

Title

Identification of genes differentially regulated by vitamin D deficiency that alter lung pathophysiology and inflammation in allergic airways disease

Running head

Vitamin D alters gene expression in allergic asthma

Authors

¹Rachel E Foong, ¹Anthony Bosco, ¹Niamh M Troy, ¹Shelley Gorman, ¹Prue H Hart,
^{1,2,3,4}Anthony Kicic, ⁵Graeme R Zosky

Author's contributions:

R.E.F, P.H.H., S.G. and G.R.Z. conception and design of research; R.E.F. and N.M.T. performed experiments; R.E.F. and A.B. analyzed data; R.E.F., A.B., A.K. and G.R.Z. interpreted results of experiments; R.E.F. prepared figures; R.E.F. drafted manuscript; R.E.F., A.B., P.H.H., S.G., A.K. and G.R.Z. edited and revised manuscript; R.E.F., A.B., N.M.T., P.H.H., S.G., A.K. and G.R.Z. approved final version of manuscript.

Affiliations

¹Telethon Kids Institute, the University of Western Australia, Perth, Western Australia, Australia;

²School of Paediatrics and Child Health, University of Western Australia, Perth, Western Australia

³Department of Respiratory Medicine, Princess Margaret Hospital for Children, Perth, 6001, Western Australia, Australia.

25 ⁴Centre for Cell Therapy and Regenerative Medicine, School of Medicine and Pharmacology,
26 The University of Western Australia, Nedlands, 6009, Western Australia, Australia.

27 ⁵School of Medicine, Faculty of Health Science, University of Tasmania, Hobart, Tasmania,
28 Australia.

29

30 **Corresponding author**

31 Rachel E Foong

32 Address: Telethon Kids Institute, 100 Roberts Road, Subiaco WA 6008, Australia.

33 Tel no.: +618 9489 7817

34 Fax no.: +618 9489 7700

35 Email: Rachel.Foong@telethonkids.org.au

36

37

38

39

40

41

42

43

44

45

46

47

48

49

50 **Abstract**

51 Vitamin D deficiency is associated with asthma risk. Vitamin D deficiency may enhance the
52 inflammatory response and we have previously shown that airway remodelling and airway
53 hyperresponsiveness (AHR) is increased in vitamin D-deficient mice. In this study, we
54 hypothesize that vitamin D deficiency would exacerbate house dust mite (HDM)-induced
55 inflammation and alterations in lung structure and function. A BALB/c mouse model of
56 vitamin D deficiency was established by dietary manipulation. Responsiveness to
57 methacholine, airway smooth muscle (ASM) mass, mucus cell metaplasia, lung and airway
58 inflammation and cytokines in bronchoalveolar lavage (BAL) fluid were assessed. Gene
59 expression patterns in mouse lung samples were profiled by RNA-Seq. HDM exposure
60 increased inflammation and inflammatory cytokines in BAL, baseline airway resistance,
61 tissue elastance and ASM mass. Vitamin D deficiency enhanced the HDM-induced influx of
62 lymphocytes into BAL, ameliorated the HDM induced increase in ASM mass and protected
63 against the HDM-induced increase in baseline airway resistance. RNA-Seq identified nine
64 genes that were differentially regulated by vitamin D deficiency in the lungs of HDM-treated
65 mice. Immunohistochemical staining confirmed that protein expression of MID1 and ADM
66 were differentially regulated such that they promoted inflammation, while and HILPDA
67 which is associated with ASM remodelling, was downregulated. Protein expression studies in
68 human bronchial epithelial cells also showed that addition of vitamin D decreased MID1
69 expression. Differential regulation of these genes by vitamin D deficiency could determine
70 lung inflammation and pathophysiology and suggest that the effect of vitamin D deficiency
71 on HDM-induced allergic airways disease is complex.

72

73 **Keywords** airway remodelling, airway hyperresponsiveness, mouse model, RNA-Seq, house
74 dust mite

75 **Introduction**

76 Vitamin D deficiency is prevalent all over the world and is a public health concern.
77 Epidemiological studies have linked vitamin D deficiency to respiratory diseases including
78 lung cancer, chronic obstructive pulmonary disease and asthma (16, 26, 43). However, while
79 there is evidence that higher vitamin D levels may reduce the risk of asthma exacerbations,
80 there is inconclusive evidence that vitamin D deficiency has the capacity to *cause* asthma (5,
81 13, 34).

82

83 Asthma is an inflammatory disorder affecting the conducting airways (22). Allergic asthma is
84 characterised by T helper (Th) 2 type inflammation, and persistent airway inflammation
85 drives structural alterations in the lung (32). These structural changes termed airway
86 remodelling, lead to reversible airflow obstruction, deficits in lung function and airway
87 hyperresponsiveness (AHR) (32). These functional deficits are ultimately linked to asthma-
88 related morbidity. Vitamin D has important immunomodulatory properties, as evidenced by
89 the widespread expression of the vitamin D receptor on immune cells, including activated T
90 and B cells, macrophages and dendritic cells (18). Studies have shown that vitamin D
91 deficiency can exacerbate allergic airways inflammation (39). Thus, in addition to its
92 potential role in airway remodelling and the development of AHR (11) (discussed below),
93 vitamin D deficiency may promote asthma symptoms by skewing the immune response
94 towards a Th2 phenotype.

95

96 We have recently shown that *in utero* vitamin D deficiency increases ASM mass and AHR in
97 mice and differentially regulates genes involved in embryonic lung development (39). Data
98 suggest that the structural alterations that are observed in the lungs of asthmatics may have
99 origins in early life (25) and, as such, vitamin D may also contribute to asthma pathogenesis

by influencing lung development. We hypothesized that vitamin D deficiency would exacerbate respiratory outcomes in allergic airways disease by altering the inflammatory response, airway structure and function. We tested this hypothesis using mouse model of chronic house dust mite (HDM) exposure and investigated the expression of pathways that vitamin D deficiency altered in response to HDM using RNA-Seq and follow-up studies in human bronchial epithelial cells.

Materials and Methods

Mouse model

Three-week-old female BALB/c mice (Animal Resource Centre, Murdoch, WA, Australia) were placed on either vitamin D₃-deficient or -replete diets containing 0 or 2280 IU of vitamin D₃, respectively, (Specialty Feeds, Glen Forrest, WA, Australia) as described previously (12). Replete diets contained 1% calcium while deficient diets were supplemented with 2% calcium for prevention of hypocalcaemia (44). Mice were housed in a room with a 12-hour ultraviolet B-free light/dark cycle. At 8-weeks of age, female mice were mated with vitamin D-replete male BALB/c mice to prevent paternal epigenetic effects of vitamin D deficiency. Vitamin D-deficient status was confirmed by measuring 25-hydroxyvitamin D levels in serum. Similar to our previous study, we focused on female mice since physiological changes were only observed in females following vitamin D deficiency (12). Female offspring were maintained on -deficient or -replete diets and intranasally inoculated with 25 µg HDM extract (Greer Laboratories, Lenoir, NC, USA) in 50 µL of saline or saline alone (control), five days a week for five weeks once mice were 8-weeks of age. This resulted in four treatment groups: saline-treated and vitamin D-replete (SalVitD+), saline-treated and vitamin D-deficient (SalVitD-), house dust mite (HDM)-treated and vitamin D-replete (HDMVitD+) and HDM-treated and vitamin D-deficient (HDMVitD-) mice. Outcomes were

assessed 72 hours after the final exposure. All procedures were approved by the Telethon Kids Institute Animal Ethics Committee and conformed to National Health and Medical Research Council of Australia guidelines.

Animal preparation and methacholine challenge

Mice were anaesthetized by intraperitoneal injection with 40 mg/mL ketamine and 2 mg/mL xylazine at a dose of 0.01 mL/g body weight. Two-thirds of the dose was administered initially to induce surgical anaesthesia prior to tracheostomy and cannulation with a polyethylene endotracheal tube (internal diameter = 0.086 cm, length = 1.0 cm). Mice (n = 7-12 per group) were connected to a flexiVent system (SCIREQ, Montreal, Canada) for assessment of responsiveness to methacholine (β -methacholine chloride, Sigma-Aldrich, MO, USA) using a modification of the low frequency forced oscillation technique (LFOT) as previously described (12). LFOT measurements were taken once a minute for 5 minutes to establish baseline lung mechanics. Mice were then challenged (10 s) with increasing concentrations of aerosolized methacholine from 0.3 mg/mL up to 100 mg/mL. Five LFOT measurements were taken after each dose of methacholine, and the maximum responses for Newtonian resistance (R_{aw} , which primarily reflects airway resistance in the mouse due to the low chest wall impedance), tissue damping (G, the parameter describing the viscous frequency dependent response in resistance which is thought to reflect resistance of the peripheral airways where airflow occurs primarily by diffusion) and tissue elastance (H, the elastic properties of the lung parenchyma) from these measurements were used to compare responses between groups.

Differential cell counts

BAL was performed after methacholine challenge and was collected from mice by washing 500 μ L of sterile saline into the lungs three times. Total cells collected in the BAL fluid were counted using a haemocytometer. Light microscopy was used to determine differential counts from cytospin cell samples stained with Leishman's stain (BDH Laboratory Supplies, Poole, England).

Measurement of cytokines in BAL fluid

Levels of interferon (IFN)- γ , tumour necrosis factor (TNF)- α , interleukin (IL)-4, IL-5 and IL-2 in the BAL was assessed using a cytometric bead array kit for mouse Th1/Th2 cytokines (BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions. Transforming growth factor (TGF)- β protein levels in the BALF were measured using a DuoSet ELISA Development kit for measuring TGF- β 1 (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

Airway histology

Lung structure was assessed in separate groups of mice ($n = 7-12$ per group) following ATS/ERS guidelines (24). After euthanasia, lungs were inflation-fixed via tracheal instillation with 4% formaldehyde solution at a pressure of 10 cmH₂O. The left lung was separated from the right lung at the main bronchus prior to tissue processing for embedding in paraffin. The right lung was embedded separately for immunohistochemical staining. The left lung was embedded with the transverse section face down in order to obtain cross sections of the first generation airway. Five μ m sections were taken at 500 μ m intervals throughout the entire lung and stained with Masson's Trichrome for visualization of the ASM layer. The cross-sectional area of the ASM and basement membrane perimeter were measured using the NewCast system (Visiopharm, Hoersholm, Denmark). The outer wall

area between the outer border of the adventitia and ASM layer, consisting of elastic and collagenous fibres, was also measured (3). Airway sections were stained with Alcian blue-periodic acid-Schiff to detect mucus-producing cells. The expression of mucus-producing cells was calculated as the percentage of positively-stained epithelial cells divided by total number of epithelial cells in the airway.

RNA-Seq protocol

Lung tissue samples from HDM and saline-treated vitamin D-deficient and -replete female mice (n = 6 per group) were stabilized in RNAlater (Sigma-Aldrich, Castle Hill, NSW, Australia). The samples were disrupted in TRIzol (Life Technologies, Mulgrave, VIC, Australia) employing a TissueRuptor rotor-stator homogenizer (Qiagen, Chadstone Centre, VIC, Australia). Total RNA was extracted from TRIzol followed by purification on RNeasy Minelute columns (Qiagen). The samples were then sent to the Australian Genome Research Facility for library preparation and sequencing. Library preparation was based on the TruSeq RNA Sample Preparation Kit v2 (Illumina, Scoresby, VIC, Australia), and the samples were sequenced on the HiSeq2000 instrument (50 bp single end reads, ~25 million reads per sample). The raw sequencing results are available at the NCBI Short Read Archive under accession SRP046087.

RNA-Seq data analysis

The sequencing reads were aligned to the reference genome (mm10) using the subread aligner (30). Reads were summarized at the gene-level using feature Counts (31). Genes with a low read count (< 5 reads/million in < 50 % of the samples) were filtered out of the analysis. Differentially expressed genes were identified using *edgeR* with False Discovery

Rate (FDR) adjustment for multiple testing (2). Biological functions, pathways, and networks enriched in the data were identified using GeneTerm Linker (10).

RT-qPCR

A subset of differentially expressed genes identified in the RNA-Seq experiment were validated using RT-qPCR. Total RNA from lung tissue samples obtained from an independent group of HDM and saline-treated vitamin D-deficient and -replete mice (n = 10 per group) were extracted as described above. Reverse transcription was carried out using the QuantiTect Reverse Transcription Kit (Qiagen). Real time PCR primer assay sequences were obtained from primerbank (40) and the primer list included in Table 1. QuantiTect SYBR Green was used for qPCR on the ABI 7900 HT instrument. Relative standard curves were prepared from serially diluted PCR products. Expression was normalized to the housekeeping gene eukaryotic translation elongation factor 2 (EEF2).

Immunohistochemistry

Immunohistochemical staining was performed using the following primary antibodies: MID1 (1:200 dilution; ab70770; Abcam, Cambridge, USA), ADM (1:100 dilution; ThermoFisher Scientific, Waltham, USA), HIG2 (1:200 dilution; Santa Cruz Biotechnology, Dallas, USA) and ANGPTL4 (1:250 dilution; Santa Cruz Biotechnology, Dallas, USA) on 5- μ m sections obtained from the right lung.

Cell culture

Transformed human bronchial epithelial cells (BEAS-2B) were cultured in bronchial epithelial cell growth medium (BEGM; Lonza, North Sydney, NSW, Australia) with serum substitute, Ultrosor G (Pall Australia, Cheltenham VIC, Australia) (2% v/v), and gentamycin

(Sigma-Aldrich) (50 µg/mL). Prior to study, 2×10^5 cells were seeded into 12-well plates for collection of RNA and 1.7×10^4 cells were seeded into 96-well black-sided plates with clear bottoms for In-cell Westerns. Once cells achieved 80% confluence, appropriate wells were supplemented with 100 nM 1,25(OH)₂D. After 24 hours, 50 µg/mL of HDM extract was added to wells with and without 1,25(OH)₂D. Cells not treated with 1,25(OH)₂D and HDM were used as controls. This resulted in four treatment groups: untreated cells (Control), 1,25(OH)₂D only (VitD), HDM only (HDM) and 1,25(OH)₂D followed by HDM (VitDHDM). RNeasy Lysis buffer (RLT buffer) (Qiagen) was added to cells in 12-well plates 4 hours after HDM exposure for RNA extraction, while 24 hours later, cells in 96-well plates were fixed for 20 minutes in 3.7% formaldehyde solution for In-Cell Westerns. All experiments were performed in triplicate.

In-Cell Westerns

After fixation, cells were washed 5 times sequentially with 0.1% Triton-X (Sigma-Aldrich) in phosphate buffered saline (PBS) for 5 min to permeabilize cells. Cells were then blocked with LI-COR Odyssey Blocking Buffer (LI-COR Biotechnology, Lincoln, USA) for 90 min at room temperature. Cells were incubated with MID1 primary antibody (ab70770; Abcam, Cambridge, USA) diluted 1:500 in Blocking Buffer at 4°C overnight. Cells were then washed with 1x PBS containing 0.1% Tween-20 (Sigma-Aldrich) sequentially 5 times for 5 min before addition of fluorescently-labelled secondary antibody IRDye 800CW Goat anti-Rabbit (1:800 dilution; LI-COR Biotechnology) containing DRAQ5 (1: 10000 dilution; LI-COR Biotechnology) and Sapphire 700(1:1000 dilution; LI-COR Biotechnology). The plate was protected from light after addition of secondary antibody and incubated for one hour prior to washing with 1x PBS containing 0.1% Tween-20 sequentially 5 times for 5 min. All liquid was removed from cells and the plate scanned with detection in both 700 and 800 nm

channels using an Odyssey Infrared Imaging System (LI-COR Biotechnology). Target protein expression was then analysed using Image Studio Software (LI-COR Biotechnology) and data normalised to the nuclear expression of DRAQ5/Sapphire 700.

Statistical analysis

Multiple group comparisons were made using two-way ANOVAs with Holm-Sidak post hoc tests. Differences between two groups were compared using non-parametric *t* tests (Mann-Whitney tests). Statistical analyses were performed using SigmaPlot software (Systat Software, California, USA) and Prism (GraphPad Software, California, USA). Data are shown as mean $\pm$ SD. *P* values of < 0.05 were regarded as statistically significant.

Results

Baseline lung function

At baseline, prior to methacholine challenge, HDM treatment caused an increase in airway resistance in vitamin D-replete mice ($p = 0.012$) (Figure 1A) and an increase in tissue elastance in both vitamin D-replete and -deficient mice ($p = 0.021$) (Figure 1B) compared to saline treated mice. Interestingly, vitamin D deficiency appeared to protect the mice from the HDM induced increase in airway resistance ($p = 0.024$) (Figure 1A) suggesting that vitamin D deficiency modifies the functional effects of HDM exposure on the airway. HDM treatment and vitamin D deficiency had no effect on tissue damping ($p > 0.05$ for all comparisons) (Figure 1C).

Methacholine challenge

Due to the differences in baseline lung function, responsiveness to methacholine was expressed as a percentage increase from saline. Vitamin D deficiency caused an increase in

the magnitude of response of airway resistance to methacholine ($p = 0.014$), but there was no effect of HDM treatment ($p = 0.059$) (Figure 2A). HDM treatment caused an increase in the magnitude of the response of tissue elastance to methacholine ($p = 0.006$) compared to saline treatment (Figure 2B). HDM treatment and vitamin D deficiency had no effect on tissue damping ($p > 0.05$ for all comparisons) (Figure 2C). Overall vitamin D deficiency did not modify the methacholine responsiveness induced by HDM exposure.

Differential cell counts

HDM treatment increased the total cell count in the BAL ($p < 0.001$) (Table 2), while vitamin D deficiency significantly increased lymphocyte cell counts ($p = 0.038$). There were significant statistical interactions between HDM treatment and vitamin D deficiency for all cell types, except eosinophils, such that the effect of vitamin D deficiency on HDM treatment was complex (Table 2). Macrophages were increased in SalVitD⁻ mice compared with SalVitD⁺ mice ($p = 0.049$), while HDM caused an increase in macrophage counts in vitamin D-deficient mice ($p = 0.007$), but not between vitamin D-replete groups ($p = 0.869$). Both HDM treatment ($p < 0.001$) and vitamin D deficiency ($p = 0.038$) caused an increase the number of neutrophils in the BAL, however, these effects were not additive such that there was no difference in the neutrophil levels in the BAL of HDMVitD⁻ mice compared to HDMVitD⁺ mice ($p = 0.899$). Similarly, both HDM treatment ($p < 0.001$) and vitamin D deficiency ($p = 0.034$) caused an increase in lymphocyte numbers in the BAL; however, in this case, these effects were additive. In contrast, HDM treatment caused eosinophilia ($p < 0.001$) while vitamin D deficiency had no effect on eosinophil levels in the BAL ($p = 0.114$). Taken together, these data suggest that vitamin D deficiency enhances the lymphocyte influx induced by HDM exposure but has limited influence on the other cell types.

Cytokine levels in BAL fluid

IFN- γ levels were not altered by vitamin D deficiency or HDM treatment ($p = 0.12$) (Figure 3A). HDM treatment increased TNF- α levels ($p = 0.003$), however there was no effect of vitamin D deficiency ($p = 0.418$) (Figure 3B). Levels of IL-4, IL-5 and IL-2 in the BAL for most samples were below the limits of detection of the cytometric bead array kit and therefore not compared between groups. TGF- β levels were increased following HDM treatment ($p < 0.001$), however within the HDM treated group, the increase in TGF- β levels induced by HDM was attenuated in the vitamin D-deficient mice ($p = 0.011$) (Figure 3C).

Airway histology

Both HDM treatment ($p < 0.001$) and vitamin D deficiency ($p = 0.009$) independently increased ASM mass. However, when the exposures were combined, vitamin D deficiency appeared to protect from the HDM induced increase in ASM mass such that the ASM mass in HDMVitD- mice was significantly lower than the ASM mass in HDMVitD+ mice ($p = 0.005$) (Figure 4).

In contrast, both HDM ($p = 0.697$) treatment and vitamin D deficiency ($p = 0.589$) had no effect on the outer wall area (*data not shown*) while HDM increased the percentage of mucous producing cells in the airway ($p < 0.001$). Vitamin D deficiency had no effect on the percentage of mucous producing cells ($p = 0.071$) (Figure 5).

RNA-Seq profiling of gene expression and validation of differentially regulated genes

Gene expression patterns were profiled using RNA-Seq in lung tissue samples from vitamin D-deficient and -replete mice, with or without HDM exposure. The results for genes differentially regulated by vitamin D alone have previously been published (11). We first describe the identification of differentially expressed genes in vitamin D-replete mice

exposed to HDM versus saline before detailing the effects of vitamin D deficiency within the HDM-treated group.

Effect of HDM alone

The data showed that 1182 genes were differentially expressed at $FDR < 0.05$ by HDM. To confirm the findings from the RNA-Seq experiment, a subset of the differentially expressed genes that are relevant to the biology of airway remodelling and respiratory disease were selected for RT-qPCR validation in an independent group of animals. Selected genes included; chloride channel regulator 3 (CLCA3), eosinophil-associated, ribonuclease A family, member 11 (EAR11), resistin like alpha (RETNLA), MUC5AC, regenerating islet-derived 3 gamma (REG3G), matrix metalloproteinase (MMP)12, secretory leukocyte peptidase inhibitor (SLPI) and trefoil factor 2 (TFF2) (Figure 7). Expression of these genes were significantly upregulated in HDM-treated mice ($p < 0.05$), with the exception of REG3G ($p = 0.086$).

Effect of vitamin D deficiency on HDM induced gene and protein expression

Gene expression patterns were then compared in lung tissue samples from HDM-treated, vitamin D-deficient and -replete mice, and this analysis demonstrated that nine genes were differentially regulated (Table 2). We performed RT-qPCR validation for expression of midline 1 (MID1), adrenomedullin (ADM), angiopoietin-like protein 4 (ANGPTL4), hypoxia-inducible lipid droplet-associated (HILDPA), acyl-CoA thioesterase 1 (ACOT1), period 2 (PER2) and 5-aminolevulinic acid synthase 1 (ALAS1) in independent animals. MID1 expression was significantly upregulated in vitamin D-deficient mice ($p = 0.029$) (Figure 7A), while ADM ($p = 0.047$) (Figure 7C) and HILDPA expression ($p = 0.026$)

(Figure 7G) was significantly downregulated. There were no differences in expression for ANGPTL4 (Figure 7E), ACOT1, PER2 and ALAS1 (*data not shown*).

Immunohistochemical staining showed more intense staining of MID1 in the airway epithelial cells, inflammatory cells and peripheral lung of vitamin D-deficient mice compared with lung sections from vitamin D-replete mice (Figure 7B). Staining for ADM was also apparent in epithelial cells, peripheral lung and inflammatory cells of vitamin D-replete mice, which was absent in vitamin D-deficient mice (Figure 7D). ANGPTL4 staining in epithelial and inflammatory cells of vitamin D-replete lung sections was more intense compared to vitamin D-deficient mouse lung sections. Strong staining for hypoxia-inducible gene product 2 (HIG2), the protein product of HILDPA, was visible throughout the lung section of vitamin D-replete mice compared with vitamin D-deficient mice. Staining was observed in epithelial cells, inflammatory cells, and the ASM layer.

MID1 expression in human airway epithelial cells

Given the above findings that demonstrate regulation of MID1 by vitamin D in the context of HDM-driven allergic airways disease in mice, we then sought to determine if vitamin D can regulate MID1 expression in human cells. To address this, we utilized BEAS-2B cells because MID1 expression is altered by HDM treatment in these cells (18). BEAS-2B cells were grown in BEGM medium with the serum substitute, Ultrosor G, to avoid potential contamination with vitamin D from animal serum. In-cell Westerns were performed to measure MID1 protein expression in BEAS-2B cells. After normalization of background fluorescence intensity, MID1 protein expression was reduced in cells treated with vitamin D plus HDM, in comparison to cells treated with either vitamin D ($p = 0.03$) or HDM alone ($p = 0.02$) (Figure 8).

372

373 **Discussion**

374 In this study we aimed to determine whether vitamin D deficiency exacerbates the
375 inflammatory, structural and functional responses induced by exposure to the aeroallergen
376 HDM. Exposure to HDM caused significant inflammation, altered airway structure (increased
377 ASM mass) and impaired lung function (increased baseline airway resistance and tissue
378 elastance). Vitamin D deficiency had an impact on all of these parameters; 1) vitamin D
379 deficiency enhanced the HDM induced influx of lymphocytes, 2) vitamin D deficiency
380 ameliorated the HDM induced increase in ASM mass and 3) vitamin D deficiency protected
381 against the HDM induced increase in baseline airway resistance. Thus, vitamin D deficiency
382 appeared to have the capacity to both exacerbate and reduce the impact of exposure to
383 HDM. We identified nine genes that were differentially expressed between vitamin D-
384 deficient and -replete mice exposed to HDM using RNA-Seq. Amongst these genes, five
385 have known roles in inflammatory cell recruitment (MID1, ACOT1 and ADM) and airway
386 remodelling (ANGPTL4 and HILDPA) in lung disease. Two additional genes, PER2 and
387 ALAS1, were also differentially regulated. PER2 is a circadian clock gene, which has
388 recently been shown to have a role in the pathophysiology of chronic airway diseases (37),
389 while ALAS1, the rate-limiting enzyme in haem biosynthesis, is also controlled by the
390 circadian clock and PER2 (27). The remaining two genes coded for immunoglobulins. Taken
391 together, these data suggest that the effect of vitamin D deficiency on HDM induced allergic
392 airways disease is complex and that the outcome may be dependent on the relative impact of
393 vitamin D deficiency on inflammation and lung structure-function outcomes.

394

395 We found that chronic HDM exposure elicited a mixed neutrophilic and eosinophilic
396 inflammatory phenotype in mice. Severe asthmatics with combined increased neutrophils and

eosinophils in sputum have lower lung function, worse asthma control and increased symptoms compared with patients exhibiting either increased neutrophilia or eosinophilia (19). Consistent with our previous study, vitamin D deficiency increased macrophage, neutrophil and lymphocyte numbers in the BAL (11). Interestingly, vitamin D deficiency, within HDM exposed mice, only enhanced the influx of lymphocytes. However, while the effect of vitamin D deficiency alone did not translate to increased numbers of macrophages and neutrophils, the combined effect of HDM treatment and vitamin D deficiency resulted in increased inflammatory cells in the lavage. This observation is consistent with other studies showing that vitamin D deficiency has the capacity to increase allergic airway inflammation (1, 39).

Employing RNA-Seq profiling, we found three genes (MID1, ACOT1, ADM) associated with inflammation in asthma that were differentially regulated by vitamin D deficiency. Differential RNA expression of MID1 and ADM was confirmed by RT-qPCR, however ACOT1 RNA expression was not significantly different between vitamin D-deficient and -replete mice. It should be noted that the log-fold change of this gene in the RNA-Seq analysis was small to begin and this may be because whole lung tissue was used for gene expression studies. Whole lung is a highly complex and heterogeneous tissue, and it is likely that using highly purified cell populations would have resulted in higher expression levels. 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 gene expression was measured in whole lung. Moreover, RNA-Seq considers reads along the full length of the mRNA transcript, whilst RT-qPCR measures a small region of the transcript consisting approximately two hundred base pairs. MID1 was upregulated by vitamin D deficiency in HDM-treated mice. MID1 plays a key role in allergic airway inflammation and is upregulated in mouse and

human bronchial epithelium following HDM exposure (7). Collison *et al.* demonstrated that MID1 expression is regulated by TLR4- and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-dependent manner to decrease protein phosphatase 2A (PP2A) activity. A decrease in PP2A leads to increased phosphorylated NF κ B and mitogen-activated protein kinases (MAPKs), which drives Th2-mediated airway inflammation (7). After confirming that vitamin D deficiency upregulated MID1 RNA expression in an independent group of HDM-treated mice, we further demonstrated that vitamin D supplementation decreased MID1 protein expression levels in human airway epithelial cells treated with HDM. Thus, these findings show that vitamin D modulates MID1 signalling in these cells and suggest that outcomes in allergic asthma may be modified by vitamin D via MID1 signalling.

We also showed that ACOT1 and ADM were downregulated in vitamin D-deficient mice. ACOT1 is a peroxisome proliferator activated receptor-alpha (PPAR α) target gene expressed in the lung (42). Activation of PPAR α is associated with reduced inflammation in airway epithelial cells following injury caused by lipopolysaccharide (21). Since ACOT1 is upregulated by PPAR α activation, reduced ACOT1 expression in vitamin D deficiency may demonstrate a reduction in PPAR α activation, which is then associated with increased inflammation following HDM exposure. ADM is an anti-inflammatory peptide and its expression in the lung has previously been shown to be decreased during allergen-induced airway inflammation (17). Thus, in addition to MID1, downregulation of ACOT1 and ADM may lead to the increase in inflammation we observed in BAL of vitamin D-deficient mice.

Consistent with our previous findings (12), we showed that vitamin D deficiency increased ASM mass in saline-treated control mice. HDM treatment also increased ASM mass in

vitamin D-replete mice. In contrast, vitamin D-deficient mice were protected against increased ASM mass following HDM exposure, and furthermore had less ASM compared with vitamin D-replete mice. This is in contrast to previous *in vitro* studies, which demonstrate inhibition of ASM cell proliferation by 1,25-dihydroxvitamin D (8). ASM remodelling processes however involve interactions between epithelial and mesenchymal cells together with inflammatory cells (23). We obtained our findings using an *in vivo* mouse model of vitamin D deficiency, which reflects interactions within a complex system involving multiple cell types and not just ASM cells, which may explain the discrepancy between studies. There was no difference between groups for the thickness of the outer wall area, suggesting no effect of vitamin D deficiency and HDM on increasing collagen in the adventitia. Neither was there an effect of vitamin D deficiency on the number of mucus-producing cells, demonstrating that the effects of vitamin D deficiency on airway wall remodelling in our model was mainly in the ASM.

RNA-Seq also identified genes that were associated with ASM remodelling. We showed that ANGPTL4 and HILPDA were downregulated with vitamin D deficiency. ANGPTL4 expression can be induced in human ASM cells by activation of MAPK and protein kinase C pathways (36), both of which have been implicated in asthma and other chronic respiratory diseases (6, 9), and may promote airway remodelling. ANGPTL4 expression can also be induced by TGF- β via crosstalk of TGF- β and PPAR signalling pathways (28). TGF- β is associated with increased ASM (4) and we found reduced TGF- β levels in BAL from vitamin D-deficient mice compared with vitamin D-replete mice following HDM treatment. A recent study also found that HILPDA expression is upregulated in human ASM cells with IL6 trans-signalling (35). Unlike classical IL6 signalling that binds to the membrane-bound IL6 receptor to activate Jak/Stat pathways, IL6 trans-signalling is an extracellular process which

involves binding of IL6 to a soluble form of the IL6 receptor, which forms a complex that can then regulate classical IL6 signalling and can alter Jak/Stat signalling in cells that do not express the IL6 receptor (35). IL6 trans-signalling has been implicated in severe asthma (20), and upregulation of HILDPA in ASM cells suggests it has a role in airway remodelling in asthma. Downregulation of both ANGPTL4 and HILDPA in vitamin D-deficient mice provide potential mechanisms that may contribute to the reduced ASM mass in airways of these mice although we did not directly test this and further studies are required.

Baseline airway resistance appeared to reflect the structural increase in ASM mass. There was an increase in airway resistance with HDM treatment in -replete mice. Similar to the observations for ASM increase, vitamin D deficiency ameliorated the HDM induced increase in baseline airway resistance. Interestingly, despite reduced baseline airway resistance in vitamin D-deficient mice exposed to HDM, vitamin D deficiency caused AHR independent of HDM exposure. We have previously demonstrated that vitamin D deficiency is sufficient to increase ASM mass and cause AHR (12). Thus, mechanisms contributing to AHR in mice with and without HDM exposure may be different and dependent on differential gene expression following HDM treatment. There may be a role for ADM in modulating AHR, as in addition to its anti-inflammatory properties, ADM also has bronchodilating effects and reduced ADM increased allergen-induced AHR in mice (41). ADM expression has also been shown to be lower in asthmatic airway epithelium and is associated with impaired epithelial repair (17), which may also contribute to AHR.

We also identified differential regulation of PER2 and ALAS1 by vitamin D deficiency in the lungs of HDM-treated mice. Both of these genes are involved in circadian clock function. Circadian clock processes regulate diurnal variations in lung physiology and inflammation,

and circadian disruption may alter lung pathophysiology and increase exacerbations in chronic lung disease (37). ALAS1 does not have a known role in lung disease, however, ALAS1 mRNA expression in the lung has been shown to be upregulated in a rat model of pulmonary hypertension (38). Data also shows that the circadian clock and haem biosynthesis reciprocally regulate each other and loss of PER2 function deregulates ALAS1 expression (27). PER2 is a core clock protein and is strongly expressed in bronchioles of both mice and humans (14). This suggests that vitamin D deficiency may alter lung pathophysiology by altering the expression of genes involved in the circadian clock system.

This study has several limitations. Firstly, a prolonged five-week chronic HDM exposure protocol was used for development of our mouse model of allergic airways disease. We chose to use HDM rather than OVA because it is a clinically relevant allergen and up to 85% of atopic asthmatics are sensitized to HDM (33). Unlike OVA models, the advantage of using HDM is that it does not require artificial systemic sensitization protocols and can be given directly via the airway, which is the natural route of exposure. The prolonged HDM exposure had a strong effect on inflammation and significantly increased mucus-producing cells in the mouse lung. However, this may have diminished the impact of vitamin D deficiency in the model. It would be interesting to examine the effect of vitamin D deficiency on asthma-related outcomes at lower doses of HDM. Secondly, our analyses focused on a single time point after chronic exposure to HDM, and it is possible that different phenotypic changes may manifest at different time points during the life course of these animals. We were also unable to assess changes in Th2 cytokines in the BAL as they were below the limits of detection for the method used. Levels of these cytokines may not be easily detected in BAL due to the relatively high dilution with saline, compared to direct measurement of cells in airway draining lymph nodes (15). Unfortunately, these cells were not collected in this study.

Finally, as previously discussed, whole lung homogenates were used for gene expression studies and it is likely that follow-up studies on highly purified cell populations would identify additional differentially expressed genes.

In summary, we have demonstrated that vitamin D deficiency exacerbates inflammation and prevents the airway structural and functional deficits induced by HDM in a mouse model. While the mechanisms of the interaction between vitamin D deficiency and HDM exposure, in terms of their impact on disease expression, are likely to be complex we identified a number of genes and pathways that were altered by vitamin D deficiency in HDM exposed mice. In particular, the MID1 pathways, which has a role in allergic airway inflammation (29) was upregulated by vitamin D deficiency, while ACOT1 and ADM were downregulated. Similarly, expression of ANGPTL4 and HILPDA, which are thought to be involved in airway remodelling processes (particularly in the ASM) was downregulated by vitamin D deficiency. Collectively, these data suggest that the interplay between vitamin D deficiency and the processes linking allergic airway inflammation with lung structure and function are complex and may be influenced by differential regulation of genes involved in inflammatory and airway remodelling processes.

Acknowledgements

We would like to acknowledge Anya Jones, Alex Gout, Kak-Ming Ling and Kevin Looi from the Telethon Kids Institute. Anya provided advice for qRT-PCR. Alex uploaded the raw sequencing data onto an online repository. Kak-Ming Ling provided support for tissue culture and Kevin assisted with the In-Cell Western experiments.

This study was funded by the National Health and Medical Research Council of Australia (1042235). RF is a recipient of an Australian Postgraduate Award, Stan and Jean Perron Top-Up Award and Asthma Foundation Western Australia Top-Up Award. AB is supported by a BrightSpark Foundation McCusker Fellowship. ACJ is a recipient of an Australian Postgraduate Award and a Top-Up Award from the University of Western Australia. SG is a recipient of the BrightSpark Foundation Fellowship.

All authors have no relevant conflict of interests to disclose.

References

1. Agrawal T, Gupta GK, Agrawal DK. Vitamin d supplementation reduces airway hyperresponsiveness and allergic airway inflammation in a murine model. Clin Exp Allergy. 43:672-683, 2013.
2. Anders S, McCarthy DJ, Chen Y, Okoniewski M, Smyth GK, Huber W, Robinson MD. Count-based differential expression analysis of rna sequencing data using r and bioconductor. Nat Protocols. 8:1765-1786, 2013.
3. Bai A, Eidelman DH, Hogg JC, James AL, Lambert RK, Ludwig MS, Martin J, McDonald DM, Mitzner WA, Okazawa M, et al. Proposed nomenclature for quantifying subdivisions of the bronchial wall. J Appl Physiol. 77:1011-1014, 1994.
4. Bossé Y, Stankova J, Rola-Pleszczynski M. Transforming growth factor- β 1 in asthmatic airway smooth muscle enlargement: Is fibroblast growth factor-2 required? Clin Exp Allergy. 40:710-724, 2010.
5. Cheng HM, Kim S, Park G-H, Chang SE, Bang S, Won CH, Lee MW, Choi JH, Moon KC. Low vitamin d levels are associated with atopic dermatitis, but not allergic

rhinitis, asthma, or ige sensitization, in the adult korean population. *J Allergy Clin Immunol.* 133:1048-1055, 2014.

6. Chung KF. P38 mitogen-activated protein kinase pathways in asthma and copd. *Chest.* 139:1470-1479, 2011.

7. Collison A, Hatchwell L, Verrills N, Wark PAB, de Siqueira AP, Tooze M, Carpenter H, Don AS, Morris JC, Zimmermann N, Bartlett NW, Rothenberg ME, Johnston SL, Foster PS, Mattes J. The e3 ubiquitin ligase midline 1 promotes allergen and rhinovirus-induced asthma by inhibiting protein phosphatase 2a activity. *Nat Med.* 19:232-237, 2013.

8. Damera G, Fogle HW, Lim P, Goncharova EA, Zhao H, Banerjee A, Tliba O, Krymskaya VP, Panettieri RA. 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.* 158:1429-1441, 2009.

9. Dempsey EC, Cool CD, Littler CM. Lung disease and pkcs. *Pharmacol Res.* 55:545-559, 2007.

10. 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.* 6:e24289, 2011.

11. Foong RE, Bosco A, Jones AC, Gout A, Gorman S, Hart PH, Zosky GR. In utero vitamin d deficiency increases airway smooth muscle mass and impairs lung function. *Am J Respir Cell Mol Biol.* 2015.

12. 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 tgfbeta expression in the lungs of female balb/c mice. *Physiol Rep.* 2:e00276, 2014.

13. Foong RE, Zosky GR. Vitamin d deficiency and the lung: Disease initiator or disease modifier? *Nutrients.* 5:2880-2900, 2013.

- 595 14. Gibbs JE, Beesley S, Plumb J, Singh D, Farrow S, Ray DW, Loudon ASI. Circadian
596 timing in the lung; a specific role for bronchiolar epithelial cells. *Endocrinology*. 150:268-
597 276, 2009.
- 598 15. Gorman S, Tan DHW, Lambert MJM, Scott NM, Judge MA, Hart PH. Vitamin d3
599 deficiency enhances allergen-induced lymphocyte responses in a mouse model of allergic
600 airways disease. *Pediatr Allergy Immunol*. 23:83-87, 2012.
- 601 16. Gupta A, Sjoukes A, Richards D, Banya W, Hawrylowicz C, Bush A, Saglani S.
602 Relationship between serum vitamin d, disease severity and airway remodeling in children
603 with asthma. *Am J Respir Crit Care Med*. 184:1342-1349, 2011.
- 604 17. Hagner S, Welz H, Kicic A, Alrifai M, Marsh LM, Sutanto EN, Ling KM, Stick SM,
605 Müller B, Weissmann N, Renz H. Suppression of adrenomedullin contributes to vascular
606 leakage and altered epithelial repair during asthma. *Allergy*. 67:998-1006, 2012.
- 607 18. Hart PH, Gorman S, Finlay-Jones JJ. Modulation of the immune system by uv
608 radiation: More than just the effects of vitamin d? *Nat Rev Immunol*. 11:584-596, 2011.
- 609 19. Hastie AT, Moore WC, Meyers DA, Vestal PL, Li H, Peters SP, Bleecker ER.
610 Analyses of asthma severity phenotypes and inflammatory proteins in subjects stratified by
611 sputum granulocytes. *J Allergy Clin Immunol*. 125:1028-1036.e1013, 2010.
- 612 20. Hawkins GA, Robinson MB, Hastie AT, Li X, Li H, Moore WC, Howard TD, Busse
613 WW, Erzurum SC, Wenzel SE, Peters SP, Meyers DA, Bleecker ER. The il6r variation
614 asp358ala is a potential modifier of lung function in subjects with asthma. *J Allergy Clin*
615 *Immunol*. 130:510-515.e511, 2012.
- 616 21. Hecker M, Behnk A, Morty RE, Sommer N, Vadász I, Herold S, Seeger W, Mayer K.
617 Ppar- α activation reduced lps-induced inflammation in alveolar epithelial cells. *Exp Lung*
618 *Res*. 41:393-403, 2015.
- 619 22. Holgate ST. Pathogenesis of asthma. *Clin Exp Allergy*. 38:872-897, 2008.

- 620 23. Holgate ST, Holloway J, Wilson S, Bucchieri F, Puddicombe S, Davies DE.
621 Epithelial-mesenchymal communication in the pathogenesis of chronic asthma. *Proc Am*
622 *Thorac Soc.* 1:93-98, 2004.
- 623 24. Hsia CCW, Hyde DM, Ochs M, Weibel ER, on behalf of the ATS/ERS Joint Task
624 Force on the Quantitative Assessment of Lung Structure. An official research policy
625 statement of the american thoracic society/european respiratory society: Standards for
626 quantitative assessment of lung structure. *Am J Respir Crit Care Med.* 181:394-418, 2010.
- 627 25. James AL, Bai TR, Mauad T, Abramson MJ, Dolnikoff M, McKay KO, Maxwell
628 PS, Elliot JG, Green FH. Airway smooth muscle thickness in asthma is related to severity but
629 not duration of asthma. *Eur Respir J.* 34:1040-1045, 2009.
- 630 26. Janssens W, Bouillon R, Claes B, Carremans C, Lehouck A, Buyschaert I, Coolen J,
631 Mathieu C, Decramer M, Lambrechts D. Vitamin d deficiency is highly prevalent in copd and
632 correlates with variants in the vitamin d-binding gene. *Thorax.* 65:215-220, 2010.
- 633 27. Kaasik K, Chi Lee C. Reciprocal regulation of haem biosynthesis and the circadian
634 clock in mammals. *Nature.* 430:467-471, 2004.
- 635 28. Kaddatz K, Adhikary T, Finkernagel F, Meissner W, Müller-Brüsselbach S, Müller R.
636 Transcriptional profiling identifies functional interactions of *tgfb* and *ppar* β/δ signaling:
637 Synergistic induction of *angptl4* transcription. *J Biol Chem.* 285:29469-29479, 2010.
- 638 29. Kent DM, Hayward RA. Limitations of applying summary results of clinical trials to
639 individual patients: The need for risk stratification. *JAMA.* 298:1209-1212, 2007.
- 640 30. Liao Y, Smyth GK, Shi W. The subread aligner: Fast, accurate and scalable read
641 mapping by seed-and-vote. *Nucleic Acids Res.* 41:e108-e108, 2013.
- 642 31. Liao Y, Smyth GK, Shi W. Featurecounts: An efficient general purpose program for
643 assigning sequence reads to genomic features. *Bioinformatics.* 30:923-930, 2014.
- 644 32. Locksley RM. Asthma and allergic inflammation. *Cell.* 140:777-783, 2010.

645 33. Miraglia del Giudice M, Pedullà M, Piacentini GL, Capristo C, Brunese FP, Decimo
646 F, Maiello N, Capristo AF. Atopy and house dust mite sensitization as risk factors for asthma
647 in children. *Allergy*. 57:169-172, 2002.

648 34. Pojsupap S, Iliriani K, Sampaio TZAL, O'Hearn K, Kovesi T, Menon K, McNally JD.
649 Efficacy of high-dose vitamin d in pediatric asthma: A systematic review and meta-analysis. *J*
650 *Asthma*. 52:382-390, 2015.

651 35. Robinson MB, Deshpande DA, Chou J, Cui W, Smith S, Langefeld C, Hastie AT,
652 Bleecker ER, Hawkins GA. Il-6 trans-signaling increases expression of airways disease genes
653 in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol*. 309:L129-L138, 2015.

654 36. Stapleton CM, Joo JH, Kim Y-S, Liao G, Panettieri Jr RA, Jetten AM. Induction of
655 angptl4 expression in human airway smooth muscle cells by pma through activation of pkc
656 and mapk pathways. *Exp Cell Res*. 316:507-516, 2010.

657 37. Sundar IK, Yao H, Sellix MT, Rahman I. Circadian molecular clock in lung
658 pathophysiology. *Am J Physiol Lung Cell Mol Physiol*. 309:L1056-L1075, 2015.

659 38. Tatsuo I, Toru T, Hiroko S, Emiko O, Taro M, Masahito K, Mamoru T, Kiyoshi M,
660 Reiko A, Fumihiko K. Increased pulmonary heme oxygenase-1 and δ -aminolevulinate
661 synthase expression in monocrotaline-induced pulmonary hypertension. *Curr Neurovasc Res*.
662 2:133-139, 2005.

663 39. Vasiliou JE, Lui S, Walker SA, Chohan V, Xystrakis E, Bush A, Hawrylowicz CM,
664 Saglani S, Lloyd CM. Vitamin d deficiency induces th2 skewing and eosinophilia in neonatal
665 allergic airways disease. *Allergy*. 69:1380-1389, 2014.

666 40. Wang X, Spandidos A, Wang H, Seed B. Primerbank: A pcr primer database for
667 quantitative gene expression analysis, 2012 update. *Nucleic Acids Res*. 40:D1144-D1149,
668 2012.

- 669 41. Yamamoto H, Nagase T, Shindo T, Teramoto S, Aoki-Nagase T, Yamaguchi Y,
670 Hanaoka Y, Kurihara H, Ouchi Y. Adrenomedullin insufficiency increases allergen-induced
671 airway hyperresponsiveness in mice. *J Appl Physiol.* 102:2361-2368, 2007.
- 672 42. Ye L, Zhao B, Yuan K, Chu Y, Li C, Zhao C, Lian Q-Q, Ge R-S. Gene expression
673 profiling in fetal rat lung during gestational perfluorooctane sulfonate exposure. *Toxicol Lett.*
674 209:270-276, 2012.
- 675 43. Zhang L, Wang S, Che X, Li X. Vitamin d and lung cancer risk: A comprehensive
676 review and meta-analysis. *Cell Physiol Biochem.* 36:299-305, 2015.
- 677 44. Zosky GR, Berry LJ, Elliot JG, James AL, Gorman S, Hart PH. Vitamin d deficiency
678 causes deficits in lung function and alters lung structure. *Am J Respir Crit Care Med.*
679 183:1336-1343, 2011.

680

681 **Tables**

682 **Table 1.** Primer list for RT-qPCR validation

Primer	Sequence
CLCA3 forward	5'-TCATCGCCATAGACCACGAC-3'
CLCA3 reverse	5'-GCCTCGTATATTCAGGCTTTGC-3'
EAR11 forward	5'-ATAACAGAGCCTATCCCCGATG-3'
EAR11 reverse	5'-ACTGGCCGGAGTTGTGAGTA-3'
RETNLA forward	5'-CCAATCCAGCTAACTATCCCTCC-3'
RETNLA reverse	5'-ACCCAGTAGCAGTCATCCCA-3'
MUC5AC forward	5'-CAGGACTCTCTGAAATCGTACCA-3'
MUC5AC reverse	5'-GAAGGCTCGTACCACAGGG-3'
REG3G forward	5'-CCGTGCCTATGGCTCCTATTG-3'

REG3G reverse	5'-GCACAGACACAAGATGTCCTG-3'
MMP12 forward	5'-GGGCTGCTCCCATGAATGAC-3'
MMP12 reverse	5'-CCAGAGTTGAGTTGTCCAGTTG-3'
SLPI forward	5'-GGCCTTTTACCTTTCACGGTG-3'
SLPI reverse	5'-GGCTCCGATTTTGATAGCATCAT-3'
TFF2 forward	5'-CCTTGGTGTTTCCACCCACTT-3'
TFF2 reverse	5'-AGCAGCAGTTTCGACTGGC-3'
ANGPTL4 forward	5'-AGGGAAAAGATGCACCCTTCA-3'
ANGPTL4 reverse	5'-CAAGAGGTCTATCTGGCTCTGA-3'
ACOT1 forward	5'-ATACCCCCTGTGACTATCCTGA-3'
ACOT1 reverse	5'-CAAACACTCACTACCCAACTGT-3'
MID1 forward	5'-AGCCCTTTACAGGCCATCG-3'
MID1 reverse	5'-AGATTA ACTGGTCATCGGTCACA-3'
HILPDA forward	5'-TGCTGGGCATCATGTTGACC-3'
HILPDA reverse	5'-TGACCCCTCGTGATCCAGG-3'
ALAS1 forward	5'-TGTCGGTCTGGTGCAGTAATG-3'
ALAS1 reverse	5'-GGCATCATCTTAGCCAGGGT-3'
ADM forward	5'-AGTGGAATAAGTGGGCGCTAA-3'
ADM reverse	5'-CAGCGAGTCCCGTAGGGTA-3'
PER2 forward	5'-CTCCAGCGGAAACGAGAACTG-3'
PER2 reverse	5'-TTGGCAGACTGCTCACTACTG-3'
EEF2 forward	5'-CCGACTCCCTTGTGTGCAA-3'
EEF2 reverse	5'-AGTTCAGGTCGTTCTCAGAGAG-3'

683

684

Table 2 Differential cell counts showing the number of macrophages, neutrophils, lymphocytes and eosinophils collected from bronchoalveolar lavage of saline and house dust mite (HDM)-treated vitamin D-deficient (VitD-) and -replete (VitD+) mice.* indicates a significant effect of vitamin D deficiency and # indicates a significant effect of HDM treatment. Data are represented as mean (SD), n = 8-12/group.

Cell types	Treatment groups			
	SalVitD+	SalVitD-	HDMVitD+	HDMVitD-
Total cells	44980.6 (24449.1)	74845.9 (17702.7)	253909.7# (178404.8)	275466.8# (235790.6)
Macrophages	36394.9 (20872.66)	55357.6* (12964.6)	76909.7 (42020.0)	80571.4# (72764.8)
Neutrophils	729.3 (677.5)	3395.4* (3029.6)	76866.3# (85133.6)	64446.0# (81016.4)
Lymphocytes	1937.5 (3407.3)	6256.2* (10087.7)	26939.4# (25037.6)	50224.4*# (44368.1)
Eosinophils	865.9 (2554.1)	924.9 (1052.8)	52242.1# (61502.2)	56268.4# (50224.9)

Table 3 Identification of differentially regulated genes in house dust mite-treated, vitamin D-deficient versus -replete mice.

Gene	Log fold change	Log counts per million	P-value	FDR
ANGPTL4	-1.47	4.78	2.16×10^{-16}	2.43×10^{-12}
ACOT1	-1.16	3.12	1.01×10^{-8}	5.66×10^{-5}

IGKV14-100	3.99	4.17	5.05×10^{-7}	0.002
IGKV14-111	-2.67	6.03	1.89×10^{-6}	0.005
MID1	1.11	5.82	3.82×10^{-6}	0.009
HILPDA	-0.52	6.51	1.17×10^{-5}	0.022
ALAS1	0.44	8.19	1.43×10^{-5}	0.023
ADM	-1.04	4.08	2.93×10^{-5}	0.041
PER2	0.81	6.23	3.46×10^{-5}	0.043

Figure Legends

Figure 1 Baseline airway resistance (R_{aw} ; A), tissue elastance (H; B) and tissue damping (G; C) in saline and house dust mite (HDM)-treated vitamin D-deficient (VitD-) and -replete (VitD+) mice.* indicates $p < 0.05$; ** indicates $p < 0.005$. Data are represented as mean (SD), $n = 9-12/\text{group}$.

Figure 2 The percentage increase from saline of airway resistance (R_{aw} ; A), tissue elastance (H; B) and tissue damping (G; C) in response 100 mg/mL of methacholine in saline and house dust mite (HDM)-treated vitamin D-deficient (VitD-) and -replete (VitD+) mice.* indicates $p < 0.05$. Data are represented as mean (SD), $n = 7-9/\text{group}$.

Figure 3 Levels of cytokines measured in bronchoalveolar lavage fluid. Interferon (IFN)- γ (A) and tumour necrosis factor (TNF)- α were measured using a cytometric bead array kit. Transforming growth factor (TGF)- β was measured by enzyme-linked immunosorbent assay. * indicates $p < 0.05$. Data are represented as mean (SD), $n = 7-14/\text{group}$.

Figure 4 Airway smooth muscle (ASM) mass in saline and house dust mite (HDM)-treated vitamin D-deficient (VitD-) and -replete (VitD+) mice. Representative images for airway sections stained with Masson's Trichrome in saline-treated, vitamin D-replete (SalVitD+) (A), saline-treated, vitamin D-deficient (SalVitD-) (B), house dust mite (HDM)-treated, vitamin D-replete (HDMVitD+) (C) and HDM-treated, vitamin D-deficient (HDMVitD-) (D) mice (Scale 60 μ m) are shown. ASM mass corrected for the basement membrane perimeter was quantified in lung sections from saline and HDM-treated vitamin D-deficient and -replete mice. *indicates $p < 0.05$; ** indicates $p < 0.005$. Data are represented as mean (SD), $n = 7-12$ /group.

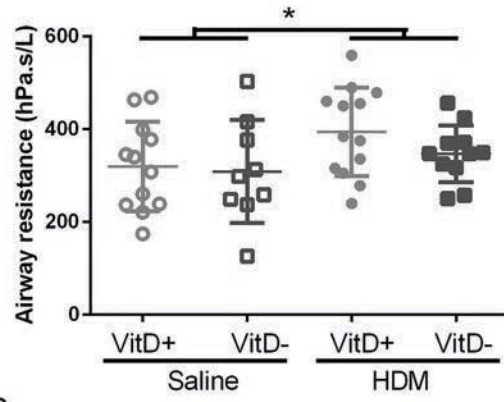
Figure 5 Representative images for airway sections stained with Alcian-blue periodic-acid Schiff in saline (A) and HDM-treated (B) vitamin D-deficient and -replete mice (scale 60 μ m) for quantification of mucus producing cells. Mucus producing cells were quantified and the percentage of mucus producing cells over the total number of epithelial cells in the airway displayed (C). **indicates $p < 0.005$. Data are represented as mean (SD), $n = 7-12$ /group.

Figure 6 Gene expression of chloride channel regulator 3 (CLCA3; A), eosinophil-associated, ribonuclease A family, member 11 (EAR11; B), resistin like alpha (RETNLA; C), mucin 5AC (MUC5AC; D), regenerating islet-derived 3 gamma (REG3G; E), matrix metalloproteinase 12 (MMP12; F), secretory leukocyte peptidase inhibitor (SLPI; G) and trefoil factor 2 (TFF2; H) in lungs from saline and HDM-treated, vitamin D-replete mice. Data are expressed as fold changes obtained by normalizing candidate genes to the eukaryotic translation elongation factor 2 (EEF2) reference gene. ** indicates $p < 0.01$; * indicates $p < 0.05$. Data are represented as mean (SD), $n = 16$ /group.

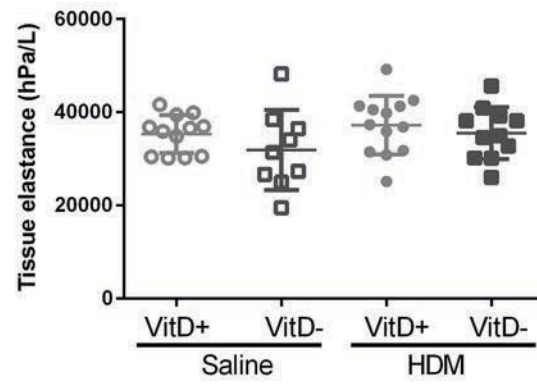
Figure 7 Gene and protein expression of midline 1 (MID1; A,B), adrenomedullin (ADM; C,D), angiopoietin-like protein 4 (ANGPTL4; E,F) and hypoxia-inducible lipid droplet-associated (HILDPA; G,H) in lungs from HDM-treated, vitamin D-deficient and -replete mice. Gene expression data are expressed as fold changes obtained by normalizing candidate genes to the eukaryotic translation elongation factor 2 (EEF2) reference gene. Representative images of lung sections immunohistochemically stained for the protein products of MID1 (B), ADM (D), ANGPTL4 (F) and HILDPA (hypoxia-inducible gene 2; H) are shown. Scale bars, 100 μ m. ** indicates $p < 0.005$. Data are represented as mean (SD), $n = 11-16$ /group.

Figure 8 Representative image of fluorescent intensity of Midline (MID1) protein expression in BEAS-2B cells detected by In-cell Westerns (A). Vitamin D (VitD), house dust mite (HDM) and HDM with vitamin D-treated cells (HDMVitD) normalized to control cells (B). VitD and HDMVitD cells were supplemented with 100 nM 1,25-dihydroxyvitamin D. After 24 hours, HDM and HDMVitD cells received 50 μ g/mL of HDM extract. Treatments were performed in triplicate. $n = 3$ independent experiments.

A



B



C

