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Vitamin D levels and vitamin D receptor gene polymorphisms in asthmatic children: a case control study*

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Abbreviations list:

GINA: Global Initiative for Asthma

ICS: inhaled corticosteroids

IQR: interquartile range

LABA: long-acting beta 2 agonists

SNP: single nucleotide polymorphisms

VDR: vitamin D receptor

25OHD: 25-hydroxyvitamin D

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ABSTRACT

Background: Vitamin D deficiency and single nucleotide polymorphisms (SNP) in the gene encoding vitamin D receptor (VDR) have been associated with asthma. **Objective:** To compare 25-hydroxyvitamin D (25OHD) levels and frequency of 3 SNPs in the VDR gene between asthmatic and healthy children. **Methods:** In persistent asthmatic and healthy control children

25OHD levels were measured using radioimmunoassay and SNPs (FokI, ApaI and TaqI) were analyzed by a PCR-RFLP assay. Relevant medical history was collected. **Results:** 75 asthmatic (median age: 9.1 yrs) and 227 healthy children (10.3 yrs) were studied. In the whole population the proportion of sufficient, insufficient, and deficient levels of 25OHD were 14.9%, 44% and 41.1%, respectively. 25OHD sufficiency status was similar in asthmatic and healthy children ($p=0.57$). However, the proportion of 25 OHD sufficient levels among asthmatics according to GINA treatment steps 2, 3 and 4 was significantly different (8.6, 16.6 and 43.7% respectively, $p=0.046$). All patients on step 4 of treatment (16/16) were heterozygous for the C allele (FokI VDR SNP). There was a lower presence of the C allele among asthmatics in step 2 (30/33), step 3 (16/24) and controls (45/50), $p=0.007$, but this significance did not persisted after logistic regression. No significant differences in ApaI and TaqI were found. **Conclusions:** We found a possible association of Vitamin D sufficiency status and FokI C allele with higher requirement of therapy to reach asthma control, suggesting that it may be involved in treatment response. Variations on VDR might also play a role in 25OHD levels.

Key words: ApaI; asthma; children; FokI; GINA; SNP; TaqI; treatment; VDR; vitamin D.

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INTRODUCTION

Asthma is a high-prevalence chronic disease.¹ Habits associated with modern lifestyles in industrialized countries, such as decreased outdoor activities and unhealthy diets, may play an important role in the global increase in asthma prevalence.² Growing evidence strongly suggests an association between development or severity of asthma and insufficient vitamin D status (evaluated by serum concentrations of 25-hydroxyvitamin D, [25OHD]), which is affected by the same habits.^{3,4,5} Possible explanations are that vitamin D plays an important role in the regulation of the immune system;⁶ or its effect on lung development, function and inflammation that have been demonstrated in vitro.^{7,8}

There is also an association of vitamin D with asthma treatment response to corticosteroids. A recent study established an inverse correlation between 25OHD levels and inhaled corticosteroids (ICS) requirements and bronchial remodeling in children with severe therapy-resistant asthma.⁹ The Childhood Asthma Management Program (CAMP) study reported that in children using ICS those with vitamin D deficiency made significantly less improvement in FEV₁ than those with vitamin D insufficiency or sufficiency.¹⁰ Moreover, a synergic effect has been observed using steroid resistant patient's PBMC, when vitamin D is added to steroid in vitro.¹¹ Together, these findings suggest that vitamin D can increase the therapeutic response to corticosteroids.

Despite of the numerous studies suggesting that vitamin D plays a role in the pathogenesis, severity and treatment response of asthma, the direct cause-effect relationship has yet to be demonstrated. Even though a number of studies claim to confirm this association, there

is still conflicting evidence. A possible explanation is the genetic background influences. Most functions of vitamin D are mediated by the transcription factor vitamin D receptor (VDR), which is distributed in various tissues, including airway smooth muscle.¹² Several VDR gene single nucleotide polymorphisms (SNP) alter vitamin D function and have been associated with different pathological conditions. A direct association between VDR SNP and the effect of vitamin D on asthma have been proposed.^{13,14}

Our aim was to evaluate serum 25OHD levels and VDR gen polymorphisms in asthmatic and healthy children. We hypothesized that uncontrolled asthmatic children exhibit lower levels of 25OHD than controlled asthmatic or healthy children; and also that VDR gene polymorphisms should be related to asthma severity and treatment requirement.

METHODS

Study design and population

A case-control study was designed. Asthmatic patients (case group) between 6 and 15 years old were enrolled between October and December 2010 in the pediatric pulmonology clinic at the Pontificia Universidad Católica de Chile. Inclusion criteria were: (1) persistent asthma of any severity diagnosed by a pediatric pulmonologist according to GINA guidelines;¹ (2) regular treatment with any dose of ICS - for at least 3 months at the time of enrollment - with or without leukotriene receptor antagonist or long-acting β_2 agonists as a coadjuvant therapy. Exclusion criteria were: other chronic diseases (e.g. cardiorespiratory, metabolic or endocrine). Patients with allergic comorbidities (i.e. allergic rhinitis, eczema) were not excluded. Healthy children between 2 and 18 years of age (control group) were recruited as part of a previous study on bone

metabolism between September 2001 and June 2005 and their characteristics were reported elsewhere,¹⁵ they were not matched with the cases. No asthmatics or controls had received vitamin D supplementation in the previous three years.

The protocol was approved by the Ethics Committee of the School of Medicine of the Pontificia Universidad Católica de Chile (#10-025). Parents or guardians of all participating children signed informed consent, and participants 7 years of age and up also provided informed assent.

Background information regarding diagnosis, treatment and monitoring of asthma and atopic comorbidities was obtained from clinical records and parents. At the time of enrollment when the blood sample was taken, the patients were classified according to GINA control status and treatment steps.¹ Allergic rhinitis and atopic dermatitis were assessed with the ISAAC questionnaire.¹⁶

Nutritional status was defined according to body mass index (BMI), children were categorized as eutrophic with BMI <85th percentile, overweight with BMI $\geq$ 85th and < 95th percentile and obese with BMI $\geq$ 95th percentile.¹⁷

25OHD measurement

Plasmatic 25OHD levels were measured using standard protocol (radioimmunoassay, RIA DiaSorin®, Stillwater, MI.). All blood samples from asthmatic patients were taken during spring to reduce seasonal variability. However, only half of blood samples from healthy patients were

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taken during spring. Nevertheless in this particular group there was no significant difference in the 25OHD levels between the results for blood samples taken during the spring versus the samples taken in another season ($p=0.45$), then we assumed that it would not behave as a confounder. To define vitamin D status, levels of 25OHD > 30 ng/ml were considered as sufficient, < 20 ng/ml as deficient and between 20 and 30 ng/ml as insufficient.²

SNPs genotyping

Three different specific and well-characterized VDR SNPs were assessed: FokI (rs10735810), ApaI (rs7975232) and TaqI (rs731236).^{13,14,18} SNP were evaluated in DNA samples from all asthma patients, and from a random selection of the controls.

The DNA from control children was extracted from lymphocytes¹⁹ and was stored at -70°C until the analysis. New sets of DNA of the case group were extracted from plasma using the QIAamp DNA kit following the manufacturer's protocol (Qiagen®). For genotyping the selected polymorphism, we used PCR-restriction fragment length polymorphism analysis; following well established protocols.²⁰

Statistical analysis

To determine the sample size, we considered finding a difference of 3 units of measurement of 25OHD (ng/mL) between the medians of independent samples (asthmatic patients and healthy controls), with a confidence of 0.95, power of 0.80, standard deviation of 8 ng/mL (obtained from the healthy children sample), and a ratio of 1/3 for cases/controls. Based on these criteria, it

was estimated that 75 patients with asthma (including controlled and uncontrolled asthma) and 225 healthy controls were required.

Categorical variables were described as number and percentages. The distribution of numerical variables was evaluated using the Shapiro–Wilk test, and described as median and interquartile ranges because they were not normally distributed. Fisher’s Exact Test, Mann–Whitney, Kruskal–Wallis and Spearman’s rho correlation coefficient were used as appropriate. Statistical analyses were performed using SPSS software version 22.0 (Armonk, NY). All p-values < 0.05 were considered significant. Two multiple logistic regression models were planned: the first using GINA control status as a dependent variable and second using the GINA treatment steps. The independent variables included in both models were those with p values < 0.1 in the univariate analysis and the age.

RESULTS

Study population characteristics

A total of 302 children between 2 and 18 years of age (median: 9.6 years, IQR: 6.5) were included in this study. Of these, 75 were asthmatic (median: 9.1 years, IQR: 3.5) and 227 were healthy children (median: 10.3 years, IQR: 7.9). There were no significant differences in age and gender between the two groups. However, there was more proportion of overweight and obese in the case group, because one of the inclusion criteria for the control group was normal nutritional status (Table 1). All but one asthmatic patient had allergic co-morbidities.

Among the asthmatic children, 23 (30.7%) were controlled, 23 (30.7%) partially controlled and 29 (38.6%) uncontrolled according to GINA at the moment of enrollment. For future analysis, we combined the partially controlled and uncontrolled patients. According to the GINA treatment step categories, 35 (46.7%) patients were on step 2, 24 (32%) on step 3 and 16 (21.3%) on step 4. There was no significant difference in the distribution of patients control status among treatment steps categories (data not shown). Regarding anti-asthmatic drugs, 43 (57.3%) patients were using ICS alone (doses up to 800 mcg of budesonide or equivalent), and 32 (42.7%) were on ICS and montelukast or long-acting β 2 agonists.

25OHD levels and VDR polymorphisms

In the whole sample 45 children (14.9%) had sufficient levels of 25OHD, 133 (44%) had insufficient levels and 124 (41.1%) were deficient. 25OHD status and levels were similar between asthmatic (median: 22 ng/ml) and healthy children (median: 21.2 ng/ml) ($p=0.57$). However, among the asthmatic, we found significant differences in the proportion of children with sufficiency levels of 25OHD between controlled and uncontrolled status (4.3% and 25% respectively, $p=0.042$); and among the GINA treatment steps (8.6%, 16.6% and 43.7% for step 2, 3 and 4, respectively, $p=0.046$) (Table 2).

The VDR SNPs were analyzed in all 75 asthmatic patients and in 50 controls randomly selected. For the FokI VDR SNP, no differences were found when comparing the distribution among healthy, controlled and uncontrolled asthmatic children (Table 3). However, all asthmatic children ($n=16$) on step 4 of the GINA treatment were heterozygous for allele C of FokI VDR SNP (as shown in Figure 1). The presence of this allele was significantly less common among

asthmatics in step 2 (30/33), step 3 (16/24) or in healthy children (45/50) ($p=0.007$) (Table 3). There were no differences in the prevalence of ApaI and TaqI VDR SNPs among healthy, controlled and uncontrolled asthmatic children or according to asthma GINA treatment steps (Tables 4 and 5). There was no significant relationship between body mass index (BMI) percentiles and VDR polymorphisms (data not shown).

Two multiple logistic regression models were performed. The independent variables included in both models were: FokI SNP (present or absent), age (younger and older than median), nutritional status (BMI less and more than 85th percentile), and 25OHD level category (normal or abnormal: insufficiency plus deficiency). For the first model, using GINA control status as a dependent variable, the only variable that remained significant was BMI > 85th percentile (OR=3.8, 95% IC [1.062 to 13.369]). For the second model, using GINA treatment steps as dependent variable, the only variable that remained significant was abnormal 25OHD level category (deficient and insufficient together) (OR=0.2, 95%IC [0.061 to 0.766]).

DISCUSSION

In this study we found a similar prevalence of 25OHD insufficiency and deficiency among healthy controls and children with persistent asthma. However, there was a higher proportion of 25OHD sufficiency in asthmatic with uncontrolled status and step 4 of treatment. Regarding the VDR genetic variants, we found that all patients with asthma in treatment step 4 had the C allele of FokI in a heterozygous form.

The findings regarding 25OHD status are in disagreement with those previously published showing lower levels among asthmatic, and an inverse correlation between its levels and control.^{5,21,22} One possible explanation for this discrepancy is that our asthmatic patients were not as severe as those included in other studies. Two studies^{9,23} found an inverse relationship between 25OHD levels and ICS doses, which contradicts our finding of a higher proportion of 25OHD sufficiency among children in step 4 of treatment. However, one of those studies also found similar 25OHD levels among healthy and asthmatic children and adults.²³ Therefore, more studies with larger samples, cohorts or meta-analysis of existing data should be done to clarify these issues.

All SNPs studied here have been investigated before in asthmatic populations. In one study FokI was not correlated with asthma diagnosis, but its C allele was associated with greater baseline airways obstruction.¹⁴ In another study they found association of FokI and risk of atopic asthma.²⁴ ApaI and TaqI were associated with asthma in 2 studies,^{13,18} but no relationship with required treatment has been reported. Nevertheless a recent study showed an association of ApaI and better asthma control.²⁵ In the present study, a possible association of FokI and treatment groups was found which is novel and supports the hypothesis that VDR plays a role in asthma treatment response and can also help to explain the differences found in 25OHD status. VDR may exert a co-action with the corticosteroid receptor when activated by its ligand, vitamin D. This possibility has been suggested by *in vitro* studies,¹¹ and a recently controlled trial that compared vitamin D versus placebo as coadjuvant of budesonide in asthmatic children found fewer exacerbations in the first group.²⁶ It is recognized that different alleles of this SNP determine different protein structures of VDR, which are known to interfere with immune

response.²⁷ In this case, we speculate that FokI SNP could determine changes in VDR that influence the corticosteroid receptor response. However, more studies are required to confirm this possibility and elucidate the exact mechanism.

VDR has an important influence on calcium metabolism, which plays a role in feedback regulation of 25OHD levels, which in turn may explain our finding. VDR may also be exerting another type of feedback to try to overcome this deficient activation, so that's could be a possible explanation for this patients to have higher levels of 25OHD, not being enough though. If these hypotheses were proven true, they would suggest that the exogenous administration of vitamin D could overcome this deficient activation and help to achieve a better response to treatment in certain genetically predisposed patients. Moreover, a correlation has been reported previously between another VDR SNP, TaqI, and 25OHD levels,²⁸ but the same study found no association for FokI. A review of VDR gene polymorphisms reported that ApaI and TaqI are in disequilibrium in Caucasian populations, but not FokI.²⁹ Therefore FokI could be used as an independent variable for treating asthmatics with vitamin D. More studies should be done to confirm this finding in independent populations.

Our study has some limitations. First, although we reached the predetermined sample size (calculated to find differences between asthmatic and healthy children in 25OHD levels), our asthmatic population is too small for subgroups analysis, with limited number of severe persistent asthmatics, although controlled and uncontrolled patients had balanced distribution. This could explain why the significant difference in FokI distribution was not found in the logistic regression. Second, we did not incorporate patients according to their specific required

treatment and it would be ideal to have patients with objective steroid resistance to determine how 25OHD levels and SNPs are distributed, but this was not an aim of our study. Third, we did not objectively evaluate atopic profile or lung function; however, all but one asthmatic patient had allergic co-morbidities (rhinitis or dermatitis); and as we know the vast majority of asthmatic children have normal spirometry.³⁰ Finally, 25OHD determinations of obese/overweight control group and measurement at different seasons would be interesting to explore. All these aspects could have helped to explain more thoroughly the complex interaction between vitamin D pathway and asthma.

In summary, this study found no difference in 25OHD levels between healthy and asthmatic children. However, we did find an association with it levels and asthma control status. We also found a possible association of vitamin D sufficiency status and VDR SNP FokI C allele with requirement of therapy to reach asthma control, showing that the group with higher requirements had a greater proportion of sufficient vitamin D and the mutated allele. This suggests that VDR might have a role on asthma treatment response, and that a possible mechanism to overcome the mutated make 25OHD levels to go up as a compensatory mechanism. Further studies need to be done to clarify the relationship between VDR SNP and asthma treatment response.

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Table 1. Demographic characteristics of the population

	Healthy controls n=227 (75.2%)	Asthmatic patients n=75 (24.8%)	Total n=302 (100%)	<i>p</i> value for trend
Age (yrs), median (IQR)	10.3 (7.9)	9.1 (3.5)	9.6 (6.5)	0.338
Male, n (%)	114 (50.2)	43 (57.3)	157 (52)	0.29
Nutritional status, n (%)*				
normal	227 (100)	44 (59.5)	271 (90)	<0.0001
overweight	0	12 (16.2)	12 (4)	
Obesity	0	18 (24.3)	18 (6)	

* There were no patients with malnutrition. One missing value among asthmatic.

Table 2. 25OHD levels (ng/ml) among healthy and asthmatic children

Group	25OHD levels median (IQR)	Category of 25OHD*		
		Sufficient	Insufficient	Deficient
		n (%)	n(%)	n (%)
Healthy (n=227)	21.2 (9.4)	31 (13.7)	101 (44.5)	95 (41.8)
Asthmatic (n=75)	22 (11.8)	14 (18.7)	32 (42.7)	29 (38.6)
<i>p</i> value	0.46		0.57	
Controlled (n=23)	22.4 (6.8)	1 (4.3)	14 (60.9)	8 (34.8)
Uncontrolled (n=52)	21.9 (14.7)	13 (25)	18 (34.6)	21 (40.4)
<i>p</i> value	0.66		0.042	
Treatment step 2 (n=35)	20.4 (9.4)	3 (8.6)	16 (45.7)	16 (45.7)
Treatment step 3 (n=24)	22.5 (12.6)	4 (16.6)	10 (41.7)	10 (41.7)
Treatment step 4 (n=16)	24.5 (19.2)	7 (43.7)	6 (37.5)	3 (18.8)
<i>p</i> value	0.11		0.046	
ICS alone (n=43)	20.7 (10.9)	6 (14)	18 (41.8)	19 (44.2)
ICS+LABA/mont (n=32)	22.8 (15.1)	8 (25)	14 (43.7)	10 (31.3)
<i>p</i> value	0.57		0.37	

**p* vale for trend

ICS= inhaled corticosteroids, LABA= long-acting beta-2 agonists, mont= montelukast

Table 3. FokI SNP distribution among healthy and asthmatic children

Group	FokI alleles [#]			<i>p</i> value
	DD	DC	CC	
Healthy (n=50)	5	45	0	
Asthmatic (n=73)	11	62	0	0.584
GINA control status:				
controlled (n=23)	2	21	0	
uncontrolled (n=50)	9	41	0	0.317*
GINA treatment steps:				
2 (n=33)	3	30	0	
3 (n=24)	8	16	0	
4 (n=16)	0	16	0	0.007*

For FokI SNP result was achieved in 73 of the 75 asthmatic.

[#]D: A or T or G (according to the Nucleotide Base Codes [IUPAC])

*p value for trend

Table 4. ApaI and TaqI SNP distribution among healthy and asthmatic children

Group	ApaI alleles				TaqI alleles			
	CC	CA	AA	<i>p</i> value	T T	TC	CC	<i>p</i> value
Healthy (n=50)	10	28	12		24	24	2	
Asthmatic (n=75)	21	35	14	0.462	35	34	3	0.988
GINA control status:								
controlled (n=23)	6	11	6		11	11	1	
uncontrolled (n=52)	15	24	8	0.656*	24	23	2	0.985*
GINA treatment steps:								
2 (n=35)	11	15	5		16	16	1	
3 (n=24)	6	14	4		12	11	1	
4 (n=16)	4	6	5	0.86*	8	7	1	0.92*

For ApaI SNP result was obtained in 70 of the 75 asthmatic patients.

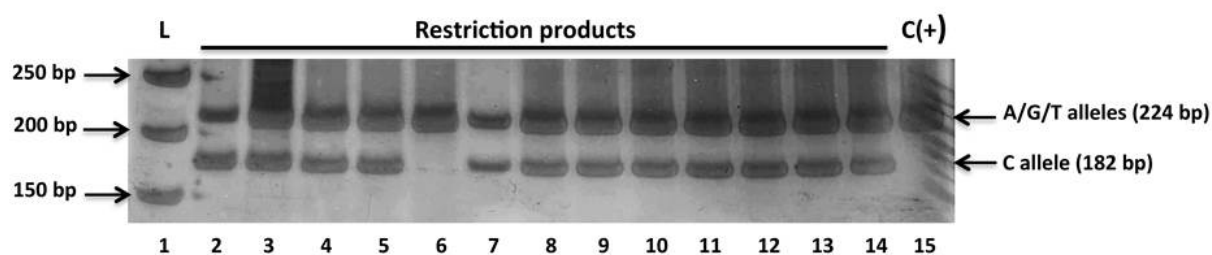
For TaqI SNP result was obtained in 72 of the 75 asthmatic patients.

**p* value for trend.

Figure Legends:

Fig 1. Genotyping of *FokI* VDR variants conducted by PCR-RFLP.

Fig 1. Genotyping of *FokI* VDR variants conducted by PCR-RFLP



Restriction pattern of *FokI* SNP variants. Lane 1: 50 bp DNA ladder (Fermentas #SM1133).
Lane 2-14 restriction products of PCR products amplified from genomic DNA of each patient.
Lane 15: positive control of PCR without *EcoI* digestion.