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Original Article

Effect of Vitamin D treatment on *Interleukin-2* and *Interleukin-4* Genes Expression in Multiple Sclerosis

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Abstract

Introduction: Multiple sclerosis is a chronic inflammatory disease of central nervous system. The etiology of MS is slightly known, but genetic and environmental factors are reported. Vitamin D regulates gene expression and affects target cell functions. The aim of this study was to investigate the expression variation of IL-2 and IL-4 genes under vitamin D supplementation in patients with multiple sclerosis.

Materials and Methods: In this study, blood samples were drawn from 32 patients before and after treatment with vitamin D. Quantitative real time PCR was used to measure IL-2 and IL-4 gene expression levels. Correlation analysis between the expression levels of genes and serum vitamin D, the Expanded Disability Status Scale (EDSS) as well as other clinical features of patients with MS was performed.

Results: No significant difference of IL-2 and IL-4 genes expression level was observed with vitamin D supplementation. We did not find significant correlation between IL-2 and IL-4 mRNA levels and EDSS score in multiple sclerosis patients.

Conclusion: We did not find any difference between the expression of IL-2 and IL-4 genes before and after treatment with vitamin D that it may have some effects on the prevention of multiple sclerosis through other inflammatory factors and signaling pathways.

Abbreviation: MS (Multiple Sclerosis), IL(interleukin), EDSS (Expanded Disability Status Scale), VD (Vitamin D), VDR (Vitamin D Receptor), qRT-PCR (Quantitative Real-TimePCR)

Keywords:
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Introduction

Multiple sclerosis (MS) is an inflammatory neurological disease of central nervous system(Goldenberg, 2012). The etiology of MS is slightly understood. Some investigators have indicated that the prevalence of MS is highest where environmental supplies of vitamin D are lowest (Burton *et al.*, 2010). Some have also showed that vitamin D not only prevents MS, but also attenuates disease activity. Burton et al. illustrated a correlation of vitamin D supplementation with peripheral T cell homeostasis and reduction of T cell proliferative response to myelin antigens in MS patients (Smolders, 2011). Central nervous system inflammation and loss of myelin cause MS. Several

studies have demonstrated the role of immune system related factors including cytokines in multiple sclerosis etiology(Arababadi *et al.*, 2010). Interestingly, 25(OH) D3 is metabolized to activate 1,25(OH)2D3 and expression of 1α-hydroxylase is performed by T cells. Vitamin D inhibits the differentiation of monocytes to dendritic cells in vitro as well as action of different transcription factors involved in cytokine gene regulation and mediates a shift of T CD4+ cells to anti-inflammatory cytokines(Correale *et al.*, 2009). Vitamin D receptors (VDRs) on immune system cells such as monocytes, macrophages and T lymphocytes increase in response to 1,25$(OH)_2$D3 exposure and the changes in transcription, proliferation and differentiation of these cells occur depending on circulating vitamin D levels in blood(Margaret H *et al.*, 2011).

IL-2 is a T cell growth factor and modulator of neural and neuroendocrine functions(Hanisch and Quirion, 1995). It also has an important role in immunoregulation of CNS (Jiang and Lu, 1998). *IL-2* penetrates the blood-barrier and regulates interactions between peripheral tissues and the central nervous system. A functional and pathological alteration in the brain is related to dysregulation of *IL-2* (Hanisch and Quirion, 1995). It has an important role in immunopathology of MS and *IL-2* is increased in the serum of active MS patients(Gallo *et al.*, 1992). Several studies have reported that 1,25(OH)2D3 decreases *IL-2*, GM-CSF and IFN-γ mRNA in Jurkat cells suggesting that direct transcription repressive effect of vitamin D on *IL-2* expression is associated to VDR(Alroy *et al.*, 1995).

IL-4 is a lymphocyte growing and survival factor that regulates immune system, proliferation, differentiation and apoptosis of different cells including dendritic cells and neuronal cells. It promotes Th2 differentiation and inhibits Th1 cell differentiation(Wurtz *et al.*, 2004). Microarray experiments from MS patients have shown the role of several cytokines in CNS inflammation. Down regulation of immune responses observed via production of anti-inflammatory cytokines such as *IL-4* during disease remission (Quirico-Santos *et al.*, 2007). Increase of *IL-4* transcripts has been shown after 1,25(OH)2D3 administration to mice(Cantorna *et al.*, 1998).

We slightly know about the effectiveness and importance of vitamin D on *IL-2* and *IL-4* gene expressions in MS. Some studies have investigated a correlation among serum VD levels and *IL-2* as well as *IL-4* expressions, but the molecular mechanism of their correlation is not determined. The aim of this study was to investigate the effects of vitamin D on the expression of *IL-2* and *IL-4* mRNA in peripheral blood mononuclear cells (PBMCs) of MS patients *in vivo.*

Materials and methods

Patients

We selected 32 RR-MS patients from MS Research Center of Sina Hospital affiliated to Tehran University of Medical Sciences (Tehran, Iran) according to McDonald criteria and MRI test. All patients were recruited from November 2012 to October 2013. They were in the remission period and did not take any steroid or immunosuppressive drugs. They also had vitamin D deficiency (<20ng/ml) with EDSS Scores ranging 0 to 5. All subjects received an oral dose of 50,000 IU of vitamin D weekly, for two months. Informed consent was obtained from all of patients prior to the blood sampling. This research was approved by the Medical Ethics Committee of Tarbiat Modares University and this trial was registered in Iranian Registry of Clinical Trials (ID: IRCT2014081818840N1R1).

25 (OH) D3 measurement

Whole blood samples were obtained from the patients before and after 8 weeks of vitamin D supplementation. To separate the serum, these samples centrifuged at 3000 rpm for 15 min at 4°C and serum levels of 25(OH)D was analyzed by vitamin D detection kits (Immunodiagnostic Systems, Inc.).

RNA extraction and cDNA synthesis

Blood samples were collected in the anti-coagulant EDTA tubes and were diluted by PBS buffer. Peripheral blood mononuclear cells were isolated by Ficoll gradient centrifugation technique (CL5020, lympholyte, Cedarlane, Netherlands) for 20 min and 3000 rpm at 4°C. RNA was extracted by RNX-plus

solution according to the manufacturer's instructions (Cinnagen, Iran). Extracted RNAs were treated with DNase I (Sigma, USA) to remove any genomic DNA contamination. Concentration, and purity of RNAs were determined by spectrophotometry. cDNA synthesis was performed by reverse transcription with 3µg of total RNA using random hexamer and oligo $(dT)_{18}$ primers through Revert Aid^{TM}, reverse transcriptase in total 20µl reaction mixture according to the manufacturer's instructions (Fermentas, Canada).

Real-Time PCR analysis

mRNA expression levels of interleukin-2 *(IL-2*) and interleukin-4 (*IL-4*) genes were measured with appropriate primers and normalized with the housekeeping gene Glyceraldehyde-3-phosphate Dehydrogenase (*GAPDH*). The sequences of primers used for gene expression were shown in Table 1. Quantitative Real-Time PCR was performed by ABI 7500 sequence detection systems (Applied Biosystem, Foster City, CA, USA) using 4 µl Eva Green qPCR Mix Plus (MREG0330, Solis Biodyne), 10ng cDNA, 200nM of each forward and reverse primers according to manufacturer's instructions in final volume of 20µl. The PCR was performed through following instructions: an initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 5s, annealing at 60 °C for 30s and extension at 72 °C for 20s. Relative quantitation of each gene was analyzed by delta-delta Ct method(Livak and Schmittgen, 2001) before and after treatment. All experiments were performed at least in duplicate.

Statistical Analysis

Statistical analysis programs (GraphPad, La Jolla, CA, USA; SPSS [Version 21.0] Chicago, IL, USA) were used. Paired Student's t test was used for comparison of mRNA expressions before and after the treatment with vitamin D. Wilcoxon signed rank test was used to analysis EDSS Scores. Pearson's correlation coefficient and standard regression tests were also used for correlation analysis. Two-tailed P values <0.05 were considered significant, and data were shown as mean ± standard deviation (SD).

Results

Demographic and Clinical Features of Patients

All 32 patients were evaluated in this study which consisted of 26 females and 6 males with a mean age 30.68 ± 7.2. Mean disease duration was 5.65±3.8 years. Patients mean EDSS before and after treatment with vitamin D were 2.21±1.03 and 1.96±1.05 respectively (p=0. 002).

Vitamin D treatment

The serum level of 25-hydroxyvitamin D increased significantly in patients after 8-week therapy when compared with baseline (12.28±5.7 nmol/L *vs* 42.43±17.42 nmol/L p<0.001). We did not find any significant difference in evaluation level of 25-hydroxyvitamin D value between females and males before and after the treatment. There was no relationship between 25-hydroxyvitamin D levels at

Table 1. The sequence of primers that were used for gene expression analysis. The relative expression of each gene was assessed in comparison with the housekeeping gene *GAPDH*. All primers were designed using PRIMER EXPRESS software (Applied Biosystems, USA).

Gene (Accession)	primer sequences F: forward, R: revers	Product size (bp)
IL-2 **(NM_000586.3)**	F: AGTTTTACATGCCCAAGAAGGC R: CATGAATGTTGTTTCAGATCCC	185
IL-4 **(NM_000589.3)**	F: TCTTTGCTGCCTCCAAGAACAC R: CTCTGGTTGGCTTCCTTCACAGG	226
GAPDH **(NM_002046.5)**	F: CCATGAGAAGTATGACAAC R: GAGTCCTTCCACGATACC	115

baseline and age (r^2=0.09, p=0.08) as well as disease duration (r^2=0.05, p= 0.21).

Measurement of gene expression levels

We did not find significant differences in *IL-2* mRNA

Table 2. The result of genes expression analysis using qRT-PCR technique in the PBMCs cells of RR-MS patients before and after supplementation with vitamin D.

subjects	ΔCt	*IL-2* gene [a]	***IL-4* gene** [a]
RRMS Patients (N= 32)	ΔCt of After Vitamin D	6.63 ± 2.20	**4.41 ± 2.05**
	ΔCt of Before Vitamin D	6.98 ± 2.42	**5.01 ± 2.44**
	ΔΔCt	-0.35	**-0.62**
	p-value [b]	0.67	**0.60**

a Values are expressed as mean ± SD
b Paired t-test

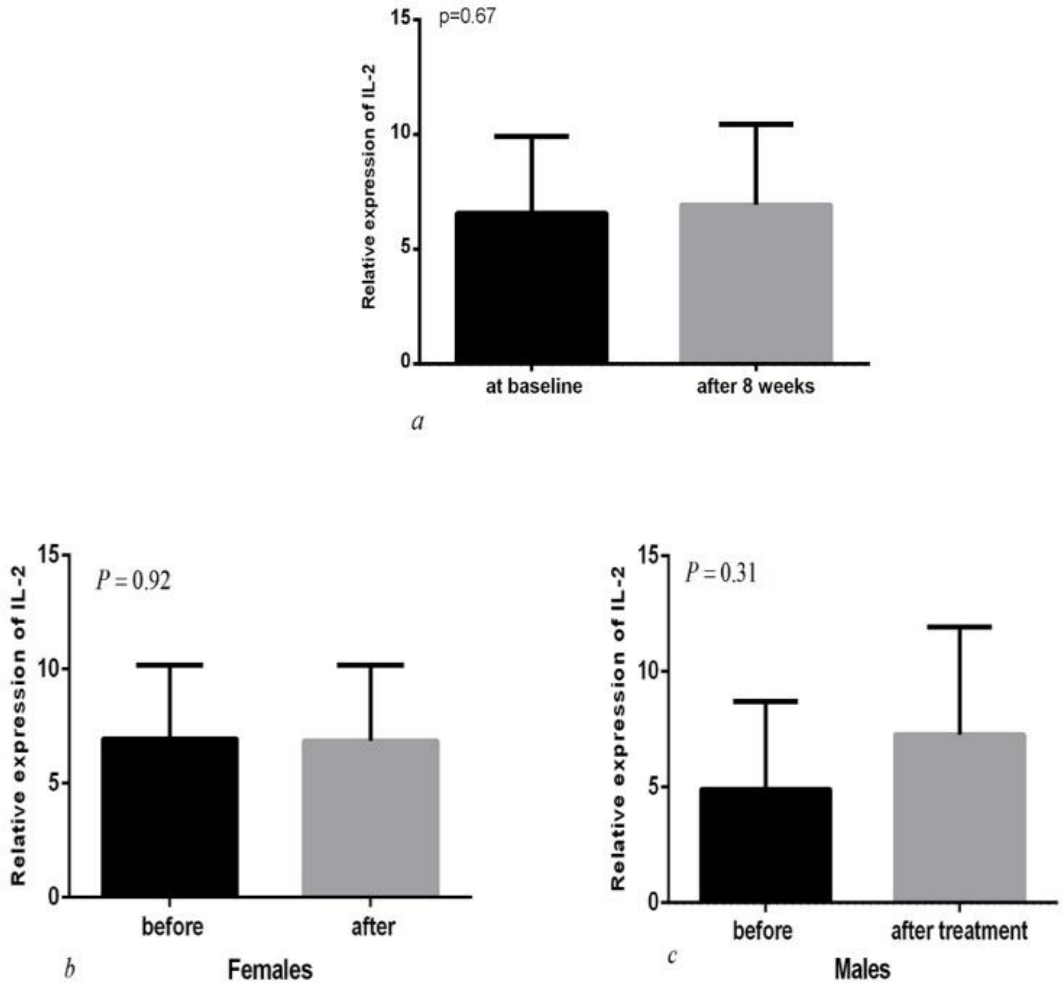

Fig 1: The result of interleukin 2 gene expression analysis in RR MS patient before and after supplementation with vitamin D. The supplementation of vitamin D do not influence the mean of IL-2 gene expression in total (a). The analysis of result based on the gender of patients showed that the mean of gene expression in the female patients do not change (b), however, the expression of IL-2 increased in male patients follow vitamin D supplementation, but it was not statistically significant(c). A *P*-value ≤0.05 was considered significant and data are shown as mean ± standard deviation (SD).

and *IL-4* mRNA expression levels before and after the treatment (p=0.67, p=0.67 respectively, Table 2). Comparison of *IL-2* mRNA expression level in patients with ∆vitD3≥20 and ∆vitD3<20 between before and after vitamin D supplementation therapy was not shown significant increasing than pre-treatment (p=0.3, p=0.8, respectively) (Figure 1a). Also, no significant difference was observed in *IL-2* mRNA between RRMS with EDSS ≤2 (p= 0.8) and EDSS >2 (p= 0.3) groups by vitamin D treatment. We did not find significant differences in *IL-2* mRNA among females (p=0.92) and males (p=0.31) before and after treatment (Figure 1b and c).

Our study did not indicate a significant difference in *IL-4* mRNA levels at baseline and 8 weeks after the treatment (p= 0.6) even in patients in two categories of EDSS ≤2 (p= 0.8) and EDSS >2 (p= 0.6) (Figure 2a). Comparison of *IL-4* mRNA expression level in patients with ∆vitD3≥20 and ∆vitD3<20 between before and after vitamin D supplementation was not shown significantly increasing than pre-treatment (p=0.67, p=0.6, respectively).

By vitamin D supplementation to the RR MS female patient the expression of *IL-4* mRNA increased while, the expression of this gene reduced in the male patients, but these changes were not statistically significant in both females (p=0.46) and males (p=0.74) groups of patients (Figure 2b and c).

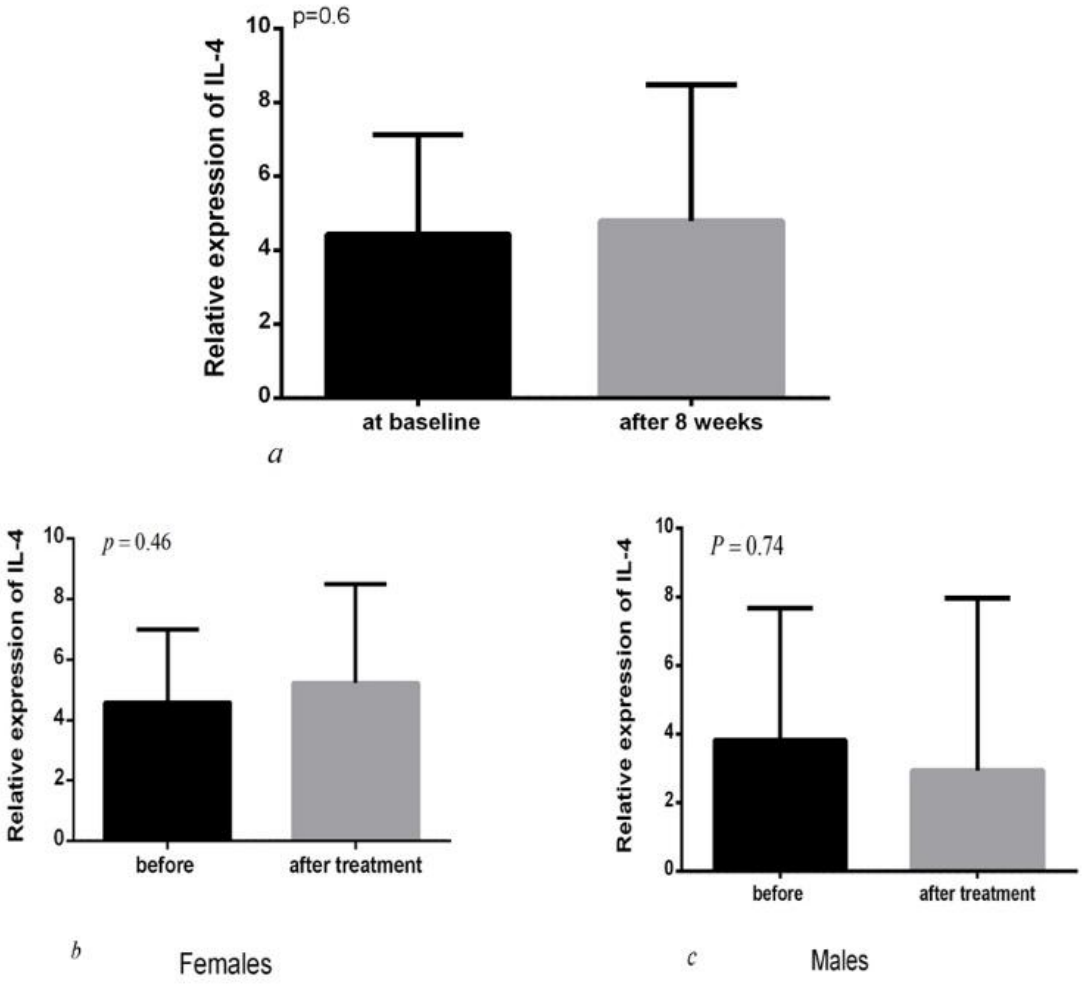

Fig 2: The result of interleukin 4 gene expression analysis in RRMS patient before and after supplementation with vitamin D. The supplementation of vitamin D did not influence on the mean of IL-4 gene expression in total (a). The response of female and male patients to 8 weeks vitamin D supplementation was different (b and c). The expressionIL-4 gene increased about 10 percent, but in the male patients the gene expression reduced about 10 percent in comparison with before vitamin D supplementation level. However, the changes in IL-4 gene expression in two groups of patients was not statistically significant.

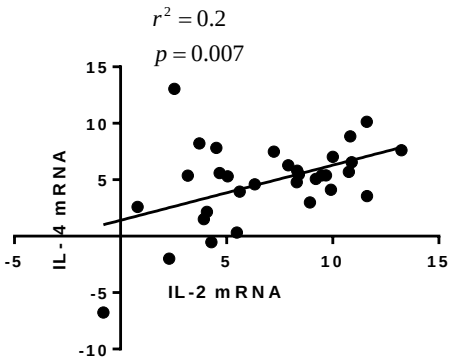

Fig 3: Correlation analysis of vitamin D supplementation with *IL-2* and *IL-4* genes expression. mRNA of IL-2 levels correlated positively with mRNA IL-4 levels in PBMCs before the treatment.

Correlation analysis between IL-2 and IL-4 with clinical features of MS patients

IL-2 levels did not show a significant correlation with EDSS scores (r^2= 0.02, p-value= 0.4), age (r^2= 0.017, p= 0.47), disease duration (r^2= 0.09, p= 0.08) and 25-hydroxyvitamin D serum levels before the treatment (r^2= 0.03, p= 0.3). There was also no significant correlation between *IL-4 expression* levels with disease duration (r^2= 0.0009, p=0.8), age (r^2=0.27, p=0.36), EDSS scores (r^2=0.0009, p=0.8) and 25-hydroxyvitamin D serum levels (r^2=0.0008, p=0.8). Our study indicated a significant correlation between IL-2 and IL-4 expression levels before supplementation (r^2= 0.2, p= 0.007, Figure 3).

Discussion

The aim of this research was to investigate vitamin D clinical effectiveness in the treatment of Iranian patients with multiple sclerosis at the molecular level. Interestingly, we did not find any statistically significant difference in *IL-2* and *IL-4* gene expression levels before and after vitamin D supplementation, but it was expected that the expression of *IL-2* and *IL-4* expression levels will decrease and rise respectively. MS and other autoimmune conditions are believed to be associated with overproduction of pro-inflammatory cytokines including *IL-2*, *IL-6*, and *TNF-α* (Erta *et al.*, 2012).

IL-2 is a T lymphocyte growth factor that promotes activated T cell proliferation *in vitro* and modulates T cytotoxic, natural killer cells and activated B cells(June *et al.*, 1989). Intracellular ionized calcium and protein kinase C activation provides a signal for *IL-2* gene activation(Ju *et al.*, 1987). Matesanz's et al studies on IL-2 polymorphisms indicated the relevance of IL-2 gene in human multiple sclerosis and other autoimmune diseases. It influences on cell growth and survival, neurotransmitter and hormone release and modulation of neuroendocrine axis in CNS. In addition, the response of oligodendrocytes and neurons to the cytokine, neurotoxicity and chronically increasing of *IL-2* was observed (Matesanz *et al.*, 2001).

IL-2 penetrates the brain-blood barrier and regulates interactions between peripheral tissues and the CNS. This cytokine binds to *IL-2* receptor (*IL-2R*) in the immune system as well as CNS and dysregulation of *IL-2/IL-2R* causes functional and pathological alterations in the brain as in the immune system(Hanisch and Quirion, 1995). Towers' study indicated that 1,25(OH)2D3 suppresses T cell proliferation and leads to a decline of *IL-2* in Jurkat cells. The result of this study suggests that vitamin D receptor (VDR) is necessary but not sufficient to *IL-2* repression(Alroy *et al.*, 1995). In addition, experimental studies have demonstrated that calcitriol inhibits synthesis of *IL-6, IL-12* and *TNF* and suppresses *IL-2* (Zittermann, 2003).Prietl showed that T cell with calcitriol treatment inhibits pro-inflammatory cytokines of Th1 (*IL-2,TNF-α,IFN-γ*), Th9 (*IL-9*), and Th22 (*IL-22*)(Prietl *et al.*, 2013). Our results in this study are not in accordance with Oursin which no significant

difference was found in *IL-2* after vitamin D exposure. Ascherio et al. reported that weekly vitamin D3 therapy (40,000 IU) (colecalciferol) for 1 year induces no significant changes in concentrations of *IL-2, IFN-γ, IL-4, IL-5,* and *IL-17*(Ascherio *et al.*, 2010). Our findings were similar to Ascherio's clinical trial results.

IL-4, Th2 cytokine regulates Th1 responses and causes spontaneous remission of MS. This cytokine binds to its receptor in T lymphocytes and causes proliferation and differentiation into Th2 cells (Paintlia *et al.*, 2006). Th1 cells promote cell-mediated immunity and Th2 cells lead to humoral immunity. The cause of successful immune responses is a balance between Th1 and Th2 cells. The role of *IL-4* in the prevention of autoimmune diseases is slightly known. This cytokine probably has regulatory function and causes spontaneous recovery from experimental autoimmune encephalomyelitis (EAE) (Choi and Reiser, 1998). It appears that overactive pro-inflammatory Th1 cells lead to inflammation and different molecular mechanisms involved in the pathogenesis of autoimmune diseases.

Some investigations have reported the inflammatory process of MS, in which 1,25(OH)2D binds to different VDRs and causes inhibition of Th1 cytokines like TNF-α and promotes Th2 cytokines such as *IL-4*(Lange *et al.*, 2009). Cantorna reported an increase in IL-4 transcripts by 3- to 25-fold with vitamin D administration to mice. 1,25-dihydroxyvitamin D3 decreases total number of lymphocytes and increase the number of IL-4 and TGFβ1 transcripts. Therefore, accumulating studies can conclude that the two anti-inflammatory cytokines block encephalomyelitis (Cantorna *et al.*, 1998, Prietl *et al.*, 2013). However, in another clinical trial, 43 patients took 40,000 IU or 20,000 IU colecalciferol per week or placebo for 1 year and no significant change was observed in IL-2, IL-4, IL-5, IL-10, 1L-13 and IFN-γ(Correale *et al.*, 2009, Ascherio *et al.*, 2010, Yusupov *et al.*, 2010). Also, we found no significant changes in IL-4 expression levels after supplementation.

We performed numerous samplings in winter. Yusupov's study indicated that seasonal differences in vitamin D cause cytokine changes in winter and summer (Yusupov *et al.*, 2010, Shaygannejad *et al.*, 2012). These data reveal that why we may not have observed a benefit of vitamin D effect on cytokine expression levels.

Some studies indicated that many autoimmune diseases are more prevalent in women than in men. There is a female –to-male preponderance approaching 2:1 in MS. The reasons for sex bias in the autoimmune diseases, such as multiple sclerosis are unclear. The factors include sex related differences in immune responsiveness, sex steroid effects, response to infection and sex differences in genetic factors (Whitacre, 2001).

Interestingly, there were no significant differences for *IL-2 andIL-4* cytokines between male and female MS subjects in this research. Also, Eikelenboom et al reported no significant differences between male and female multiple sclerosis patients in these cytokines (Eikelenboom *et al.*, 2005).

The disagreement in our results with previous studies is probably due to methodology of this study, Iranian's genetic background, dosage of vitamin D, period of research and different pathogenesis of MS in Iran. This study like other investigations involves some limitations as no placebo-control group and small sample size. The effects of vitamin D on other genes and epigenetic mechanisms in large populations with placebo-control groups in the long period by different doses of vitamin D warrant consideration.

In summary, we demonstrated that vitamin D supplementation may not lead to a reduction in MS risk by down regulation of pro-inflammatory cytokine of *IL-2* and up-regulation of anti-inflammatory cytokine of *IL-4* genes expression. Overall, there is still a need for improved therapeutic approaches, especially vitamin D effectiveness on other genes with large sample size and a placebo-group.

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Conflict of interest

The authors declare that they have no conflict of interest.

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