To whom it may concern

Date : 15/06/2020

Subject : Vitamin D Regulates the Expression of Glucocorticoid Receptors in Blood of Severe Asthmatic Patients

I am Dr.Bassam Mahboub ,Principle investigator for the study (Vitamin D Regulates the Expression of Glucocorticoid Receptors in Blood of Severe Asthmatic Patients) . I am confirming that the document is relevant to the study in the record.

Title :Vitamin D Regulates the Expression of Glucocorticoid Receptors in Blood of Severe Asthmatic Patients

Introduction

Vitamin D (VitD) deficiency is a public health concern affecting up to 80% of the population in certain countries and of all age groups [1]. Hypovitaminosis D is prevalent in the Middle East and North Africa (MENA) [2], and VitD level is suboptimal in the residents of sunny United Arab Emirates (UAE) [3]. In addition to its well-established role in calcium and bone metabolism, the potential roles of VitD in cancer development, cardiovascular and chronic lung diseases are being increasingly appreciated [4]. The increased awareness of VitD deficiency and related health problems made vitamin D supplementation a routine action plan even without laboratory confirmations leading to a higher risk of exogenous hypervitaminosis D [5]. Black et al. were among the first to show a positive correlation between VitD status and pulmonary function [6]. VitD deficiency is associated with features of asthma, such as decreased lung function, increased airway remodeling, and airway hyperresponsiveness [7]. Several reports suggested the association of VitD levels with the severity of asthma [8]. Different cross-sectional studies indicated that low serum VitD in patients with mild to moderate asthma is associated with poor asthma control, more exacerbations, reduced lung function, and increased medication use [9-11]. Inverse relationships have also been reported between serum VitD levels and airway remodeling, IgE, eosinophil numbers, as well as airway hyperresponsiveness. Interestingly, Gupta et al. demonstrated a role for active VitD in correcting IL-10 levels in a paediatric population of moderate and severe therapy-resistant asthma [12]. On the other hand, experimental studies demonstrated that vitamin D receptor (VDR)-deficient mice fail to develop airway inflammation (decreased infiltration of lymphocytes and eosinophils) and experimental allergic asthma [13].

Steroid resistance, as documented in cases of severe asthma, is associated with increased expression of the glucocorticoid receptor (GR)-β, which is an alternative splicing isoform and dominant-negative regulator of the GR-α [14]. The abundance of GR-α mRNA is higher than GR-β mRNA in all normal tissues and cells. Normally, default splicing pathway leads to GR-α, while the alternative

splicing pathway that leads to GR-β is minimally activated [15]. The latter pathway, however, seems to be upregulated in severe asthmatic patients [16]. In chronic inflammatory diseases like asthma, upregulated blood levels of inflammatory cytokines can participate in glucocorticoid resistance and impaired GR function by modulating its translocation, DNA binding, and GR phosphorylation status [18].

As data from observational studies suggested a better control of asthma following restoration of VitD levels.

Hypothesis:

Correcting VitD levels in adult asthmatic patients could enhance steroid responsiveness by regulating blood GR-α/GR-β ratio levels.

Primary Out comes:

1.To find out the top pathways regulate Vitamin D

identify VitD targeted enriched pathways, we analyzed Comparative Toxicogenomics .Enriched Ontology Clustering for the identified genes was performed using the Metascape (a web-based tool used for comprehensive gene list annotation and analysis resource)Database from thousands of experiments.[Time Frame: 8 weeks]

2.To examine asthma severity in relation to steroid receptors genes [Time Frame: 8 weeks]

3.study the effect of vitamin D on GR-α receptor [Time Frame: 8 weeks]

4. tudy the effects of Vitamin D on cytokines. [Time Frame: 8 weeks]

Inclusion Criteria:

- Diagnosis of asthma
- age group between 18 and 65 years
- low level of 25-hydroxyvitamin D3 (25 D3) level less than 20ng/mL

Exclusion Criteria:

- smoker patients
- less than 18 years old
- patient on Vitamin D supplementation already

Materials and Methods

In silico identification of top genes regulated by vitamin D supplementation

Using Comparative Toxicogenomics Database to identify the top genes influenced by vitamin D supplementation in thousands of experiments. One hundred five genes with at least five documented interactions with VitD were identified. Moroever, to explore if the identified genes are sharing common pathways, Enriched Ontology Clustering for the identified genes will performed using the Metascape (a web-based tool used for comprehensive gene list annotation and analysis resource) .

In silico determination of effect of vitamin D treatment on the expression of NR3C1 and NR3C2 in monocytes, macrophages, and dendritic cells

In order to investigate the effect of VitD supplementation on the mRNA expression of NR3C1, NR3C2 and VDR, publicly available datasets (GSE52819, GSE13762, GSE56490) we will explored as they were designed to examine the global transcriptional effect of VitD supplementation on monocytes, macrophages, and dendritic cells.

In silico detemination of the mRNA expression levels, and correlations, of NR3C1, NR3C2, and VDR in the blood of asthmatic patients compared to healthy controls

We will extract from publicly available datasets (GSE69683) with a large number of participants and their detailed clinical information. To find Gene expression profiling of blood from these groups was analyzed, and the gene expression of GR (NR3C1 and NR3C2) which determined.

Patients

To do double-blinded, randomized, placebo-controlled study of VitD supplementation on 45 asthmatics with VitD deficiency will performed. Moderate to severe asthmatics between 18 and 65 years of age who had clinician-diagnosed asthma with 25-hydroxyvitamin D3 (25 D3) level less than 20ng/mL at the screening visit will be recruited at the Rashid Hospital, Dubai, U.A.E., and the Zayed Military Hospital, Abu Dhabi, U.A.E.

mRNA expression and cytokine expression

Whole blood will collected in PAXgene tubes (Qiagen, Germany) for the isolation of cellular RNA. Blood specimens will assessed for mRNA expression of Glucocorticoid receptor (GR-β and

GR-α) using qRT-PCR. Bioplex multiplex immunoassay (BioRad, CA,USA) will use to assess the protein expression of IL-17A, IL-17-F, IFN-γ, IL-4, IL-5 and TNF-α in serum obtained from patients before and after treatment.

Statistical Analysis

Standard statistical t-tests and one-way ANOVA will be performed to test for statistical significance between data groups using GraphPad Prism 8 (GraphPad, San Diego, CA, USA). $p < 0.05$ was considered significant.

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Results :

Top Vitamin D regulated pathways are involved in response to Steroids

To identify VitD targeted enriched pathways, we analyzed Comparative Toxicogenomics Database from thousands of experiments. Interestingly, the top enriched pathways regulated by VitD were involved in several metabolic and inflammatory processes including response to steroids, lipopolysaccharide as well as Interleukin-4, Interleukin-13 and AGE-RAGE signalling (Suppl. Figure 1). Many of those VitD targeted pathways were also targets of dexamethasone. These include pathways involved in inflammation; proliferation; pro-apoptosis, beside others (Figure S1).

VDR, NR3C1 and NR3C2 expression levels in different types of immune cells

The level of expression of GR coding gene (NR3C1), mineralosteroid receptor coding gene (NR3C2) [17], and VDR were then determined in different immune cells. NR3C1 had a significantly higher level of expression compared to VDR and NR3C2 in different types of immune cells (Figure 1). Among all cells, its expression was significantly higher on Th2 CD4 cells (215.9 TPM). Classical (94.5 TPM) and non-classical monocytes (46.7 TPM) had the highest VDR expression among all inflammatory cells. Moroever, the expression of all three genes was higher in non-classical monocytes compared to classical ones (2.0, 1.49, and 1.21 folds, respectively). Naïve CD8 T (74.1 TPM) cells and Th1/17 CD4 cells on the other hand (64.7 TPM) had the highest NR3C2 mRNA expression among all immune cells. On the other hand, activation of T cells seems to induce the expression of VDR and NR3C1, but not NR3C2 genes. Activated CD8 T cells express higher VDR and NR3C1 (10.6 and 1.47 respectively) and lower NR3C2 (0.12-fold), compared to naïve cells. Similarly, activated CD4 T cells express higher VDR, and NR3C1 (3.95 and 1.25 respectively) and lower NR3C2 (0.11-fold), compared to naïve CD4 T cells (Figure 1).

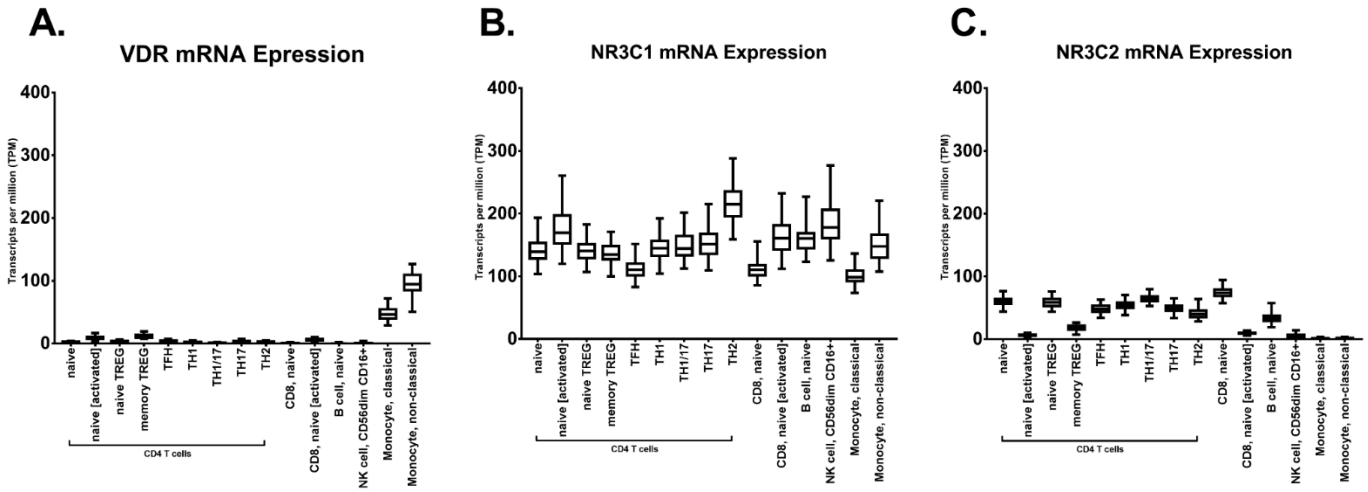

Figure 1: NR3C1, NR3C2 and VDR mRNA expression level in immune cells. Data was extracted using publicly available "Database of Immune Cell Expression, Expression quantitative trait loci (eQTLs) and Epigenomics".

VDR and steroid receptors genes are downregulated in asthma relative to severity

Activated T cells was shown to upregulate VDR, while treatment with VitD suppressed the proliferation of these cells, as well as their ability to produce IFN-γ and IL-17 cytokines. [18]. The level of expression of VDR in the blood of asthmatic and its association with the expression of steroid receptors relative to disease severity was not determined. To investigate that, we analyzed the expression profile of publicly available datasets (GSE69683) which has a large number of participants and detailed clinical information. A total number of 498 participants, of which 87 healthy controls, 77 moderate asthma, and 246 non-smokers severe asthma were included in the U-BIOPRED study. The differential expression of NR3C1, NR3C2, and VDR genes in the blood of patients from all groups was then determined. Patients with moderate asthma had higher levels of NR3C1 mRNA expression compared to health controls, although not to a significant level (log2 intensity 10.17±0.29; p=0.07) (Figure 2A). When compared to moderate asthmatics, the expression of this gene, however, was significantly downregulated in severe asthmatic (log2 intensity 10.14±0.23 versus 10.26±0.21; p<0.01)). NR3C2 mRNA expression was significantly downregulated in severe asthmatic compared to moderate asthma (log2 intensity 5.397±0.727 versus 5.62±0.5011, p<0.01), and to healthy controls (log2 intensity 5.575±0.5894 versus 5.397±0.727, p=0.03) (Figure 2B). Moderate asthmatics showed higher NR3C2 mRNA expression than healthy controls, although the difference was not significant. On the other hand, VDR mRNA expression was significantly upregulated in severe asthmatics compared to healthy controls (log2 intensity 8.386±0.46 versus 8.206±0.44 p<0.01), but not to moderate asthmatics (Figure 2C). Interestingly, NR3C1 mRNA expression correlated positively (Spearman r =0.11, 95% confident interval=0.02007 to 0.1988 and p = 0.01) (Figure 2D), while NR3C2 expression correlated

negatively (Spearman r =-0.55, 95% confident interval=-0.5724 to -0.438 and p <0.0001) with the levels of VDR expression in blood (Figure 2E).

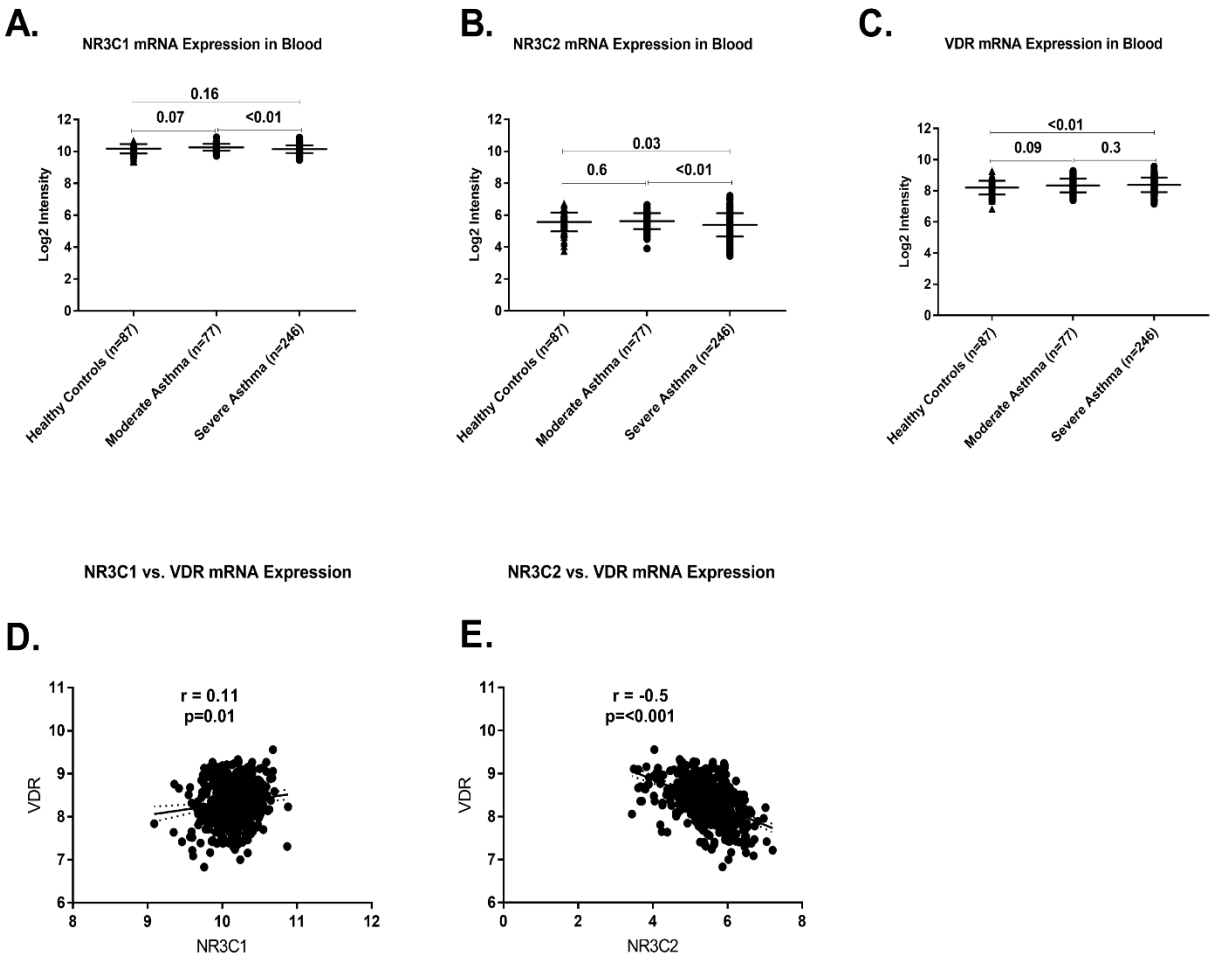

Figure 2: NR3C1, NR3C2 and VDR mRNA expression level in the blood of subjects with severe asthma, moderate asthma, and non-asthmatics. Data was collected in the U-BIOPRED study (GSE69683).

Supplementation of vitamin D selectively favours the upregulation of GR-α receptor

To validate the *in silico* findings, a double-blinded, randomized, placebo-controlled study was performed to test the effect of VitD supplementation on 45 vitamin D deficient asthmatics. Moderate to severe asthmatics between 18 and 65 years of age who were diagnosed with asthma and VitD (25D3) levels less than 20ng/mL at the screening visit were recruited at two hospitals in UAE (Rashid Hospital, Dubai, U.A.E. and Zayed Military Hospital, Abu Dhabi, U.A.E.). The patients were divided into two groups, an experimental group and a placebo group. The baseline serum levels of VitD in the experimental and placebo groups were comparable (11.74 ± 0.77 ng/mL, 12.96 ± 1.29 ng/mL, respectively). The experimental group received 50, 000 IU of VitD

oral supplementation weekly for eight weeks [19]. Following treatement, a 2.84±0.01-fold increase in blood VitD D levels (33.34 ± 2.04 ng/mL) was observed (Figure 3). The level of VitD in the placebo group did not change (14.45 ± 1.61 ng/mL) (Table1 and Figure 3). This result indicated that the regimen used was effective to correct VitD deficiency.

Table 1: Clinical characteristics of patients received vitamin D or placebo for eight weeks.

Variable	*Total*	*Placebo*	*Vitamin D*
Total	*54*	*20*	*34*
Male	*19 (35%)*	*4 (20%)*	*15 (44%)*
Female	*35 (64.8%)*	*16 (80%)*	*19 (55.8%)*
Age (years)	*38.64±1.90*	*40.60±2.97*	*37.45±2.48*
Body Mass Index	*32.27±0.92*	*32.32±5.52*	*32.32±1.28*
Pre-treatment 25D$_3$ levels (ng/mL)		*12.96±1.29*	*11.74±0.77*
Post treatment 25D$_3$ levels (ng/mL)		*14.45±1.61*	*33.34±2.04*

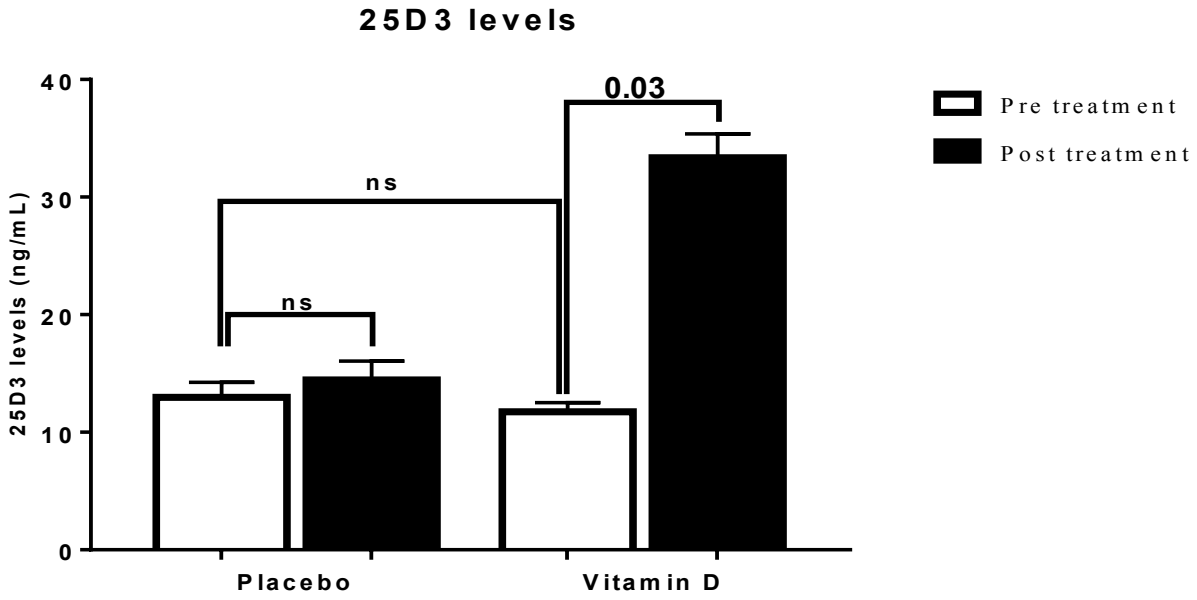

Figure 3: Pre-treatment and post-treatment levels of serum vitamin D (25D3) in patients who received vitamin D or placebo for eight weeks. Severe asthmatic patients with vitamin deficiency were divided into 2 groups. 23 patients received 50000 IV of Vitamin D orally over 8 weeks, while 22 patients received placebo. Vitamin D levels were measured in the blood of these patients before and after the treatment.

We next determined whether VitD supplementation affects the gene expression of steroid receptor GR-α or GR-β. Correction of VitD levels lead to a significant increase in the expression of GR-α

when compared to baseline level. A significant increase was observed following treatment with VitD compared to pre-treatment levels (1.89 ± 0.56, 5.34 ± 1.40, respectively; $p<0.05$) (Figure 4A). Moroever, there was a significant increase in GR-α expression in patients who received VitD compared to those who received the placebo control (5.34 ± 1.40 versus 1.64 ± 0.76-fold; $p<0.05$). Interestingly, no change in GR-β expression was observed following treatment with VitD (1.49 ± 0.27 versus1.30 ± 0.25) (Figure 4B). Relatively, the GR-α /GR-β ratio increased significantly following VitD supplementation compared to pre-treatment levels (3.58 ± 0.83 folds; $p<0.05$); or patients who received a placebo (Figure 4C).

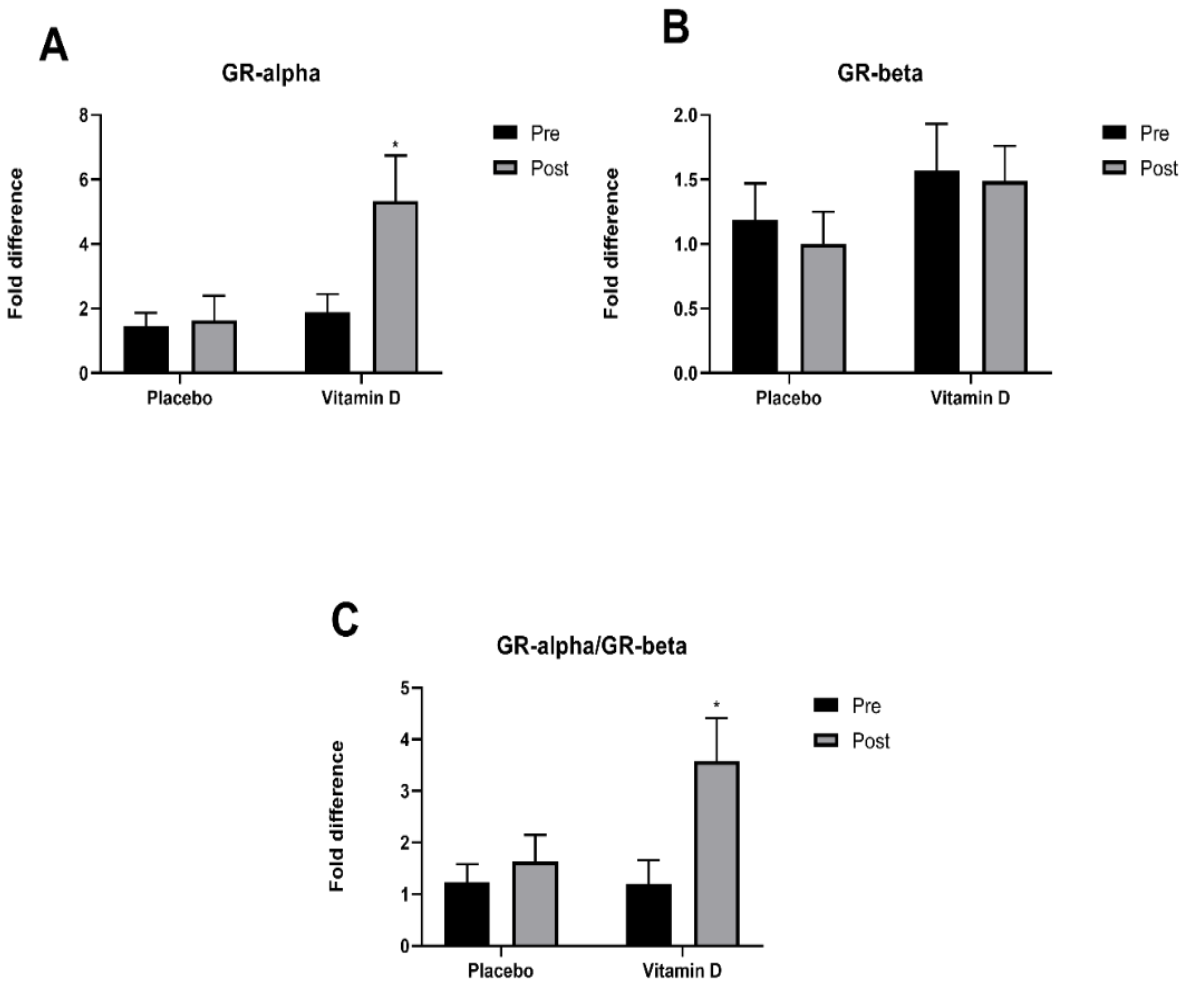

Figure 4: Vitamin D treatment increases the expression of GR- α with no effect on GR- β. Asthmatic subjects were treated with 50, 000 IU of vitamin D orally or placebo for eight weeks. Blood specimens obtained following 8 weeks of treatment were analyzed for mRNA expression of GR-α (A), GR-β (B) using RT qPCR and the ratio of GR-α /GR-β was calculated (C). *$p<0.05$

Vitamin D supplementation downregulated the blood levels of IL-17 and IL-4 cytokines in asthmatic patients

The effect of VitD supplementation on the blood levels of asthma related pro-inflammatory cytokines was then determined (Figure 5)[20]. Blood levels of IL-17A, IL-17F, IFN-γ, IL-4, IL-5, and TNF-α were determined before and after VitD supplementation using ELISA assay (Figure 5). VitD preferentially suppressed the blood levels of IL-17F cytokine (p=0.04) without affecting IL-17A; while there was no significant difference in the placebo group (p=0.17). A decrease in blood IL-4 cytokine levels was obsereved following treatment, however, not to a significant level (p=0.07). No significant effect of VitD supplementation was observed on the rest of the cytokines tested.

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