



Free 25(OH)D concentrations are associated with atopy and lung function in children with asthma

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ABSTRACT

Background: Evidence suggests free mono-hydroxyvitamin D (25[OH]D) concentrations are more strongly linked to certain outcomes than total concentrations; however, no studies have examined the relation between free 25(OH)D and respiratory or allergic disease.

Objective: To examine associations between total and free 25(OH)D concentrations and asthma outcomes.

Methods: We quantified total and free 25(OH)D concentrations in 137 Peruvian children with asthma and 152 children without asthma and examined associations with asthma outcomes.

Results: Mean age $\pm$ SD was 13 ± 2.5 years, and 50.2% were boys. Mean total and measured free 25(OH)D concentrations were 29 ± 9.5 ng/mL and 5.0 ± 1.3 pg/mL, respectively. Lower free but not total 25(OH)D concentrations were significantly associated with atopy in all children (total, odds ratio [OR] 1.3 per 10-ng/mL decrease, 95% confidence interval [CI] 0.95–1.7, $P = .12$; vs free, OR 1.3 per 1-pg/mL decrease, 95% CI 1.0–1.6, $P = .02$) and children with asthma (total, OR 1.1 per 10-ng/mL decrease, 95% CI 0.75–1.7, $P = .57$; vs free, OR 1.6 per 1-pg/mL decrease, 95% CI 1.0–2.5, $P = .04$). Free but not total 25(OH)D levels were significantly associated with pre-bronchodilator forced expiratory volume in 1 second (total, 0.11 L, -0.12 to 0.34 , $P = .34$; vs free, 0.20 L, 0.021 – 0.39 , $P = .03$) and forced vital capacity (total, 0.13 L, -0.12 to 0.37 , $P = .31$; vs free, 0.22 L, 0.026 – 0.42 , $P = .03$) Z-scores in children with asthma.

Conclusion: Atopy, forced expiratory volume in 1 second, and forced vital capacity were more strongly linked to free than to total 25(OH)D concentrations, suggesting the free form might be more relevant in modulating allergic disease risk and pulmonary function in children with asthma.

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Introduction

One billion individuals worldwide are estimated to have vitamin D deficiency.¹ Recent evidence has linked vitamin D deficiency to several diseases, including chronic respiratory diseases such as asthma.² Vitamin D can affect asthma through several mechanisms.

It is believed to play a role in modulating the inflammatory response and has been shown to reverse corticosteroid resistance in individuals with asthma.^{2–4} Vitamin D deficiency also has been implicated in lung function impairment, airway remodeling, and atopic disease.^{3,5} However, results from studies of vitamin D and asthma have been contradictory, with several studies showing an

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inverse relation between vitamin D and asthma risk or severity and other studies showing no such effect.²

The free hormone hypothesis states that hormones free from their respective binding proteins are biologically active, whereas the bound form is inactive. From 85% to 90% of circulating 25-hydroxyvitamin D₃ (25[OH]D) and 1,25-dihydroxyvitamin D₃ is bound tightly to vitamin D binding protein (DBP), 10% to 15% is bound to albumin, and less than 1% is in its free form.⁶ The free hormone hypothesis also predicts that changes in DBP concentrations influence the bound fraction and total 25(OH)D concentrations, but not the concentration of free 25(OH)D.^{7,8} Similarly, only the free, unbound form of 25(OH)D is metabolized and undergoes clearance.⁹

Nearly all studies of vitamin D and disease have used total circulating 25(OH)D levels as an indicator of vitamin D status. Recently, however, a handful of studies have demonstrated a stronger association of free and bioavailable 25(OH)D levels with bone mineral density^{10,11} and mineral metabolism¹² compared with total 25(OH)D levels, prompting the Institute of Medicine to question the use of total 25(OH)D concentrations to define vitamin D deficiency.¹³ These studies provide evidence supporting the free hormone hypothesis and highlight the importance of elucidating the role of free vitamin D metabolites in modifying disease risk.

No studies to date have examined the association between free 25(OH)D and asthma. Furthermore, most studies of free 25(OH)D have relied on calculated estimates based on DBP concentration instead of direct measurement. To investigate the relation between free 25(OH)D concentrations and asthma outcomes, we measured total and free 25(OH)D levels in 137 children and adolescents with asthma and 152 without asthma in Lima, Peru. We present an analysis of the relation between these measurements and asthma, allergy, and pulmonary function.

Methods

Study Setting

We conducted a cross-sectional analysis nested within an unmatched case-control study of children and adolescents in Peru. Participants for this study were selected from the community of Villa El Salvador, located in the coastal capital city of Lima approximately 25 km south of the city center. Villa El Salvador is located at sea level, and altitude does not vary significantly within the community's geographic limits. The institutional review boards at the Johns Hopkins University School of Medicine (Baltimore, Maryland) and AB PRISMA (Lima, Peru) approved this study.

Study Design

This is an ancillary, subset analysis of children and adolescents enrolled in an unmatched case-control study examining the role of gene-by-environment interactions in determining asthma risk. We enrolled children and adolescents 9 to 19 years of age, and we obtained written informed consent from all parents and written assent from all participants enrolled in the study. We excluded individuals with ocular, abdominal, or thoracic surgery or a history of hospitalization for cardiac reasons in the past 3 months, had a diagnosis or were currently receiving treatment for tuberculosis, had a chronic respiratory condition other than asthma, or who were pregnant. We recruited participants using community censuses conducted less than 3 years before recruitment. We identified and visited all potential asthma cases 9 to 19 years old using birthdate and a positive response to a census question identifying children and adolescents with wheeze or asthma medication use in the past 12 months or physician diagnosis of asthma. We identified potential children and adolescents without asthma using a simple

random sample of age-eligible children in our census responding negatively to the asthma-related census questions. We confirmed asthma status at enrollment and evaluated severity according to National Asthma Education and Prevention Program Expert Panel Report 3 (NAEPP-3) guidelines.¹⁴ For this analysis, we defined asthma as any self-reported or parent-reported occurrence of wheezing in the chest or any use of asthma medications in the past year. Children and adolescents without asthma had no occurrence of self- or parent-reported respiratory symptoms consistent with asthma in the past year and no use of asthma medications in the past year. For this analysis, we analyzed data from a random subset of 137 children with asthma and 152 children without asthma living in Villa El Salvador.

Questionnaires

We administered a baseline questionnaire to all participants. This questionnaire included demographic information, socioeconomic status (SES), asthma medication use, history of allergic rhinitis and eczema, and smoking history.

Clinical Measurements

We conducted spirometry on all study participants at enrollment. We used a flow-based portable spirometer (SpiroPro, Jaeger/ERT, Hoechberg, Germany) and obtained at least 3 acceptable and reproducible spirometry maneuvers for a maximum of 8 in accordance with American Thoracic Society and European Respiratory Society guidelines.¹⁵ Spirometric evaluations were generally carried out in the morning and early afternoon hours. We used criteria derived by the Global Lung Health Initiative to obtain predicted values and Z-scores.¹⁶ We calculated body mass index (BMI) using height and weight measurements at enrollment. Asthma Control Test score was measured using a validated questionnaire and scored using age-specific criteria.¹⁷ We collected a fasting blood sample once for each participant using standard phlebotomy techniques. All samples were processed within 2 hours of extraction and stored at -80°C . We measured immunoglobulin E (IgE) antibody to mixes of 3 common allergens (animal, mold, and dust mite) using the ImmunoCAP 250 (ThermoFisher Scientific, Waltham, Massachusetts). An IgE level higher than 0.1 kUa/L indicated a positive IgE antibody response.

We analyzed total serum 25(OH)D levels in duplicate at Nemours Children's Health System (Jacksonville, Florida) using the ALPCO 25OH Vitamin D Total enzyme-linked immunoassay kit (ALPCO, Salem, New Hampshire) and analyzed free serum 25(OH)D levels in duplicate using the DAsource Free 25OH Vitamin D enzyme-linked immunoassay RUO (Louvain-la-Neuve, Belgium).

Definitions

We defined vitamin D categories as deficient (<20 ng/mL), insufficient ($20\text{--}30$ ng/mL), and sufficient (≥ 30 ng/mL).¹ Atopy was defined as the presence of IgE antibody to mixes of at least 1 of 3 common allergens (mold, dust mite, and animal).^{18,19} Asthma severity was defined according to NAEPP-3 guidelines.¹⁴ Summer season was defined as blood drawn in the months of January through April and spring was defined as October through December. No blood samples were collected from May through September.

Biostatistical Methods

Our objective was to compare different measurements of 25(OH)D levels (total and free) and determine associations with asthma outcomes. We used Pearson correlation coefficients to determine correlations among different measurements of 25(OH)D.

Table 1
Characteristics of Children With and Without Asthma

	Asthma	No asthma	P value
Sample size	137	152	
Vitamin D measurements, mean (SD)			
Total 25(OH)D (ng/mL)	29.4 (10.5)	28.2 (8.5)	.28
Free 25(OH)D (pg/mL)	5.2 (1.4)	4.8 (1.2)	.03
Calculated free 25(OH)D (pg/mL)	11.0 (6.0)	11.1 (6.6)	.90
Bioavailable 25(OH)D (ng/mL)	4.1 (2.2)	4.2 (2.4)	.90
Demographics			
Age (y) at blood draw, mean (SD)	13.3 (2.47)	13.2 (2.59)	.70
Boys, n (%)	68 (50)	76 (50)	.95
Anthropometry, mean (SD)			
Height (cm)	151 (11.5)	150 (11.2)	.38
BMI (kg/m ²)	22 (5.0)	22 (4.2)	.22
Socioeconomics, n (%)			
Maternal education ≥6 y	102 (82)	99 (69)	.02
≥6 household members	67 (54)	75 (52)	.76
Smoking			
Current smoker	0	0	
Pulmonary function, allergy, eNO			
Pre-bronchodilator FEV ₁ Z-score, mean (SD)	1.1 (1.4)	1.5 (1.1)	.004
Pre-bronchodilator FVC Z-score, mean (SD)	1.3 (1.5)	2.3 (1.2)	.86
Pre-bronchodilator FEV ₁ /FVC Z-score, mean (SD)	−0.29 (1.1)	0.40 (0.88)	<.001
Either parent with history of physician-diagnosed asthma, n (%)	28 (23)	15 (11)	.01
Atopy, n (%)	102 (77)	91 (62)	.006
Pollen allergy (self-report), n (%)	12 (7.4)	2 (1.9)	.002
Animal dander allergy (self-report), n (%)	28 (22)	8 (5.5)	<.001
Ever having allergic rhinitis (self-report), n (%)	98 (71)	45 (25)	<.001
Either parent with history of allergic rhinitis (self-report), n (%)	39 (31)	28 (20)	.03
Ever having eczema (self-report), n (%)	10 (8.0)	4 (2.8)	.05
Either parent with history of eczema (self-report), n (%)	3 (2.4)	1 (1.0)	.26
Exhaled nitric oxide (ppb), mean (SD)	28.6 (28.8)	20.2 (23.1)	.02
Season of blood draw, n (%)			
January–March (summer)	76 (55)	40 (26)	.001
April–June			
July–September			
October–December (spring)	61 (45)	112 (74)	

Abbreviations: 25(OH)D, mono-hydroxyvitamin D; BMI, body mass index; eNO, endothelial nitric oxide; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity.

We used the χ^2 test to compare proportions of characteristics of those with vs without asthma and used *t* tests to compare means. We generated a composite score using demographic and household variables for SES using principal component analysis and used random forest methods to impute missing observations (<10% of data). We used multivariable logistic regression to determine the adjusted association of total and free 25(OH)D with odds of asthma and atopy. We adjusted for potential confounders including age, sex, BMI, SES, and season of blood draw. We used multiple linear regression to assess the adjusted association of free 25(OH)D with Asthma Control Test score and pulmonary function measurements (forced expiratory volume in 1 second [FEV₁] and forced vital capacity [FVC]), assuming a significance level of .05. Analyses were conducted in R (<http://www.r-project.org>) and STATA 11 (Stata Corp, College Station, Texas).

Table 2
Unadjusted and Adjusted Associations of Total 25(OH)D and Free 25(OH)D With Asthma-related Outcomes in 281 Peruvian Children^a

	Total 25(OH)D (n = 281)				Free 25(OH)D (n = 281)			
	Single variable, OR (95% CI)	P value	Multivariable, OR (95% CI)	P value	Single variable, OR (95% CI)	P value	Multivariable, OR (95% CI)	P value
Asthma	0.86 (0.66–1.2)	.27	0.88 (0.66–1.2)	.37	0.81 (0.67–1.1)	.02	0.83 (0.67–1.03)	.10
Atopy	1.2 (0.90–1.6)	.22	1.3 (0.95–1.7)	.12	1.3 (0.96–1.7)	.10	1.3 (1.0–1.6)	.02

Abbreviations: 25(OH)D, mono-hydroxyvitamin D; CI, confidence interval; OR, odds ratio.

^aCoefficients represent odds ratios per 10-ng/mL decrease (total 25[OH]D) and 1-pg/mL decrease (free 25[OH]D).

Results

Participant Characteristics

Of 1,177 participants enrolled, we conducted measurements of free and total 25(OH)D concentrations in 289 (137 children with asthma and 152 healthy controls). Participants with free 25(OH)D measurements did not differ significantly from participants without measurements by sex (50.2% vs 53.7% boys with vs without sample, respectively, *P* = .25), atopy (68.7% vs 70.5%, *P* = .59), pre-bronchodilator FEV₁ Z-score (1.3 vs 1.4, *P* = .32), or BMI (22.1 vs 21.7 kg/m², *P* = .25). Participants with free 25(OH)D measurements did differ in age at enrollment (13.0 vs 13.5 years, *P* = .002).

We summarized characteristics of participants with and without asthma in our study (Table 1). Children with asthma on average were older, taller, and had greater lean mass. As expected, children with asthma had lower pre-bronchodilator FEV₁ Z-scores and FEV₁/FVC Z-scores and were more likely to have atopy.

Distribution of Total and Free 25(OH)D Levels

Of 289 participants, the prevalence of vitamin D deficiency was 13.8%. Mean $\pm$ SD total 25(OH)D concentration was 29 $\pm$ 9.5 ng/mL and mean measured free 25(OH)D was 5.0 $\pm$ 1.3 pg/mL. The correlation between total and free 25(OH)D concentrations was 0.65 (95% confidence interval 0.58–0.71).

Total and Free 25(OH)D and Asthma, Atopy, and Lung Function

In single-variable and multivariable logistic regression analyses, neither total nor free 25(OH)D concentrations were associated with odds of asthma after adjusting for season, age, sex, BMI, atopy, and SES score (Table 2). SES, season of blood draw, and atopy were significantly associated with odds of asthma in multivariable analyses.

In logistic regression, free but not total 25(OH)D concentrations were inversely associated with odds of atopy in all children (Table 2) and in children with asthma (Table 3) after adjusting for season, age, sex, BMI, asthma status, and SES score. Pre-bronchodilator FEV₁ Z-score was associated with free but not with total 25(OH)D concentrations in children with asthma in multivariable analyses (Table 3). Uncontrolled asthma, as defined by an Asthma Control Test score lower than 20, was not associated with either form of 25(OH)D in children with asthma (Table 3).

Discussion

In this study of Peruvian children and adolescents, we found that atopy was more strongly associated with free than with total 25(OH)D levels in all children and children with asthma. Pre-bronchodilator FEV₁ and FVC Z-scores were more strongly linked to free 25(OH)D than to total 25(OH)D in children with asthma. Furthermore, we did not identify a relation between either form of 25(OH)D and asthma.

We did not observe a significant relation of total or free 25(OH)D with odds of asthma in our study population. The lack of association of total 25(OH)D concentrations with asthma is consistent with several other studies.² However, in a previous study

Table 3

Unadjusted and Adjusted Associations of Total 25(OH)D and Free 25(OH)D With Asthma-related Outcomes Among 137 Peruvian Children With Asthma

	Total 25(OH)D				Free 25(OH)D			
	Single variable	P value	Multivariable	P value	Single variable	P value	Multivariable	P value
Atopy, OR (95% CI) ^a	1.1 (0.77–1.6)	.56	1.1 (0.75–1.7)	.57	1.3 (0.98–1.7)	.07	1.6 (1.0–2.4)	.04
Uncontrolled asthma, OR (95% CI) ^a	0.78 (0.55–1.1)	.16	0.76 (0.52–1.1)	.17	0.93 (0.71–1.2)	.59	0.92 (0.68–1.3)	.62
Pre-bronchodilator FEV ₁ Z-score, difference in liters (95% CI) ^b	0.063 (–0.16 to 0.29)	.59	0.11 (0.12–0.34)	.34	0.10 (–0.68 to 0.28)	.23	0.20 (0.021–0.39)	.03
Pre-bronchodilator FVC Z-score, difference in liters (95% CI) ^b	0.099 (–0.15 to 0.35)	.43	0.13 (–0.12 to 0.37)	.31	0.15 (–0.034 to 0.34)	.11	0.22 (0.026–0.42)	.03

Abbreviations: 25(OH)D, mono-hydroxyvitamin D; CI, confidence interval; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; OR, odds ratio.^aCoefficients represent differences or odds ratios per 10-ng/mL decrease in total 25(OH)D concentration or 1-pg/mL decrease in free 25(OH)D concentration.^bCoefficients represent differences or odds ratios per 10-ng/mL increase in total 25(OH)D concentration or 1-pg/mL increase in free 25(OH)D concentration.

conducted in an adjacent community with higher levels of deficiency, the odds of asthma increased by a factor of 1.8 for every 10-ng/mL decrease in total 25(OH)D levels.²⁰ A possible explanation for this difference is the low prevalence of vitamin D deficiency in our study population (13.8%). Because this study is cross-sectional, we did not measure 25(OH)D levels at the moment the children acquired asthma, or in utero or early life, which is hypothesized to be the etiologically relevant period of exposure.²¹ Therefore, the lack of association between asthma and 25(OH)D concentrations does not necessarily indicate that vitamin D deficiency does not play a role in the etiology of asthma or that free 25(OH)D levels are irrelevant in determining asthma risk.

We found that atopy was associated with a significant decrease in free but not total 25(OH)D levels in all children and those with asthma. This result suggests that free 25(OH)D concentrations might be more biologically relevant than total 25(OH)D with respect to allergic disease. The free hypothesis implies that in certain tissues cells acquire unbound 25(OH)D through passive diffusion.⁶ There is prior evidence that passive diffusion of 25(OH)D occurs in dendritic cells, which regulate the balance of the T-helper cell type 1 and 2 response.²² This mechanism contrasts with megalin-mediated endocytosis, whereby tightly bound DBP enhances acquisition of 25(OH)D by cells, and which occurs in some tissues including the kidney, osteoblasts, and mammary cells. Jeffery et al²² found that addition of DBP restricted the availability of 25(OH)D to dendritic cells, indicating that dendritic cells likely acquire 25(OH)D through passive diffusion of the free form, thus supporting the concept that circulating free 25(OH)D represents the driving hormone of the vitamin D system. These data provide mechanistic evidence that could help explain the stronger association of free 25(OH)D with allergy compared with total 25(OH)D.

We also found that increases in pre-bronchodilator FEV₁ and FVC Z-scores were independently significantly associated with higher free but not with total 25(OH)D levels in children with asthma, suggesting that passive diffusion of free 25(OH)D could be an important mechanism by which cells acquire 25(OH)D in lung tissue. Previous research using total 25(OH)D as an indicator of vitamin D status have shown conflicting results on the effect of vitamin D deficiency on pulmonary function.^{23,24} A study conducted by our research group in an adjacent community showed no association between total serum 25(OH)D levels and pulmonary function.²⁰ Thus, our results suggest that free 25(OH)D concentrations might be a better marker of vitamin D's biological activity in the lung than total levels, at least in children with asthma.

This study has important limitations. This study was cross-sectional and therefore cannot prove a causal relation between lower free 25(OH)D and atopy or decreased lung function. Moreover, this study was carried out in a relatively small subsample, and therefore these results might due to chance. In addition, diurnal variation in pulmonary function could have resulted in bias, although we do not believe that this bias was of large magnitude

because most measurements were carried out in the morning hours. Two additional limitations of our study were that we did not quantify sun exposure and we were unable to quantify dietary intake of vitamin D. However, in a subset of 225 children (80%), we assessed the mean daily frequency of consuming 2 vitamin D-rich food groups (oily fish and vitamin D-fortified milk) in the 2 weeks before blood draw using a food-frequency questionnaire. Sensitivity analyses showed that including these 2 food groups to regression models as confounding factors did not significantly change the direction, magnitude, or statistical significance of the primary results (data not shown).

This study is the first to report associations of free 25(OH)D concentrations with respiratory or allergic disease. Other studies have reported that free and bioavailable 25(OH)D levels are more closely linked than total 25(OH)D levels to bone mineral density¹¹ and mineral metabolism.¹² In addition, studies have found that free 25(OH)D levels do not differ between African Americans and Whites despite lower total serum 25(OH)D levels in African Americans.^{10,25–28} Although our results do not represent proof of the role of the free form of 25(OH)D in allergy and pulmonary function, our results build on prior evidence and suggest that passive diffusion of the free form might be an important mechanism by which vitamin D exerts hormonal activity and modifies disease risk. Furthermore, they suggest that concentrations of total 25(OH)D could be a spurious indicator of vitamin D status and for assessing the relation between respiratory outcomes and vitamin D deficiency. Given the growing evidence supporting the free hormone hypothesis and conflicting results from studies relating total vitamin D concentrations to disease risk, large-scale studies incorporating measurements of free 25(OH)D concentrations are needed. Furthermore, there is a need for studies examining the mechanism through which 25(OH)D is acquired by cells in the lung tissue, leukocytes, and other tissues.

In summary, we found that measured free but not total 25(OH)D concentrations were associated with atopy and lung function in children with asthma. This result suggests that free 25(OH)D concentrations might be a better indicator of vitamin D activity than total levels with respect to allergy and pulmonary function. The results of this study could have important implications for future research and clinical practice because total 25(OH)D is currently the standard biomarker in research and clinical assessment of vitamin D deficiency. There is a need for larger-scale studies of vitamin D and disease that include free 25(OH)D measurements to confirm these results in a larger sample. Furthermore, studies are needed that elucidate the mechanism of action of vitamin D metabolites in lung tissue and cells involved in the allergic response.

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