

A mutated vitamin D receptor in hereditary vitamin D-resistant rickets prevents induction of bronchial hyperreactivity and inflammation

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Context: Previous studies have reported an association between vitamin D deficiency and asthma. Hereditary 1,25-dihydroxyvitamin D resistant rickets (HVDRR) patients provide a natural model to assess the role of the vitamin D receptor (VDR) in regulating human lung immune responses and airway hyperreactivity (AHR).

Objectives: The aim of the study was to determine the role of the VDR on lung functions, airways and systemic markers of inflammation, and allergy in HVDRR patients.

Design and Methods: Thirteen HVDRR patients (aged 6–37 years) and 17 normal controls (aged 6–38 years) underwent spirometry, a methacholine challenge test (MCT), blood tests, allergy skin tests, determination of fractional exhaled nitric oxide (FeNO), and measurement of serum and exhaled breath condensate (EBC) cytokines, including interleukin-4 (IL-4), IL-5, IL-10, IL-17 and interferon- γ levels.

Results: All HVDRR patients had negative MCT results, while six controls (35.3%) had positive MCT results ($P < .014$). Serum immunoglobulin E levels, eosinophil counts, and FeNO and allergy skin test results were similar for the HVDRR patients and controls, as were the serum cytokines concentrations. The HVDRR patients had different cytokines levels in their EBC (increased IL-4 and IL-17 and decreased IL-5, IL-10 and interferon- γ levels) compared to the controls ($P < .005$).

Conclusions: HVDRR patients show diverse exhaled cytokine profiles but seem to be protected against provoked bronchial hyperreactivity and clinical asthma. These findings suggest that an intact VDR has an important role in asthma pathophysiology.

Asthma is a chronic condition of the lower respiratory tract associated with airway hyperreactivity (AHR), airway inflammation and allergy. Accumulating epidemiological and clinical studies show that vitamin D deficiency has been associated with poor asthma control, asth-

matic exacerbations and reduced lung functions in asthmatic patients (1–5). However the mechanisms that link vitamin D and asthma are not yet clear.

Vitamin D had been traditionally associated with systemic calcium homeostasis and bone mineralization.

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Abbreviations: VDR, vitamin D receptor; VDR-KO, vitamin D receptor knockout; HVDRR, vitamin D-resistant rickets; AHR, airway hyperactivity; Th1, T helper cell type 1; NKT, natural killer T cells; iNKT, invariant NKT cells; MCT, methacholine challenge test; FeNO, fractional exhaled nitric oxide; EBC, exhaled breath condensate; SPT, skin prick test; IFN γ , interferon γ ; PC₂₀-FEV₁, provocative concentration causing a 20% drop in forced expiratory volume in the first second; FEF_{25–75}, forced expiratory flow between 25–75 % of forced vital capacity; IgE, immunoglobulin E; KO, knockout; TNF- α , tumor necrosis factor α ; hsCRP, high-sensitivity C-reactive protein; interleukin-4, IL-4

However, understanding of the physiologic functions of vitamin D has expanded over the last three decades to include many nonclassic biological actions (6). It is now recognized that the vitamin D receptor (VDR) and the key enzyme 25-hydroxyvitamin D3-1 α -hydroxylase (CYP27b1) are expressed in immune system cells (7) as well as in respiratory tract epithelial cells (8, 9), and that vitamin D is involved in regulating various components of the innate and adaptive responses (10, 11). Liu et al (12) showed that pathogen activation of monocytes toll-like receptors increased CYP27B1 and VDR expression. The increased intracellular production of 1,25(OH)₂D₃ enhanced the VDR-mediated expression of cathelicidin and defensin, which are two antibacterial proteins that provide the first line of defense against infections (13–15). As such, Vitamin D-induced production of cathelicidin and defensin in airway macrophages and epithelial cells may fend off infections known to induce asthmatic exacerbations. Vitamin D also significantly affects the adaptive immune responses, 1,25(OH)₂D₃, suppress the production of interferon- γ (IFN- γ) (16) and interleukin-17 (IL-17) (17), and increases the production of IL-4 and IL-10 in immune cells (10). Since these cytokines are involved in the immune processes leading to airway inflammation and asthma, it is reasonable to suggest that vitamin D and VDR might have an effect on clinical asthma.

Hereditary 1,25-dihydroxyvitamin D-resistant rickets (OMIM 277440; HVDRR) is a rare disease, with fewer than 100 reported patients (18–20). These patients have a mutated, nonfunctioning VDR resulting in target organ resistance to the action of 1,25(OH)₂D₃. We had previously demonstrated that macrophages and lymphocytes obtained from these patients showed impaired innate and adaptive immune functions in vitro (21, 22). As such, HVDRR patients provide a “natural” experimental model for studying the role of VDR and 1,25(OH)₂D₃ in the pathophysiology of airway inflammation hyper-responsiveness, and lung function

Materials and Methods

Subjects

Thirteen HVDRR patients (6 females and 7 males aged 6 to 37 years) and 17 normal controls (9 females and 8 males aged 6 to 38 years) were enrolled into the study (Table 1). All participants had normal serum calcium levels at the time of study entry. Patients younger than 18 years of age maintained normal serum calcium levels by calcium supplementation, while older patients required no supplementation. These patients' phenotypes and genotypes have previously been described (19). Although serum calcium levels in the patient group were near-normal, their parathyroid hormone levels varied significantly. These patients all belong to an extended pedigree of Arab ethnicity with a nonsense

Table 1. Characteristics and Baseline Laboratory Values of Patients with Hereditary 1,25-dihydroxyvitamin D Resistant Rickets (HVDRR) and Normal Controls

	HVDRR (n = 13)	Controls (n = 17)	P value
Age, y	23.2 $\pm$ 7.6	22 $\pm$ 8	0.68
Sex (female/male)	6/7	9/8	
Calcium, mg/dl (8.5–10)	8.9 $\pm$ 0.3	9.7 $\pm$ 0.3	<0.001
Phosphorous, mg/dl (3–4.5)	3.5 $\pm$ 0.6	3.7 $\pm$ 0.4	0.28
Parathyroid hormone, ng/liter (15–65)*	124 $\pm$ 105	30 $\pm$ 7.4	<0.001
25(OH)D, ng/ml (>20)	10.7 $\pm$ 4.4	16.9 $\pm$ 8.4	0.026
1,25(OH) ₂ D pg/ml (25–76)*	161 $\pm$ 76	50.6 $\pm$ 12.3	<0.001

*The numbers in the parentheses are the normal ranges.

mutation in exon 8 c.C885A. This results in a stop codon Tyr295stop that subsequently expresses a truncated receptor unable to bind 1,25(OH)₂D₃ and which is devoid of any biological function (18, 23). All the HVDRR patients maintained a normal lifestyle, had normal liver and kidney function, and were not being treated with any medication other than calcium. Both the HVDRR patients and the normal controls live in the same village. They come from the same families (genetic background, share the same standards of living and are exposed to same degree of air pollution). With respect to medical services, the only difference is that the HVDRR patients were treated with calcium. Although HVDRR patients and controls had almost normal calcium and phosphorous levels due to the huge calcium supplementation that HVDRR patients need until the end of puberty, the young HVDRR patients had slightly lower levels of calcium compared to the control group. Exclusion criteria for the control group were any known chronic lung disease and acute illness (fever or pneumonia) at the time of study entry. None of the study participants was being treated with anti-inflammatory drugs, bronchodilators or theophylline derivatives.

The Rambam Health Care Campus Helsinki Committee approved the current study. Written informed consent was obtained from participants over 18 years of age and from the parents of minors.

Clinical evaluation

Details on personal and family history of respiratory diseases, allergic symptoms, and smoking history were recorded. Each patient underwent spirometry, the methacholine challenge test (MCT), blood tests, allergy skin tests, determination of fractional exhaled nitric oxide (FeNO), and collection of exhaled breath condensate (EBC) for the investigation of cytokines. All parameters were evaluated in the morning.

Spirometry

Spirometry was performed in accordance with the American Thoracic Society/European Respiratory Society Task Force protocol using a KoKo spirometer (n-Spire Healthcare Inc., Longmont, CO, USA) (24). The results were expressed as percent predicted according to Quanjer et al (25).

Methacholine challenge test (MCT)

The MCT was performed according to the published guidelines of the American Thoracic Society (26). The solutions were administered using a pulmonary dosimeter (nSpire Healthcare, Inc. Longmont, CO, USA) according to the manufacturer's instructions. Nebulized methacholine was inhaled for two minutes with 5-minute intervals between doses until either maximal concentration or the endpoint was reached. Provocative concentration that had caused a 20% drop in forced expiratory volume (FEV) in the first second (PC20-FEV₁) was defined by the provocative concentration that reduced FEV₁ by 20% from baseline. Upon completing the MCT, 200 mcg of albuterol inhaler was given to all patients and normal subjects by a spacer device in order to restore airway caliber. The patients were defined as having a negative MCT when there was no response to a methacholine concentration of 13.95 mg/ml.

Blood tests

Blood samples were analyzed for complete blood count (CBC), and for serum immunoglobulin E (IgE), C-reactive protein (CRP), and calcium and phosphorous levels. Serum 25OHD concentrations were measured by competitive binding radio assay (DiaSorin, Inc., Stillwater, MN). Serum 1,25-(OH)₂D was measured by RIA kit (DiaSorin, Inc.). Plasma parathyroid hormone was measured using an immunoradiometric assay (IRMA) (Nichols Institute Diagnostics, San Juan Capistrano, CA). Serum IL-4, IL-17, tumor necrosis factor (TNF)- α (TNF- α) and IFN- γ concentrations were determined by an ELISA kit (PeproTech, Rocky Hill, NJ), and IL-10 was determined by an R&D Systems ELISA kit (Minneapolis, MN). All kits were used according to the manufacturer's instructions.

Fractional exhaled nitric oxide (FeNO)

FeNO was measured by a portable electrochemical analyzer NIOX MINO (Aerocrine AB, Smidesvågen, Sweden) (27) according to the American Thoracic Society's recommendations (28). Measurement was by deep inhalation to total lung capacity followed by exhalation for 10 seconds at a mouth flow rate of 50 mL/sec and a pressure of 10 cmH₂O. Due to inability to perform the test, only 10 HVDRR patients and 16 controls underwent FeNO measurements.

Exhaled breath condensate (EBC)

EBC samples were collected in RTubes (Respiratory Research, Charlottesville, VA) according to the manufacturer's instructions and the European Respiratory Society's recommendations (22) and stored at -80°C until analysis. Samples were analyzed for IL-4, IL-5, IL-10, and IL-17 (high-sensitivity ELISA kits; R&D Systems, Inc. Minneapolis, MN), as well as IFN- γ (high-sensitivity ELISA kit; eBioscience, San Diego CA). All samples were assayed in duplicate at two dilutions and at plate reader absorbance of 450 nm for all assays. Results were analyzed with a 4-parameter logistic curve fit. The intra-assay and interassay variability's were < 10%, and specificity ranged from 94% to 98%. The limit of detection of the assays was 0.11 pg/ml for IL-4, 0.06 pg/ml for IL-5, 0.09 pg/ml for IL-10, 15.0 pg/ml for IL-17, and 0.15 pg/ml for IFN- γ . Due to technical restraints, TNF- α was measured only in serum while IL-5 was measured only in EBC.

Skin prick test (SPT) for inhaled allergens

The SPT was performed according to the guidelines of the European Academy of Allergology and Clinical Immunology (29). The reactions were assessed after 15 minutes by outlining the wheal contour with a pen.

Analysis and statistics

The MCT was taken as the primary result, and all other parameters were considered as being secondary results. We used the Java application "Exact power for the Fisher Exact Test" (Hedwig.mgh.harvard.edu/sample_size/fisher/fishapp.html) to perform power calculations for our sample of 13 cases and 17 controls with a 5% type I error rate. Our hypothesis is that the HVDRR patients will show no response, and the best estimate from the literature is that the controls will have a response rate of 52% at a dose of 8 mg/ml methacholine (30), a conservative estimate of the response rate for the general population at our higher dose of 13.95 mg/ml methacholine. Using these proportions, our sample has 98% power to detect the effect. Lowering the control response rate for even more conservative power calculations, the results showed a power of 94% at a 45% response rate, and a power of 79% at a 36% response rate.

Statistical analysis was performed by the paired Student's *t* test for parametric values, the Fisher Exact test and the Mann-Whitney U test for nonparametric values for EBC, and the analysis of variance (ANOVA) was assessed by the Newman-Keuls post hoc tests, as appropriate. The results are expressed as mean $\pm$ SD, median, and range. A *P* value < 0.05 was considered statistically significant.

Results

The characteristics of the patients and their baseline blood test results are presented in Table 1. Pulmonary function tests and the SPT, eosinophil, IgE, CRP and FeNO levels were similar for the HVDRR patients and controls (Table 2). All HVDRR patients had vitamin D insufficiency or deficiency (<30 ng/ml). Six (35.3%) control subjects had low vitamin D levels (<10 ng/ml). The eosinophil counts were normal in all subjects except for one in the control group who also had positive SPT and MCT findings. The IgE levels were similar for the two groups, although two control subjects had elevated IgE levels (2820 IU/mL and 575 IU/mL with a positive MCT). The CRP level was similar for the two groups. Six of the 17 controls (35.3%) had a positive MCT (<16 mg/ml) compared to all 13 HVDRR patients who had a negative MCT (*P* < .014). FeNO levels as a marker of eosinophilia airway inflammation were similar for the two groups, but there was a trend towards higher levels in the controls, possibly due to the two subjects who had a positive MCT and high IgE and eosinophil counts.

Cytokine concentrations in serum and EBC in HVDRR patients and controls

No differences were observed in serum cytokine concentrations between the HVDRR group and the control

Table 2. Laboratory Findings in the Hereditary 1,25-dihydroxyvitamin D Resistant Rickets (HVDRR) Patients and Normal Controls

<i>P</i> Value	Controls (<i>n</i> = 17)	HVDRR (<i>n</i> = 13)	Parameter
0.22*	6/12 (50%)	3/13 (23.1%)	Positive SPT
0.95 [†]	85.3 ± 164.1{36.7}	40.8 ± 29.0{41.}	IgE, IU/ml
0.25 [†]	0.19 ± 0.16	0.11 ± 0.67	Eosinophil count 10 ³ /ml
0.23 [†]	5.5 ±	3.8 ± 3.9 {2.35}	Hs-CRP, mg/liter
0.68 [‡]	5.1{4.01} 98.6 ± 8.4	97.3 ± 7.2	Baseline FEV ₁ %
0.24 [‡]	101.8 ± 15.5	112.0 ± 25.7	Baseline FEF _{25–} 75 %
0.42 [†]	13.2 ±	6.3 ± 2.2 (<i>n</i> =	FeNO, ppb
0.0149*	14.9 (<i>n</i> = 16){5} 6 (35.3)	10){5} 0 (0)	Positive MCT, <i>n</i> (%)

Abbreviations: SPT, skin prick test; IgE, immunoglobulin E; HsCRP, high-sensitivity C-reactive protein; FEV₁ %, forced expiratory volume in the first second; FEF_{25–75 %}, forced expiratory flow between 25–75% of forced vital capacity; FeNO, fractional exhaled nitric oxide; MCT methacholine challenge test.

*Fisher's exact test

[†]Mann-Whitney *U* test; {Median}

[‡]Student's *t* test

group (Table 3). There were, however, significant differences in the EBC cytokine values: IL-4 and IL-17 concentrations were significantly higher for the HVDRR patients compared to the controls ($P < .005$), while the IL-5, IL-10 and IFN- γ concentrations were significantly lower for the HVDRR patients compared to the controls ($P < .005$) (Figure 1).

Discussion

The role of the vitamin D and VDR in airway lung function and in the development of bronchial asthma has not been

Table 3. Serum Cytokines in the Hereditary 1,25-dihydroxyvitamin D Resistant Rickets (HVDRR) Patients and Normal Controls

Parameter	HVDRR (<i>n</i> = 13)	Controls (<i>n</i> = 17)	<i>P</i> Value
IFN- γ , pg/mL	76.7 [54.1; 91.2]	86.4 [48.6;129]	0.65
TNF α , pg/ mL	443 [389; 503]	445 [383;515]	0.88
IL4, pg/mL	0.11 (0.09)	0.16 (0.10)	0.27
IL10, pg/mL	429 [286; 587]	451 [209;571]	0.88
IL17, pg/mL	20.0 [6.19; 57.5]	38.9 [23.8;124]	0.13

Abbreviations: IFN, interferon; TNF, tumor necrosis factor; IL, interleukin.

investigated in depth in humans. The association between low vitamin D levels and asthma has been based on three lines of evidence: genetic studies, epidemiological and clinical observations and immunological data. Genome-wide association studies for asthma have identified a possible linkage to chromosome 12, region q13–23, which houses the VDR gene, and VDR variants were suggested as being genetic risk factors for asthma/atopy (31). A range of clinical studies have indicated that low vitamin D levels are associated with poor asthma control, asthmatic exacerbations, and reduced lung functions, as well as with higher serum IgE concentrations, eosinophil counts and incidence of positive SPTs (10–12, 14). However, other studies showed no correlation between serum 25-hydroxyvitamin D concentration and asthma in either adolescents (32) or adults (33). These controversial findings may be due to the heterogeneity of the causes and forms of bronchial asthma, or to the fact that most clinical studies on vitamin D and asthma are cross-sectional and therefore subject to potential confounding factors. Furthermore, trials involving supplementation with vitamin D are beginning to emerge with variable results that partly reflect differences in study design. Therefore, more large-scale well-designed studies are needed to demonstrate whether vitamin D supplementation prevents or attenuates asthmatic severity or exacerbations. Accumulating data indicate that vitamin D is involved in regulating various com-

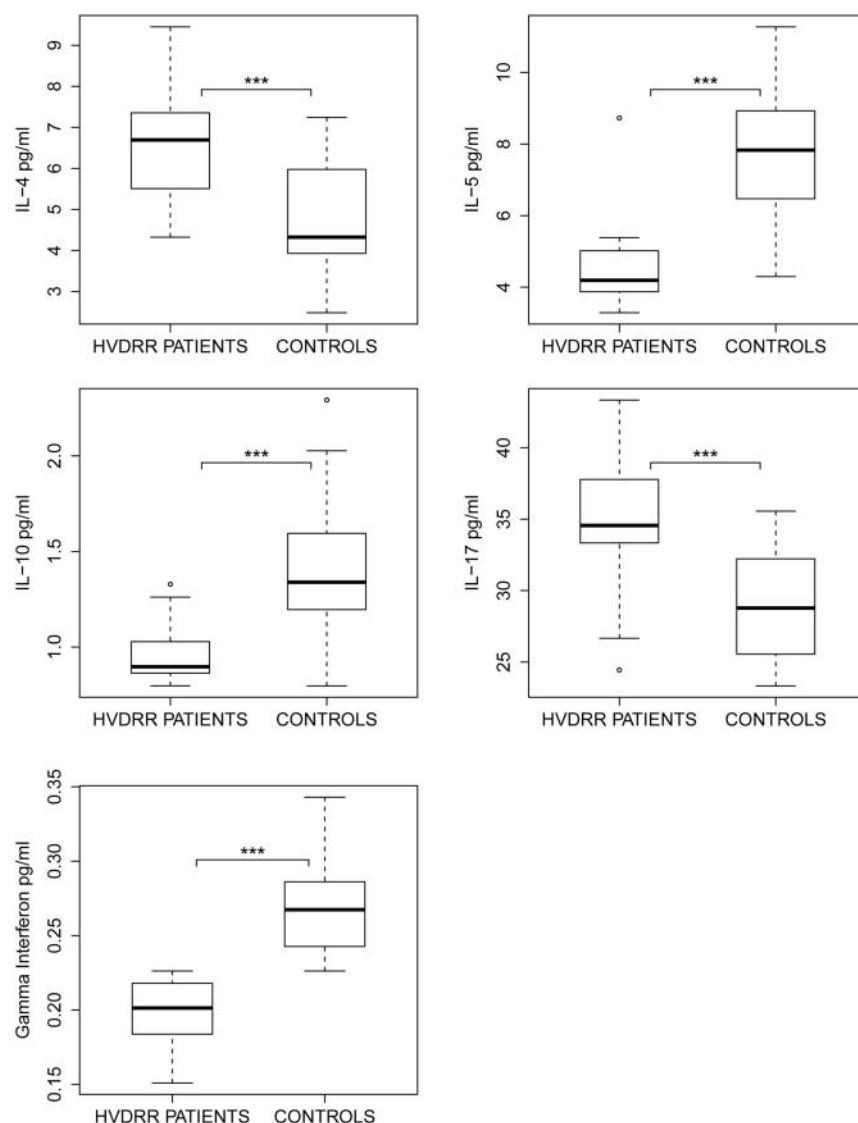


Figure 1. Cytokine concentrations in exhaled breath condensate in hereditary 1,25-dihydroxyvitamin D resistant rickets (HVDRR) patients and controls. The (•) represents the minimum and the maximum outliers *** $P < 0.005$. Abbreviation: IL, interleukin

ponents of the innate and adaptive immune system (10, 11). 1,25(OH)2D3 enhances the expression of cathelicidin and β defensin in macrophages, in gut mucosa, trophoblasts, keratinocytes and airway epithelial cells, indicating that 1,25(OH)2D3 has an important role in innate immunity in barriers sites (34). As such, 1,25(OH)2D3-induced cathelicidin in airway macrophages and airway epithelial cells may inhibit infections which are known to induce asthmatic exacerbations.

1,25(OH)2D3 enhances IL-4, IL-10 and INF- γ generation and suppresses TNF- α and IL-17. Given that IL-4, IL-5, IL-13 and IL-17 are key cytokines in the development of allergic airway inflammation (35), it is plausible that vitamin D exerts some effects on adaptive immunity and on inflammation in the lung through the regulation of the expression of these cytokines. Interestingly, however, de-

spite a distinct EBC cytokine profile, which demonstrated increased concentrations in IL-4 and IL-17 and a decrease in the concentration of the anti-inflammatory cytokine IL-10, no increases in allergy and inflammation parameters (ie, IgE, blood eosinophils, FeNO and positive SPT) were observed in our HVDRR patients. As to the MCT, when MCT was applied in the general population the positive results ranged from 25% to 52%. Henriksen et al delivered a maximal cumulative dose of 2 mg methacholine during a bronchial provocation test in 213 healthy controls (aged 13 ± 22 years) and found that 25% of the asymptomatic control subjects had a positive test (36). In another study (30) that enrolled 197 healthy controls (aged 8.6 ± 0.6 years), when the methacholine test was performed while doubling the concentration from approximately 0.03 to 8.0 mg/ml methacholine, 52% of the normal controls were positive. In the current study, we defined a negative MCT when there was no response to a methacholine concentration of 13.95 mg/ml. Therefore, the 35.3% positive MCT finding for the control subjects who live in the same neighborhood and under the same environment conditions as our HVDRR patient is in line with those previous observations.

Furthermore, while 35.3% of the normal controls showed bronchial hyperreactivity responses following MCT challenge, none of our HVDRR patients generated airway hyperreactivity responses following MCT challenge as reported in VDR-KO mice (37). Asthma-induced VDR knockout mice failed to develop airway inflammation, eosinophilia, or airway hyperreactivity despite high IgE concentrations and elevated Th2 cytokines (37). One plausible mechanism that may explain why VDR-KO and HVDRR patients do not develop airway hyperreactivity and inflammation is based on the relationship between VDR, invariant natural killer (NK) (iNKT) cells and IL-5. VDR is required for the proliferation, maturation and function of iNKT cells (38), and iNKT cells are required for the development of airway hyperreactivity response and airway inflammation (39).

In VDR-KO mice, the absence of functional VDR leads to defective iNKT cells which fail to produce cytokines, such as IL-5 and IL-13, and to generate experimental allergic asthma and airway hyper-reactive response (37). The EBC cytokine profile in our HVDRR patients showed low IL-5 concentrations. IL-5 involvement is critical in the differentiation of bone marrow precursor cells to eosinophils as well as in eosinophil survival (40). Trials using antiblocking antibodies specific for IL-5 demonstrated reduced eosinophil numbers in the lungs and inhibited allergen responses in both animal studies and in patients with severe asthma associated with eosinophilia (41, 42). The low levels of IL-5 in the EBC of our HVDRR patients suggest that normal VDR activity may be needed for IL-5 generation in airway inflammation and airway hyperreactivity.

The finding of low IFN- γ levels in the EBC of our HVDRR patients is in agreement with the findings of a defect in iNKT cells in VDR-KO mice (39). In their seminal study, Pryke et al (43) showed that TNF- α and IFN- γ enhanced the induction of 1-hydroxylase in alveolar macrophages in humans to produce local 1,25(OH) $_2$ D $_3$. Here, we show that IFN- γ concentration in the EBC of HVDRR patients is reduced in the absence of 1,25(OH) $_2$ D $_3$ activity.

Interestingly, experimentally induced vitamin D deficiency in mice failed to mirror the VDR-KO phenotype, suggesting that there might be a difference between the absence of vitamin D and the absence of a normal VDR (44). It is possible that a low vitamin D level impairs airway epithelial innate immunity and increases the propensity of lung infections and subsequent asthmatic exacerbation and severity in asthmatic patients who have a normal VDR, whereas in the presence of a mutant VDR, as found in VDR-KO mice and HVDRR patients, the bronchial immune cascade does not progress to the development of airway hyperreactivity and inflammation.

In conclusion, our findings in HVDRR patients correlate with the findings of others in VDR-KO mice and indicate that an intact VDR plays an important role in bronchial inflammation and airway hyperreactivity. HVDRR patients, like VDR-KO mice, fail to develop airway hyperreactivity. However, it is possible that low vitamin D levels in asthmatic patients with an intact VDR affect the innate and adaptive immune responses in their airways in a way that increases the number of exacerbations as well as disease severity.

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