Evaluation of IGF-1, TNF-α, and TGF-β Gene Expression after Oral Vitamin D Supplementation in School-Aged Children with Chronic Bronchial Asthma

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Abstract

BACKGROUND: Airway remodeling in children with bronchial asthma is due to the effect of inflammatory mediators and growth factors on the bronchial epithelium. Vitamin D (VitD) has immunomodulatory effect in many inflammatory diseases as bronchial asthma. The ant-inflammatory and anti-fibrotic role of VitD could prevent or improve air way remodeling in asthmatic patients.

AIM: The study investigated the effect of VitD supplementation on the expression of transforming growth factor-beta (TGF-β), tumor necrosis factor-alpha (TNF-α), and insulin growth factor 1(IGF-1) and to correlate them with asthma severity and level of control.

METHODS: The serum level of VitD and the mRNA expression of IGF-1, TGF-β, and TNF-α were estimated in 50 patients and 20 healthy controls control subjects using quantitative PCR in real-time. Asthmatic patients with VitD deficiency received VitD supplementation for 2 months followed by remeasurement of serum VitD and the genes expression TGF-β, TNF-α, and IGF-1.

RESULT: Pre-intake of VitD and serum level of VitD were lower in all patients than control subjects (p = 0.005). VitD level was directly correlated with IGF-1 mRNA expression, which was indirectly correlated with TGF-β, r = 0.5 and −0.57; p = 0.0001 and 0.002, respectively. After VitD supplementation, the expression of the TGF-β mRNA gene was the only gene that decreased significantly (p = 0.04) together with improved asthma control and spirometric parameters.

CONCLUSIONS: VitD supplementation down regulated the gene expression of TGF-β and improved asthma control level, but it did not significantly affect the gene expression of TNF-α and IGF-1.

Introduction

Asthma is a chronic sustained inflammatory airway disease that ultimately leads to airway remodeling. Although the treatment guidelines help to improve symptoms of asthma, they did not affect airway remodeling, and treatment, now, is directed to be personalized for each patient according to individual biomarkers to prevent airway remodeling [1].

Transforming growth factor-beta (TGF-β) is involved in both the inflammatory and remodeling pathways of bronchial asthma [2]. It is considered a pro-inflammatory and pro-fibrotic mediator that are released from sensitized airways by allergens in response to the proliferation of T-helper 2 cells and the release of inflammatory cytokines and interleukins (IL-4, 5, and 13) [3]. TGF-β binds to its receptor complex that induces several pathways that end by gene induction, which finally leads to apoptosis of airway epithelial cells, and proliferation of goblet cells that aggravate asthma [4]. Furthermore, TGF-β induces fibrosis through activation of epithelial-mesenchymal transition in the airway epithelial cell [5]. TGF-β1 has a unique role in stimulating extracellular matrix accumulation. Therefore, TGF-β1 is suggested to be involved in progression of airway remodeling [6].

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine that has a role in asthma and has been suggested as a therapeutic option for patients with steroid-resistant asthma [7]. However, the response to anti-TNF-α is controversial and the mechanism of action of TNF-α is still not well understood as it may be

a direct effect on airway smooth muscles or through its effect on stimulating other cytokines and leukotrienes, in addition to its role as a chemoattractant for neutrophils and eosinophils and increasing the cytotoxicity of eosinophils on airway endothelial cells, leading to airway hyper-responsiveness [8].

Insulin growth factor 1(IGF-1) is produced by alveolar macrophages that are induced by IL-33 [9]; it increases in asthma and has a role in promoting inflammation, AHR, and inducing subepithelial fibrosis and smooth muscle hyperplasia. On the other hand, IGF-binding protein 3 inhibits the development of asthma by decreasing airway inflammation and AHR. Both IGF-1 and IGFBP3 are, now, considered therapeutic targets for asthma [10].

Vitamin D (VitD) is a dietary, immunomodulatory factor affecting the inflammatory process in asthmatic patients and although there was controversy regarding its role in improving asthma, its role in improving inflammation is well documented through decreasing many cytokines and interleukins, and also its effect on the cellular and humoral immune response [11].

The present study aims to find the change in the gene expression of TGF-β, TNF-α, and IGF-1 after 2 months of oral intake of VitD and to correlate them with asthma severity and level of control.

Methods

Study populations

First stage

A cross-sectional study was conducted on 50 children with bronchial asthma not on vitamin D for the past 6 months and 20 healthy controls from Children's Hospital, Ain Shams University after obtaining the informed written consent by the caregivers. The study protocol was approved by the Ethical Committee of the National Research Centre (NRC), Cairo, Egypt (Ethical Approval Number: 16336).

Clinical Assessment was done according to Global Initiative for Asthma (GINA) Guidelines levels of asthma control [12] and Childhood Asthma Control Test (C-ACT) questionnaire [13]. The permission was obtained to use the C-ACT questionnaire. Pulmonary Function Test was performed according to American Thoracic Society (ATS) criteria, with an electronic spirometer (Master Screen IOS; Jaeger, Ho¨chberg, Germany) [14].

Quantitative determination of serum 25(OH) D was performed by a "Vitamin D3(25-OH)" assay (Roche Diagnostics) on a Cobas e411 by electrochemiluminescence (ECLIA). The Roche Diagnostics vitamin D total assay is a competitive electrochemiluminescence protein binding assay intended for the quantitative determination of the total 25-OH vitamin D in human serum and plasma. The assay employs a vitamin D binding protein (VDBP) as capture protein, which binds to both 25-OH D3 and 25-OH D2 (Roche Diagnostics USA, n.d.).

Vitamin D deficiency was defined as 25(OH) D < 30 ng/mL and further categorized into three intensities: Mild, moderate, and severe vitamin D deficiencies; 25-OHD values of 20–30 ng/mL, 10–20 ng/mL, and <10 ng/mL, respectively [15], [16].

Measurement of the IGF-1, TGF-β, and TNF-α expression level: Total RNA from fresh venous blood samples was extracted using Qiagen QIAamp RNA Blood Mini Kit (Catalog number: 52304) according to the manufacturer's instructions, followed by assuring RNA quality and purity using Nanodropper 2000 (ThermoScientific) [17].

cDNA synthesis: Total RNA was reverse transcribed into first-strand complementary DNA (cDNA) using a High Capacity cDNA Reverse Transcription Kit (Invitrogen, Life Sciences) using Bio-Rad T100TM Thermal Cycler.

Relative quantitative real-time PCR was performed by the LightCycler480 system using Maxima SYBER Green Q PCR Master Mix, 10 ng cDNA, and 200 nM of each forward and reverse primers according to manufacturer's instructions in a final volume of 20 μl. The PCR was performed through the following instructions: Initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for the 30 s, and extension at 72°C for 20 s. The values were normalized based on the expression level of the endogenous housekeeping gene beta-actin.

Primers were designed with Roche probe library ("Roche – Universal ProbeLibrary," n.d.) 0 its sequences which were revised using primer blast (http://www.ncbi.nlm.nih.gov/BLAST/).1-VDR primer sequence: Forward primer 5′ CATGCATTTGTCTTTGTAATGTCAC -3′, reverse primer 5′- AGGAGTTCCCCGAAGAAGG -3′ and 2- beta-actin primer sequence: Forward primer 5′: TGTATGAAGGCTTTTGGTCTCC – 3′, Reverse primer 5′ CTGGTCTCAAGTCAGTGTACAGGT -3′.

The second stage

It was an intervention study and included only 25 asthmatic subjects who agreed to continue, all patients with serum vitamin D level <20 ng/ml were asked to receive oral vitamin D3 (cholecalciferol) syrup (1000IU/D) for 2 months duration [18], as an add-on therapy to their current antiasthma medications. The compliance of the patient was evaluated weekly. They were also regularly encouraged through mobile calls. The Pan African Clinical Trials Registry (PACTR.org); trial ID: PACTR201811682685226.

Table 1: General characters of the studied groups

Variables	Asthmatic (N = 50)		Control (N = 20)		P
Age (years) (mean, SD)	9	(6–11)	11.05	(5.13–13.35)	0.33^
Sex (male) (n, %)	32	64	16	80	0.31#
BMI (Kg/m) (mean, SD)	15.6	3.5	14.5	3.8	0.2^
VitD (ng/mL) (median, IQR)	9.68	(6.36–14.31)	13.94	(11.56–17.77)	0.005^^
IGF-1 mRNA (median, IQR)	1.21E-04	(3.45E-05–6.65E-04)	0.0000341	(2.31E-05–1.17E-04)	0.03^^
Fold change	10.36		1.69		
TGF-β mRNA (median, IQR)	1.42E-03	(1.21E-04–8.73E-03)	1.81E-04	(7.86E-05–3.74E-04)	0.002^^
Fold change	33.91		2.22		
TNF-α mRNA (median, IQR)	1.90E-02	(7.52E-03–2.98E-02)	4.79E-03	(2.32E-03–1.59E-02)	0.009^^
Fold change	1.9		1.4		

N: Number, SD: Standard deviation, BMI: Body mass index; VitD: Vitamin D, IGF-1: Insulin growth factor 1, TGF-β: Transforming growth factor-beta, TNF-α:Tumor necrosis factor-alpha. IQR: inter quartile range, continues data represented as mean and SD or median and IQR, categorical data represented as number and %, ^: Independent t-test, ^^: Mann Whitney test, #: Chi square test, P considered significant if<0.05.

After 2 months of VitD supplementation, the VitD serum level and the gene expression of IGF-1, TGF-β, and TNF-α were reassessed.

Statistical analysis

The accuracy of serum VitD, IGF-1, TGF-β, and TNF-α expression level was assessed with receiver operating curve analysis, assuming that the null hypothesis of AUROC was 0.5, at power 80%, CI 95%, and prevalence 23% of bronchial asthma [19], the sample size was calculated with a minimum total number of 40 using MedCalc windows (MedCalc Software bvba 13, Ostend, Belgium). The best cutoff value of serum VitD IGF-1, TGF-β, and TNF-α expression was selected with maximum sensitivity and specificity for prediction of asthma. The AUROC was also calculated, criteria to qualify for AUC were as follows: 0.90–1 = excellent, 0.80–0.90 = good, 0.70–0.80 = fair, 0.60–0.70 = poor, and 0.50–0.60 = fail. All tests were two-sided. p-value below 0.05 was considered significant. Another statistical analysis was performed using *Minitab 17.1.0.0 for windows (Minitab Inc., 2013, Pennsylvania, USA)*. All tests were two-sided. p < 0.05 was considered significant. Data normality was checked for using the Shapiro–Wilk test. Independent t-test or Mann–Whitney U-tests were used for comparison between two groups of continuous data nature, and Chi-square test for comparison between two or more groups of categorical data. One-way (ANOVA) test or Kruskal–Wallis test was used to compare between more than two groups with multiple comparisons using Tukey methods, Person correlation coefficient test was used to examine the linear relationship between different continues data, (+) sign indicate positive correlation and (−) sign indicate a negative correlation. Paired *t*-test was applied to compare the parameters before and after treatment application.

Results

Stage I

The patients and control were matched as regarding age, sex, and BMI (p = 0.33, 0.31, and 0.2), respectively. Demographic and clinical criteria of the patients are summarized in Table 1. The median level of VitD in asthmatic patients was significantly lower compared to controls (p = 0.005) (Table 1), while, the qRT-PCR test showed that expression levels of IGF-1, TGF-β, and TNF-α mRNA were significantly increased in patients than the control group (p = 0.03, 0.002, and 0.009, respectively) (Table 1).

The diagnostic accuracy of serum VitD level, TGF-β, and TNF-α expression in prediction of bronchial asthma were good; AUC = 0.72, 0.74, and 0.7, respectively, and p = 0.004, 0.001, and 0.009, respectively, at cutoff value of VD < 11.52 (ng/mL), the sensitivity and specificity were 64% and 80%, respectively, the +LR = 3.2 and – LR = 0.45, while, in TGF-β and TNF-α expression the cutoff values >2.48E-04 and 6.96E-03, respectively, the sensitivity and specificity were 70 and 65% and 78 and 70%, respectively, the +LR = 2 and 2.6, respectively, and –LR = 0.46 and 0.31, respectively. Finally, the accuracy of IGF-1 expression was near to be fairly acceptable as the AUC was 0.67 and *P* = 0.02 (Table 2 and Figure 1).

Table 2: Variable tests predicted the bronchial asthma

Parameters	VitD (ng/mL)	IGF-1	TGF-β	TNF-α
AUC	72%	67%	74%	70%
SE	0.06	0.06	0.06	0.07
95% CI	0.5957–0.8383	0.5420–0.7960	0.6237–0.8543	0.5669–0.8351
p	0.00	0.03	0.00	0.01
Cutoff	<11.52	>6.99E-05	>2.48E-04	>6.96E-03
Sensitivity	64%	66%	70%	78%
95% CI	0.4919–0.7708	0.5123–0.7879	0.5539–0.8214	0.6404–0.8847
Specificity	80%	65%	65%	70%
95% CI	0.5634–0.9427	0.4078–0.8461	0.4078–0.8461	0.4572–0.8811
LR +	3.20	1.89	2.00	2.60
LR -	0.45	0.52	0.46	0.31
PV +	41%	29%	31%	36%
PV -	91%	90%	91%	94%

VitD: vitamin D, IGF-1: Insulin growth factor 1; TGF-β: Transforming growth factor-beta; TNF-α: Tumor necrosis factor-alpha.

The correlation between IGF mRNA expression and the level of VD in patients was significantly positive (r = 0.5; p = 0.001), while TGFB mRNA showed significant negative correlation with it (r = −0.57; p = 0.002) (Figure 2).
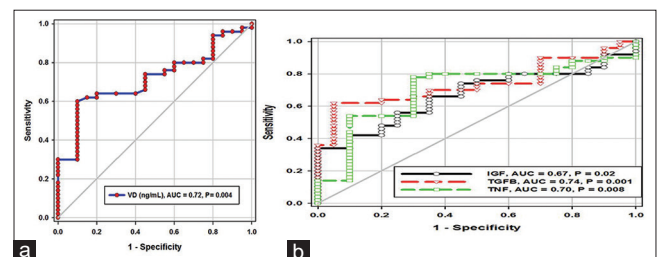

Figure 1: ROC curve of VitD, and IGF-1, TGF-β, and TNF-α mRNA gene expression. (a) VitD. (b) mRNA expression of IGF-1, TGF-β, and TNF-α
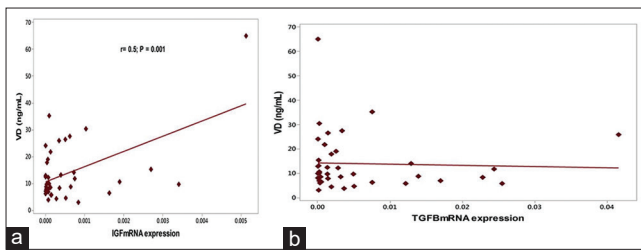
*Figure 2: Correlation between VD level in patients and mRNA expression of IGF-1 (a) and TGF-β (b). *The test of significant was person correlation coefficient, p < 0.05 considered significant, the sign before "r" denote the direction of relationship*

Stage II

FEV1, FVC, and ACT showed significant statistical elevation after supplementation of VitD (p = 0.004, 0.003, and 0.02). Furthermore, the number of cases with poor control asthma decreased and the good controller increased significantly (p = 0.02) (Table 3).

Table 3: Clinical evaluation after VD supplementation

Variables	Before (n = 25)		After (n = 25)		p
FEV1 (%) (mean, SD)	92.1	5	99.5	10	0.004^\$
FVC (%) (mean, SD)	98.05	4	110	9	0.003
ACT (median, IQR)	15.5	(10–19.25)	19.5	(15–21.5)	0.02
GINA (n, %)					
Well controlled	8	34.78	15	65.22	0.002
Not well controlled	10	70.83	7	29.17	
Poorly controlled	7	85	3	15	

N: Number, SD: Stranded deviation, FEV1: Forced expiratory volume in the first second, FVC: Forced vital capacity, ACT: Asthma Control Test. IQR: inter quartile range, continues data represented as mean and SD or median and IQR, categorical data represented as number and %, ^\$: Paired *t*-test, ^^: Mann Whitney test, #: Chi square test, P considered significant if<0.05.

VitD level was significantly elevated after the 2 months of supplementation, p < 0.001, while the expression of TGF-β mRNA gene was the only gene that showed a significant decrease after VitD treatment, p = 0.04 (Table 4).

Table 4: VitD and mRNA expression changes after treatment supplementation

Variables	Before (n = 25)		After (n = 25)		p
VitD (ng/mL) (median, IQR)	9.68	(6.36–14.31)	24.65	(18.25–52.29)	<0.001
IGF-1 mRNA (mean, SD)	4.05E-04	7.87E-04	5.51E-04	1.85E-03	0.3
Fold change	3.95		5.6		
TGF-β mRNA (mean, SD)	7.94E-03	1.43E-02	2.51E-03	8.72E-03	0.04
Fold change	23.13		10.8		
TNF-α mRNA (mean, SD)	1.89E-02	1.74E-02	2.00E-02	2.10E-02	0.7
Fold change					

VitD: Vitamin D, IGF-1: Insulin growth factor 1; TGF-β: Transforming growth factor-beta; TNF-α: Tumor necrosis factor-alpha.

Discussion

The role of serum level of VitD and its implication on numerous gene expression biomarkers in asthma was discussed controversially [20], [21], and [22]. This was corroborated by Alyasin *et al.*, and Whiting *et al.*, who found that serum VitD levels were proven to be inversely correlated with asthma severity and that VitD levels and pulmonary function test (PFT) outcome had a direct and significant association [23], [24]. This is in line with a recent research of our group that showed a significant reduction of asthma symptoms and increase spirometry parameters when VitD was added to the asthma treatment [25]. However, these findings were limited by investigating solely vitamin D receptor expression.

Before administration of VitD, TGF-β, IGF-1, and TNF-α *gene expression* were higher in asthmatic patients compared to the control subjects. The studies of various international groups, reported (TNF-alpha) genes, are already identified and the known locus of it might influence the inflammatory activity. Consequently, latest studies reported that TNF-alpha is one of the genes that play a leading role in the disease development [26]

The study by Chen and Xu (2018) was against our observation, which found that TNF-α was higher, while TGF-β was lower in asthmatics than control [27], and surprisingly, a recent study done by Han *et al.* (2021) showed that IGF-1 has a beneficial role in asthmatic adults and is associated with better pulmonary functions [28]. However, the study of Mu *et al.* (2019) proved that IGF-1 is elevated in the lungs and BAL of asthmatic mice and it inhibits phagocytosis and development of apoptotic cells after lung injury, and after using blocking antibodies to IGF-1, phagocytosis, and apoptosis improved that ultimately lead to a decrease inflammatory cells infiltration and improvement of asthma [9].

Regarding TGF-β, Manuyakorn *et al.* found significantly higher serum TGF-β1 in pediatric patients with atopic asthma compared to controls, but they did not find a correlation between it and asthma duration, treatment, or pulmonary functions [29]. Moreover, Redington *et al.* (1997) found a significant increase of TGF-β1 in bronchoalveolar lavage (BAL) of asthmatic patients that increased more on allergen exposure, suggesting the role of TGF-β1 on airway remodeling [30]. Hereby, the expression of TGF-b1 is likely to be inducible and transient in nature, and that up-regulation from baseline level requires an antigen challenge [31].

Furthermore, Joseph *et al.* and Ozyilmaz *et al.* reported that TGF-β1 was significantly higher in asthmatic cases versus the control group [32], [33]. Furthermore, Ivanovna *et al.* showed a highly significant increase in the serum TGF-β1 in both asthmatic groups with mild and severe grades versus control group [34]. Interestingly, El-Sayed *et al.* reported a significant increase in the serum TGF-β1 level in the mild bronchial asthma group and a significant decrease in the serum TGF-β1 level in severe asthma [35].

In our study, TGF-β gene expression correlated negatively with vitD levels and its level of gene expression was decreased after VitD levels increased due to the identified suppression of TGF-β1 gene expression. This agrees with the study of Isik *et al.* who suggested the role of TGF-β1 in inducing fibrosis in patients with VitD deficiency [36]. This suggested that the level of TGF- β 1 expression might correlate with severity and exacerbation.

Many studies such as Al-Alawi *et al.*, [3] Makinde *et al.*, [4], and Howell *et al.* [37] suggested the potential therapeutic role of decreasing TGF-β in preventing remolding in asthmatic patients and that it can be used as individualized therapy by biological therapy as steroids did not decrease its level in the study done by Chakir *et al.* [38]. Thus, sensitive monitoring of TGF-β gene expression might asses asthma severity.

Concerning the reported positive correlation between the increased level of IGF-1 and VitD, Bereket *et al.* [39] and Soliman *et al.* [40] confirmed the same conclusion. This was explained by the well-established experimental data that IGF-1 stimulates the synthesis of 1, 25(OH) 2D in the kidney, causing the increase of the blood levels of both VitD and IGF-1 reciprocally, but the mechanism that interplay between VitD and IGF-1 is complicated [41].

On the other hand, we did not find a significant change in the gene expression of TNF-α or that of IGF-1 after VitD supplementation. Furthermore, Sinha-Hikim *et al.* (2015) in their study on inflammatory markers in pre-diabetic patients did not find a significant effect of VitD on TNF-α or IGF-1 levels [42]. Other studies on the effect of Vit D on IGF-1 showed different results. Some go with our results as Trummer *et al.* in their study on patients with arterial hypertension [43], and Kamycheva *et al.* a study on obese patients [44]. Furthermore, the meta-analysis done by Kord-Varkaneha *et al.* (2020) showed no significant increase in IGF-1 after VitD supplementation in eight different studies [45]. In contrast, Hyppönen *et al.*, in their study on patients with metabolic syndrome, found that IGF-1 increased after VitD supplementation to 75-85 nmol/l but not more [46].

Bogazzi *et al.* reported positive correlation between VitD and IGF-1 especially in patients with severe VitD deficiency (<20 ng/ml) [47]. In addition, Ameri *et al.* concluded that VitD increased the levels of IGF-1 in adults with growth hormone deficiency after 12 weeks supplementation of 7000 IU/week vitamin D3 [48].

Regarding TNF-α, Chandler *et al.* did not find a significant correlation between VitD levels and soluble tumor necrosis factor-alpha receptor type 2 (sTNF-R2) in African-Americans [49]. Furthermore, the results of Dadaei *et al.* (2015) showed that although the level of TNF-α decreased in Iranian patients with inflammatory bowel diseases after VitD supplementation, the decrease was not statistically significant [50]. However, Haddad *et al.* (2018) showed that VitD caused downregulation in the gene expression of TNF-α in diabetic hemodialysis patients [51]. Interestingly, Kuo *et al.* (2010) found that Vit D3 decreases TNF-α and T- helper 1 related chemokines in asthmatic patients at lower doses, but higher doses and excessive use of VitD supplementations enhance T-helper 2 related chemokines and worsen asthma, autoimmune diseases, and other T-helper 2 related allergic [52].

Conclusions

VitD intake is of beneficial value in asthma control. The mRNA gene expression of TGF-β, TNF-α, and IGF-1could be potential candidate genes in the pathogenesis and assessment level of bronchial asthma control.

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