevant for studying the effects of vitamin deficiency; thus, it seemed logical to measure gene expression in whole lung. Second, lung structure and function measurements were all performed in adult mice. We are therefore unable to ascertain exactly when the lung function and structure changes occur, although we were able to isolate responses that were specific to *in utero* deficiency. Finally, this is largely a descriptive study demonstrating associations between altered lung structure and function with inflammation, and with altered gene expression *in vivo*. Unfortunately, we are unable to determine if inflammation correlated with changes in airway structure, as BAL fluid was not collected from this subset of mice, although we observed altered expression of S100A8, S100A9, and phosphorylated I $\kappa$ B $\alpha$  in the airway epithelium of vitamin D-deficient mice. Further *in vitro* studies using airway epithelial cells exposed to vitamin D to

investigate if these molecules are released may be warranted.

In summary, maternal vitamin D deficiency during pregnancy was associated with increased ASM mass, baseline airway resistance, airway inflammation, and altered lung structure in adulthood. Expression of S100A8/A9 was increased in adulthood in mice as a result of vitamin D deficiency. Because neutrophilic inflammation influences the expression of many genes found to be differentially expressed in our RNA-Seq analysis, including S100A8, S100A9, MMP8, and MMP9, neutrophils may be the link between vitamin D deficiency and altered lung structure and function. Postnatal “supplementation” with vitamin D protected against AHR, but did not resolve the other deficits. It is possible that, although postnatal supplementation may reduce inflammation, it was not sufficient to alter lung structure defects, which are likely to be established early in life. This could explain why vitamin D supplementation studies in patients with asthma have not revealed beneficial effects (35). ■

**Author disclosures** are available with the text of this article at [www.atsjournals.org](http://www.atsjournals.org).

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## References

- Chinellato I, Piazza M, Sandri M, Peroni D, Piacentini G, Boner AL. Vitamin D serum levels and markers of asthma control in Italian children. *J Pediatr* 2011;158:437–441.
- Janssens W, Bouillon R, Claes B, Carremans C, Lehouck A, Buysschaert I, Coolen J, Mathieu C, Decramer M, Lambrechts D. Vitamin D deficiency is highly prevalent in COPD and correlates with variants in the vitamin D-binding gene. *Thorax* 2010;65:215–220.
- Gonem S, Raj V, Wardlaw AJ, Pavord ID, Green R, Siddiqui S. Phenotyping airways disease: an A to E approach. *Clin Exp Allergy* 2012;42:1664–1683.
- Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. *Am J Respir Crit Care Med* 2001;164:S28–S38.
- Foong RE, Shaw NC, Berry LJ, Hart PH, Gorman S, Zosky GR. Vitamin D deficiency causes airway hyperresponsiveness, increases airway smooth muscle mass, and reduces TGF- $\beta$  expression in the lungs of female BALB/c mice. *Physiol Rep* 2014;2:e00276.
- Gupta A, Sjoukes A, Richards D, Banya W, Hawrylowicz C, Bush A, Saglani S. Relationship between serum vitamin D, disease severity, and airway remodeling in children with asthma. *Am J Respir Crit Care Med* 2011;184:1342–1349.
- Damera G, Fogle HW, Lim P, Goncharova EA, Zhao H, Banerjee A, Tliba O, Krymskaya VP, Panettieri RA Jr. Vitamin D inhibits growth of human airway smooth muscle cells through growth factor-induced phosphorylation of retinoblastoma protein and checkpoint kinase 1. *Br J Pharmacol* 2009;158:1429–1441.
- Marin L, Dufour ME, Nguyen TM, Tordet C, Garabedian M. Maturation changes induced by 1  $\alpha$ ,25-dihydroxyvitamin D3 in type II cells from fetal rat lung explants. *Am J Physiol* 1993;265:L45–L52.
- Sakurai R, Shin E, Fonseca S, Sakurai T, Litonjua AA, Weiss ST, Torday JS, Rehan VK. 1 $\alpha$ ,25(OH) $_2$ D3 and its 3-epimer promote rat lung alveolar epithelial-mesenchymal interactions and inhibit lipofibroblast apoptosis. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L496–L505.
- Kho AT, Sharma S, Qiu W, Gaedigk R, Klanderman B, Niu S, Anderson C, Leeder JS, Weiss ST, Tantisira KG. Vitamin D related genes in lung development and asthma pathogenesis. *BMC Med Genomics* 2013;6:47.
- Zosky GR, Hart PH, Whitehouse AJO, Kusel MM, Ang W, Foong RE, Chen L, Holt PG, Sly PD, Hall GL. Vitamin D deficiency at 16 to 20 weeks' gestation is associated with impaired lung function and asthma at 6 years of age. *Ann Am Thorac Soc* 2014;11:571–577.
- Zosky GR, Berry LJ, Elliot JG, James AL, Gorman S, Hart PH. Vitamin D deficiency causes deficits in lung function and alters lung structure. *Am J Respir Crit Care Med* 2011;183:1336–1343.
- Wang X, Spandidos A, Wang H, Seed B. PrimerBank: a PCR primer database for quantitative gene expression analysis, 2012 update. *Nucleic Acids Res* 2012;40:D1144–D1149.
- Bosco A, McKenna KL, Devitt CJ, Firth MJ, Sly PD, Holt PG. Identification of novel Th2-associated genes in T memory responses to allergens. *J Immunol* 2006;176:4766–4777.

15. Zosky GR, Janosi TZ, Adamiczka A, Bozanich EM, Cannizzaro V, Larcombe AN, Turner DJ, Sly PD, Hantos Z. The bimodal quasi-static and dynamic elastance of the murine lung. *J Appl Physiol* (1985) 2008;105:685–692.
16. Hsia CCW, Hyde DM, Ochs M, Weibel ER; ATS/ERS Joint Task Force on Quantitative Assessment of Lung Structure. An official research policy statement of the American Thoracic Society/European Respiratory Society: standards for quantitative assessment of lung structure. *Am J Respir Crit Care Med* 2010;181:394–418.
17. Fontanillo C, Nogales-Cadenas R, Pascual-Montano A, De las Rivas J. Functional analysis beyond enrichment: non-redundant reciprocal linkage of genes and biological terms. *PLoS One* 2011;6:e24289.
18. Bosco A, Ehteshami S, Panyala S, Martinez FD. Interferon regulatory factor 7 is a major hub connecting interferon-mediated responses in virus-induced asthma exacerbations *in vivo*. *J Allergy Clin Immunol* 2012;129:88–94.
19. Zhang M, Shi J, Huang Y, Lai L. Expression of canonical WNT/ $\beta$ -CATENIN signaling components in the developing human lung. *BMC Dev Biol* 2012;12:21.
20. Sharma S, Tantisira K, Carey V, Murphy AJ, Lasky-Su J, Celedón JC, Lazarus R, Klanderman B, Rogers A, Soto-Quirós M, *et al*. A role for Wnt signaling genes in the pathogenesis of impaired lung function in asthma. *Am J Respir Crit Care Med* 2010;181:328–336.
21. Larriba MJ, González-Sancho JM, Bonilla F, Muñoz A. Interaction of vitamin D with membrane-based signaling pathways. *Front Physiol* 2014;5:60.
22. Kumawat K, Koopmans T, Gosens R.  $\beta$ -catenin as a regulator and therapeutic target for asthmatic airway remodeling. *Expert Opin Ther Targets* 2014;18:1023–1034.
23. Tsurikisawa N, Oshikata C, Tsuburai T, Saito H, Sekiya K, Tanimoto H, Takeichi S, Mitomi H, Akiyama K. Bronchial hyperresponsiveness to histamine correlates with airway remodelling in adults with asthma. *Respir Med* 2010;104:1271–1277.
24. Foell D, Wittkowski H, Vogl T, Roth J. S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. *J Leukoc Biol* 2007;81:28–37.
25. Lu X, Jain VV, Finn PW, Perkins DL. Hubs in biological interaction networks exhibit low changes in expression in experimental asthma. *Mol Syst Biol* 2007;3:98.
26. Zhang P, Zhang P, Zhou M, Jiang H, Zhang H, Shi B, Pan X, Gao H, Sun H, Li Z. Exon 4 deletion variant of epidermal growth factor receptor enhances invasiveness and cisplatin resistance in epithelial ovarian cancer. *Carcinogenesis* 2013;34:2639–2646.
27. Xie X, Ghadimi MPH, Young ED, Belousov R, Zhu QS, Liu J, Lopez G, Colombo C, Peng T, Reynoso D, *et al*. Combining EGFR and mTOR blockade for the treatment of epithelioid sarcoma. *Clin Cancer Res* 2011;17:5901–5912.
28. Song Y, Qi H, Wu C. Effect of 1,25-(OH) $_2$ D $_3$  (a vitamin D analogue) on passively sensitized human airway smooth muscle cells. *Respirology* 2007;12:486–494.
29. Shen Z, Zhang X, Tang J, Kasiappan R, Jinwal U, Li P, Hann S, Nicosia SV, Wu J, Zhang X, *et al*. The coupling of epidermal growth factor receptor down regulation by 1 $\alpha$ ,25-dihydroxyvitamin D $_3$  to the hormone-induced cell cycle arrest at the G1-S checkpoint in ovarian cancer cells. *Mol Cell Endocrinol* 2011;338:58–67.
30. Ali S, Hirschfeld AF, Mayer ML, Fortuno III ES, Corbett N, Kaplan M, Wang S, Schneiderman J, Fjell CD, Yan J, *et al*. Functional genetic variation in NFKBIA and susceptibility to childhood asthma, bronchiolitis, and bronchopulmonary dysplasia. *J Immunol* 2013;190:3949–3958.
31. Cataisson C, Pearson AJ, Torgerson S, Nedospasov SA, Yuspa SH. Protein kinase C  $\alpha$ -mediated chemotaxis of neutrophils requires NF-kappa B activity but is independent of TNF  $\alpha$  signaling in mouse skin *in vivo*. *J Immunol* 2005;174:1686–1692.
32. Kang JH, Hwang SM, Chung IY. S100A8, S100A9 and S100A12 activate airway epithelial cells to produce MUC5AC via extracellular signal-regulated kinase and nuclear factor- $\kappa$ B pathways. *Immunology* 2015;144:79–90.
33. Zhang H, Chu X, Huang Y, Li G, Wang Y, Li Y, Sun C. Maternal vitamin D deficiency during pregnancy results in insulin resistance in rat offspring, which is associated with inflammation and I $\kappa$ B $\alpha$  methylation. *Diabetologia* 2014;57:2165–2172.
34. Hansdottir S, Monick MM, Lohan N, Powers L, Gerke A, Hunninghake GW, Vitamin D. Vitamin D decreases respiratory syncytial virus induction of NF-kappaB-linked chemokines and cytokines in airway epithelium while maintaining the antiviral state. *J Immunol* 2010;184:965–974.
35. Castro M, King TS, Kunselman SJ, Cabana MD, Denlinger L, Holguin F, Kazani SD, Moore WC, Moy J, Sorkness CA, *et al*. National Heart, Lung, and Blood Institute's AsthmaNet. Effect of vitamin D $_3$  on asthma treatment failures in adults with symptomatic asthma and lower vitamin D levels: the VIDA randomized clinical trial. *JAMA* 2014;311:2083–2091.

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