



Effect of vitamin D replacement on immunological biomarkers in patients with multiple sclerosis



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ABSTRACT

We aimed to investigate the immunologic effects of vitamin D replacement in RRMS patients. In a controlled single center study, patients deficient in 25-hydroxyvitamin D (serum level < 25 ng/ml) received 10,000 IU/week cholecalciferol for 3 months. Sufficient vitamin D patients (serum level > 35 ng/ml) were followed for the same period. Assessments were performed at baseline and at 3 months. 25-hydroxyvitamin D levels increased significantly from baseline to month-3 in the deficient group after treatment and remained stable in the sufficient group. We observed a decreased interferon- γ (IFN γ) secretion by CD4⁺ T cells in vitamin D deficient group but not in the sufficient group, and a negative correlation between baseline serum vitamin D and IFN γ production. There was no change in the frequency of T helper or regulatory T cell subsets in either group. Increasing serum levels of 25-hydroxyvitamin D are associated with decreased production of IFN γ by CD4⁺ T cells.

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1. Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease that affects the brain and spinal cord, associated with several genetic and environmental risk factors including vitamin D deficiency [1]. Vitamin D has immunomodulatory and anti-inflammatory effects that have been demonstrated *in vitro* [2–4] and *in vivo* in animal models [5,6].

Vitamin D deficiency has been associated with different inflammatory, neurologic, and autoimmune diseases [7]. Moreover, several studies suggested that administration of vitamin D decreases inflammation and improves motor function in animal models of MS [8,9] as well as in patients with inflammatory CNS disease [10]. Vitamin D levels appear to be lower in MS patients than controls [3,11], and low vitamin D levels in blood were correlated with increased MS incidence and relapses [12–15]. Moreover, vitamin D deficiency in pregnant women or newborns was linked to an increased risk of multiple sclerosis in siblings [16], in contrast to earlier studies [17]. The immunomodulatory, anti-

proliferative and anti-inflammatory effects of vitamin D in MS are well established *in vitro* on peripheral blood- or cerebrospinal fluid- derived CD4⁺ T cells [3,18], antigen presenting cells [19], and CD8⁺ T cells [20].

Studies investigating the effect of vitamin D supplementation in MS have demonstrated a clear improvement in the course of the disease [21], along with a good safety profile and tolerability [22–25].

The aim of the present study was to explore the *in vivo* immunological effects of high dose vitamin D supplementation in patients with RRMS.

2. Methods

2.1. Study sample and design

A controlled before-and-after high dose vitamin D supplementation study was performed evaluating immunological profiles of patients with relapsing remitting multiple sclerosis (RRMS) at baseline and at 3 months. The study was performed at the Nehme and Therese Tohme Multiple Sclerosis Center at the American University of Beirut Medical Center (AUBMC), between 2012 and 2014. Eligible patients were adults diagnosed with RRMS based on the revised 2010 McDonald's criteria [26] clinically stable for at least 4 weeks prior to enrollment, and treated with Interferons (IFN beta-1a subcutaneously 44 mcg three times per week, IFN beta-1b subcutaneously 250 mcg each other day, or IFN beta-1a 30 mcg intramuscularly weekly). Patients were excluded if shifted to another treatment, had any radiological (new or enhancing

Abbreviations: MS, multiple sclerosis; RRMS, relapsing remitting multiple sclerosis; PBMC, peripheral blood mononuclear cells; FACS, fluorescence activated cell sorting; CBA, cytometric bead array; IFN γ , interferon gamma; IL, interleukin; TNF α , tumor necrosis factor alpha; GM-CSF, granulocyte macrophage colony stimulating factor; Th, T helper; Treg, T regulatory.

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T2 lesion or gadolinium enhancing lesion) or clinical (MS exacerbation) evidence of disease activity, or received intravenous steroids or other immune suppressant medications during the 3-months follow up period. Patients diagnosed with progressive forms of MS or neuromyelitis optica were also excluded (Fig. 1, CONSORT flowchart of patient disposition). The study was approved by the American University of Beirut Institutional Review Board, and written informed consent was obtained from all participants.

All participants underwent serum 25-hydroxyvitamin D measurement at the Endocrine Core Laboratory at AUBMC using Roche Diagnostics total assay, and were classified at baseline into vitamin D deficient (<25 ng/ml) or sufficient (>35 ng/ml). Participants with baseline vitamin D levels between 25 and 35 ng/ml were excluded. The patients categorized as vitamin D deficient were treated with high dose vitamin D (10,000 IU orally daily for 3 months), while those with normal vitamin D maintained their usual medical care. Weekly calls were performed to ensure the deficient patients were compliant with their vitamin D replacement. Blood samples were obtained from both groups at baseline and at 3 months to measure 25-hydroxyvitamin D levels and for immunological assays. The immunology laboratory personnel were blinded to treatment group during the study. Clinical data was collected including demographics, medications and vitamin D supplements, disease duration, and disease modifying therapy.

2.2. Sample size calculation

The sample size was calculated by extrapolation from previous *in vitro* studies of vitamin D effect on the percentage of interleukin-(IL)-17⁺ T cells [2]. Assuming a rate of 25% of IL-17⁺ T cells after *in vitro* stimulation in the control group and a 2% IL-17⁺ T cells in the vitamin D groups, with a type 1 error of 5% and study power of 80%, a sample of 38 per group was calculated. A planned recruitment goal was 43 subjects per group after accounting for drop-outs.

2.3. Immunological tests: *in vitro* T-cells stimulation

Peripheral blood mononuclear cells (PBMC) were isolated from patients and cryopreserved according to Immune Tolerance Network protocol (www.immunetolerance.org). PBMC were thawed on the day of processing, and T cells were sorted for purity on a BD fluorescence

activated cell sorting (FACS) Aria SORP using anti-CD3 and anti-CD4 (BD Biosciences) and cultured in serum-free X-VIVO 15 medium (1041, Lonza, Verviers, Belgium) with anti-CD3/anti-CD28 (Life Technologies, Carlsbad, CA) at a cell to bead ratio of 4:1 for 6 days. Cells were treated with recombinant human IL-2 (10 ng/ml; R&D) on the 3rd day. After 6 days of activation in culture, supernatants were collected for cytokine measurement.

2.4. Cytometric bead array

BD Cytometric Bead Array (CBA) human Th1/Th2/Th17 cytokine kit was used for the simultaneous measurement of interferon- γ (IFN γ), tumor necrosis factor- α (TNF α), and interleukins (IL-17, IL-10, IL-6, IL-4, and IL-2) concentrations in serum samples or cell supernatants after 6 days of *in vitro* activation. 50 μ l of allophycocyanin (APC)-conjugated capture beads, with distinct fluorescence intensities specific to each cytokine, were incubated with recombinant standards or test samples (sera or supernatants). Phycoerythrin (PE)-conjugated detection antibodies were then added to form a sandwich complex that was analyzed by flow cytometry on BD FACS Aria SORP, and the results were generated using CBA analysis FCAP Array Version 3.0 software (BD Bioscience-Pharmingen).

2.5. PBMC isolation and *ex vivo* cell staining assays

Cryopreserved PBMC were thawed on the day of processing, and cells were washed and stimulated with phorbol-12-myristate-13-acetate (PMA) (50 ng/ml; Sigma) and ionomycin (1 μ g/ml; Invitrogen) in the presence of Brefeldin A (0.2% golgiplug; BD) for 4 h. Cells were re-suspended in cell staining media with live/dead stains (life technologies) and multiple fluorochrome-conjugated antibodies against different T cell surface markers. Cells were then fixed and permeabilized with BD Cytofix/Cytoperm or BD Transcription factor buffer set according to the manufacturer's instructions, and finally stained intracellularly with fluorochrome-conjugated antibodies against cytokines or transcription factors (Supplementary Table 1). We explored different T-cell populations using 3 different panels of antibodies (Supplementary Table 2). Panel 1 was for the pathogenic T helper 17 (Th17) profile and included antibodies against Th17 cell surface markers (CCR6, CD161, and IL23R) and the cytokines IL-17 and granulocyte macrophage

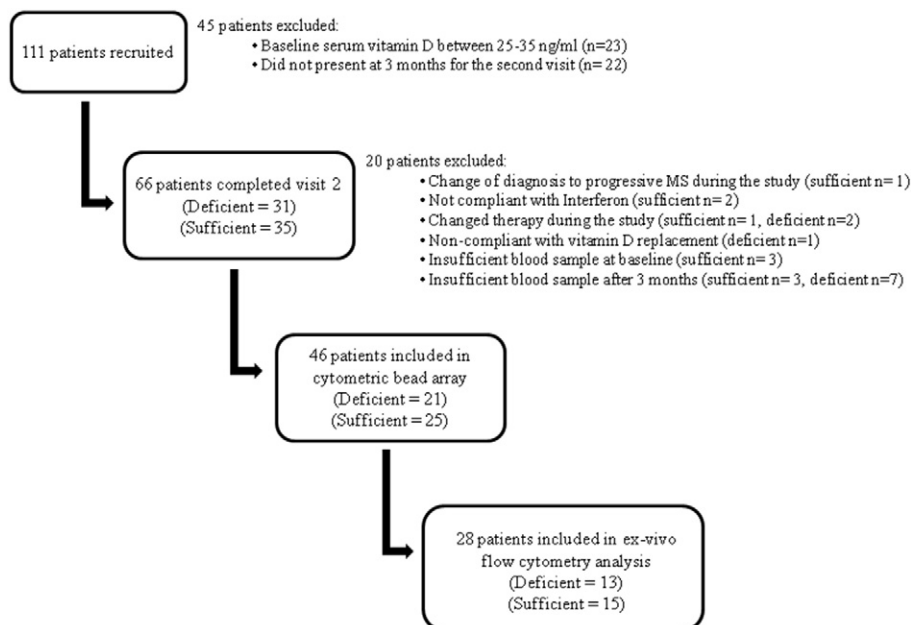


Fig. 1. Flowchart of patient disposition.

Table 1
Clinical and demographic characteristics of the study participants.

Variable	Deficient (N = 21) Mean (SD) or n (%)	Sufficient (N = 25) Mean (SD) or n (%)
Vitamin D level at baseline (ng/ml)		15.9 (6.4)
Vitamin D level after 3 months (ng/ml)		51.4 (8.5)
Age (years)		34.7 (12.8)
Female (%)		13 (52)
Disease duration at baseline (years)		3.9 (4.2)
Expanded Disability Status Scale (EDSS) at baseline		1.2 (0.8)
Body weight (Kg)		73.9 (12.7)
Current tobacco smoker (%)		11 (52.4)
Type of MS treatment:		
1. Interferon beta-1a SC 3/week (%)		10 (47.6)
2. Interferon beta-1b SC EOD (%)		4 (19)
3. Interferon beta-1a IM once weekly (%)		7 (33.3)

colony stimulating factor (GM-CSF). Panel 2 included antibodies for naïve and memory T cell markers as well as IL-17 and IFN γ antibodies. Panel 3 was for the T regulatory (Treg) profile using antibodies against CD25, Foxp3, and cytotoxic T-lymphocyte associated protein 4 (CTLA4). Data were acquired (minimum of 200,000 events per sample) on a BD FACS Aria SORP, and subsequently analyzed using FACS DiVa software. Unstained controls, compensation controls (using AbC Anti-Mouse Bead kit; life technologies), FMOs (Fluorescence minus one); and specific isotype controls were applicable.

2.6. Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences software (IBM Corp. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp). Continuous variables were expressed as mean \pm standard deviation, whereas categorical variables were reported as frequencies and percentages. The normality of distributions was evaluated through the Shapiro-Wilk test. Demographic characteristics and baseline immune activation markers were compared between the two groups using independent *t*-test or Mann-Whitney Test as applicable for continuous variables, and Chi-square Test or Fisher Exact Tests for categorical ones. Different immune activation markers were compared at baseline (visit 1) and after 3 months (visit 2) using paired *t*-test or Wilcoxon test as applicable among both groups. A *p*-value \leq 0.05 (two sided) was used to indicate statistical significance.

3. Results

3.1. Clinical and demographic characteristics of the patients

From 111 initially recruited patients, 55 were excluded either because of 25-hydroxyvitamin D levels between 25 and 35 ng/ml at baseline ($n = 23$), or they did not complete the second visit after 3 months ($n = 25$). Another 20 patients were excluded because of non-compliance with treatments or insufficient blood sample. The remaining 46 patients (deficient = 21, sufficient = 25) completed the study for evaluation of immune markers by cytometric bead arrays. In a subgroup of 28 patients (deficient = 13, sufficient = 15) sufficient blood samples were available for *ex vivo* flow cytometry analysis (Fig. 1).

All 46 participants (vitamin D deficient = 21, vitamin D sufficient = 25) had RRMS, with a mean (SD) age of 34.6 (11.9) years, disease duration of 3.8 (4.3) years, and EDSS of 1 (0.86). 22 (47.8%) patients were treated with interferon beta-1-a subcutaneously three times per week, 9 (19.6%) with interferon beta-1-b every other day, and 15 (32.6%) with interferon beta-1-a intramuscularly weekly. There were no significant differences in any of the clinical and demographic characteristics between vitamin D deficient and sufficient patients (Table 1).

As expected, vitamin D deficient patients had a significantly lower mean vitamin D level at baseline (15.9 ± 6.4 ng/ml) as compared to vitamin D sufficient patients (58.2 ± 18.3 ng/ml, $p < 0.0001$). All deficient patients were treated with high-dose vitamin D, raising their serum levels after 3 months to 51.3 ± 8.4 ng/ml ($p < 0.0001$), while vitamin

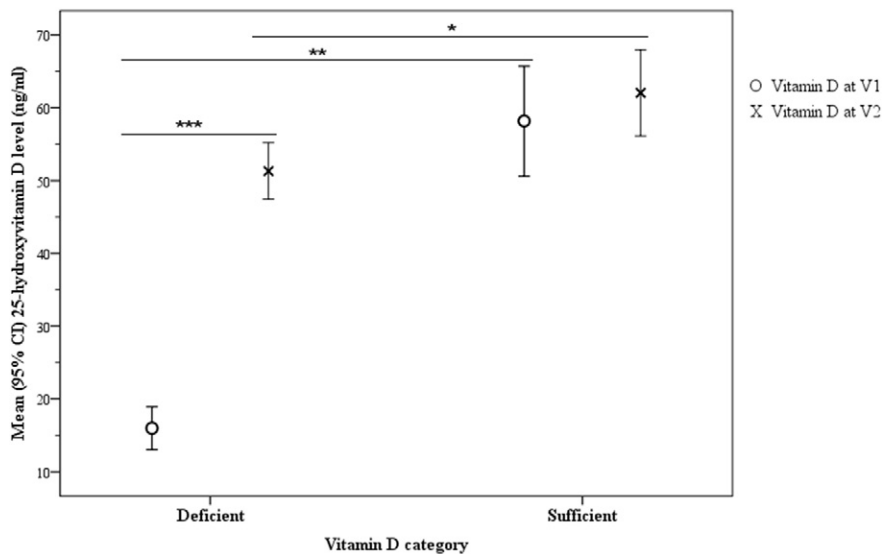


Fig. 2. Change in vitamin D levels at baseline (V1) and after 3 months (V2) among vitamin D deficient and sufficient patients. Mean vitamin D level \pm SD at baseline (V1) and after 3 months (V2) in both groups. * $p = 0.004$ Mann-Whitney Test, $Z = -2.86$; ** $p < 0.0001$ Mann-Whitney Test, $Z = -5.79$; *** $p < 0.0001$ Wilcoxon-Signed Rank Test, $Z = -4.02$. CI = confidence interval.

D sufficient patients were kept on their usual care, maintaining adequate vitamin D levels after 3 months (62.1 ± 14.3 ng/ml). The mean vitamin D level in the deficient patients (51.3 ± 8.4 ng/ml) remained lower than that among sufficient patients (62.1 ± 14.3 ng/ml; $p = 0.004$) at visit 2 (Fig. 2).

We also measured serum levels of IL-17, IFN γ , TNF α , IL-10, IL-6, IL-4, and IL-2 but none of these cytokines was within the limit of detection (20 pg/ml–5000 pg/ml).

3.2. Cytokine production by stimulated T cells

Supernatants of activated T-cells were collected after 6 days of *in vitro* activation, and IL-17, IFN γ , TNF α , IL-10, IL-6, and IL-4 concentrations were measured. IFN γ was significantly higher among vitamin D deficient subjects compared to vitamin D sufficient patients at baseline (883.9 ± 595 pg/ml versus 558 ± 477.1 pg/ml, $p = 0.024$). Furthermore, despite some decrease in IFN γ after 3 months among vitamin D

deficient patients, its level remained higher as compared to vitamin D sufficient patients (694.8 ± 414.7 pg/ml versus 495.1 ± 447 pg/ml, $p = 0.032$) (Fig. 3A). On the other hand, no significant differences in IL-17, TNF α , or IL-10 were evident between or within groups. IL-6 and IL-4 were below the detection limit (<20 pg/ml).

Given the baseline differences in IFN γ production between groups and a trend for decreased IFN γ among vitamin D deficient patients after vitamin D therapy, we performed a subgroup analysis excluding patients whose IFN γ level was below the 25th percentile of the group at baseline (5 out of 21 deficient and 9 out of 25 sufficient patients were excluded in this analysis). The analysis showed a statistically significant drop in IFN γ after 3 months among deficient patients (1072.5 ± 558 pg/ml to 714.3 ± 442.8 pg/ml, $p = 0.039$), with no significant changes in the vitamin D sufficient group (Fig. 3B). We further explored the relationship between vitamin D level as a continuous variable among all patients and the baseline IFN γ production. This analysis revealed a negative correlation between

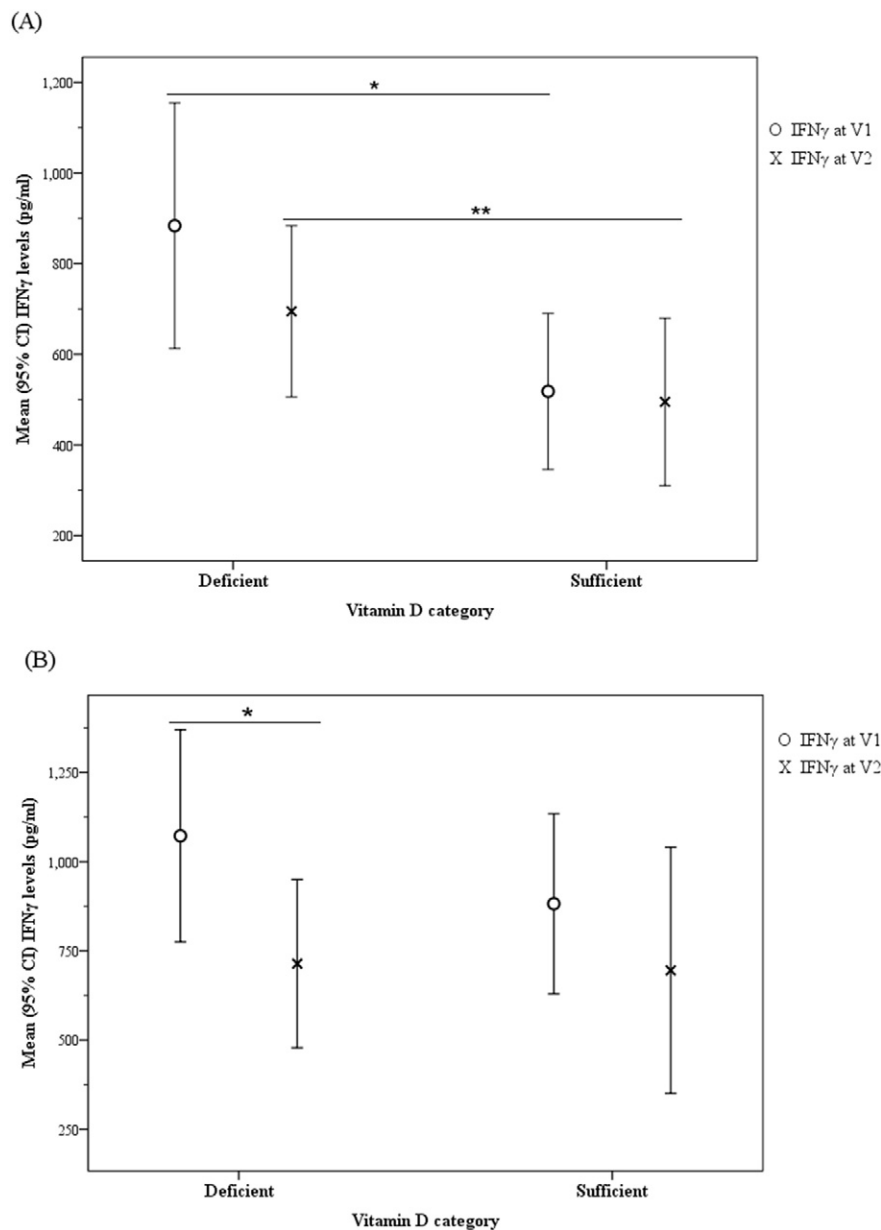


Fig. 3. Changes in IFN γ levels at baseline (V1) and after 3 months (V2) among vitamin D deficient and sufficient patients. (A) Mean IFN γ level \pm SD at baseline (V1) and after 3 months (V2) in both groups. * $p = 0.024$ Mann-Whitney Test, $Z = -2.26$; ** $p = 0.032$ Mann-Whitney Test, $Z = -2.14$. (B) Mean IFN γ level \pm SD at baseline (V1) and after 3 months (V2) in both groups after excluding patients with baseline IFN γ below the 25th percentile. * $p = 0.039$ Wilcoxon-Signed Rank Test, $Z = -2.07$. CI = confidence interval.

vitamin D levels and IFN γ at baseline (standardized Beta = -0.32 , $p = 0.031$) (Fig. 4).

3.3. Flow cytometric results from *ex vivo* immunophenotyping

We measured the frequencies of naïve (CD45RO $^-$ CCR7 $^+$), central memory (CD45RO $^+$ CCR7 $^+$) and effector memory CD4 $^+$ (CD45RO $^+$ CCR7 $^-$) T cells, and explored pathogenic Th17, Th1, and Treg cell markers in 28 patients (13 deficient, 15 sufficient). The percentages of CD3 $^+$ CD4 $^+$ and CD3 $^+$ CD4 $^-$ cells did not change after vitamin D supplementation and there were no significant changes in naïve and memory CD4 $^+$ subsets. There were no statistically significant differences within or between groups in the frequency of Th17 populations (CCR6 $^+$ -IL23R $^+$ CD161 $^+$ IL-17 $^+$ GMCSF $^+$) or in any of its single markers *ex vivo* (data not shown). In addition, no significant differences were found in the frequency of Th1 and Treg *ex vivo*.

4. Discussion

In spite of a large body of evidence suggesting the importance of vitamin D as a risk factor for MS [13,27], and possible link with disease activity [14,28–30], there are few studies addressing immune changes in patients receiving vitamin D supplements. We investigated the immunologic effects of vitamin D supplementation in patients with RRMS in a controlled prospective study. All the patients were clinically stable, had similar demographic and clinical characteristics, and were treated with the same class of disease modifying therapy (DMT). Serum level of vitamin D was significantly increased after supplementation and this was associated with decrease in IFN γ production by T cells. We also found a significant correlation between IFN γ production and serum vitamin D levels at baseline in all subjects.

A detrimental role of IFN γ in the pathogenesis of MS was demonstrated when exacerbation of the disease was observed in seven of 18 patients treated with IFN γ [31]. Increased production of IFN γ by stimulated cells was reported to correlate with increased risk of relapses in MS [32]. IFN γ is produced by Th1 cells as well as by Th17.1 cells that co-produce IL-17 and IFN γ [33,34]. Increased levels of IFN γ -secreting Th1-like Foxp3 $^+$ Tregs were also reported in MS [35]. Thus, we cannot determine whether the cellular source of IFN γ detected in the supernatants in our study. Another group reported increased transforming

growth factor-beta and interleukin-10, but the stimulation conditions were different than our study in that they use phytohaemagglutinin (PHA)-stimulated PBMC [36].

Unlike previous reports that showed significant changes in serum cytokine levels upon vitamin D supplementation [37–39], we could not detect serum cytokines in our patients similar to a recent report [25]. Serum cytokines can vary throughout the day and are sensitive to changes related to sample collection and processing [40] and this may explain the discrepant reports [25,37–39].

In vitro studies showed that vitamin D receptor (VDR) blocks binding of the transcription factor of activated T cells NFAT1 to the promoter of the human IL-17 gene leading to a decrease in IL-17 production [6]. So, one might expect a change in Th17 cell frequency after high dose vitamin D supplementation. However, unlike the report by Sotirchos et al. [25], we did not detect any changes in Th1 or Th17 cell frequency by flow cytometry in our study. This is most likely related to the small sample size, as only 13 deficient and 15 sufficient subjects had available samples for flow cytometry. It is also possible that a longer duration of treatment with high dose vitamin D is required to detect changes in IL-17 and other pro-inflammatory Th17 markers.

Our findings are in agreement with the SOLARIUM study, where Muris et al. reported that Vitamin D supplementation neither affected the proportion of lymphocytes with a regulatory phenotype, nor the proportion of T helper cells producing pro-inflammatory cytokines [41].

We are aware of several limitations of our study. A larger sample would have allowed detecting more correlations between vitamin D and inflammatory markers, since we observed significant heterogeneity between subjects in the levels of these markers. The duration of treatment was only 12 weeks, and it is possible that with longer treatment duration other immunological effects would have been detectable.

Randomized trials of add-on vitamin D are underway [42–44] thus it is important to understand the effects of vitamin D supplementation on immune function *in vivo*. Standardization of sample collection and immunologic protocols become crucial [45] to optimize these studies.

5. Conclusion

A 12-week course of high dose vitamin D supplementation in MS decreased IFN γ production by stimulated T cells and the IFN γ production was negatively correlated with baseline serum vitamin D. Our findings

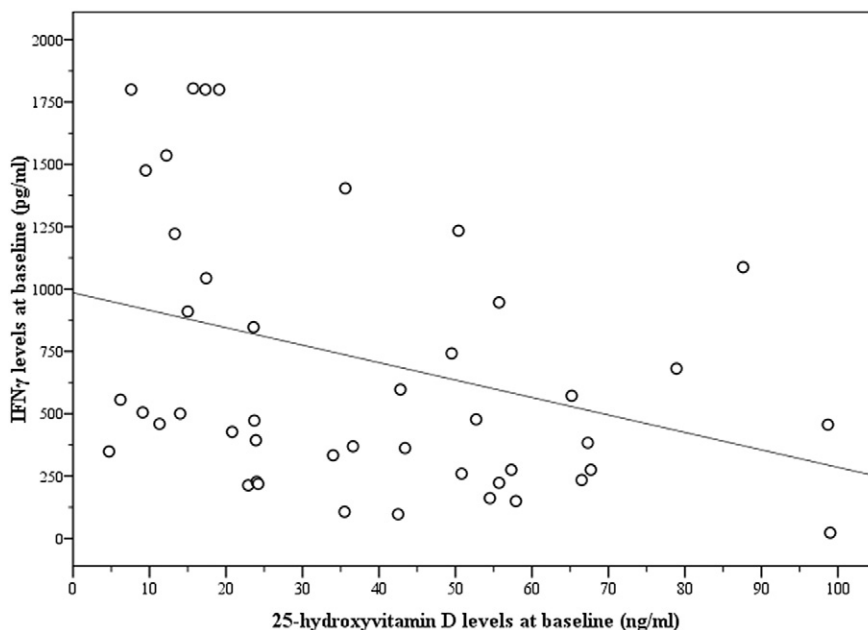


Fig. 4. Correlation between vitamin D and IFN γ levels at baseline (V1) among all participants. Scatter plot showing relation between serum 25-hydroxyvitamin D concentrations and IFN γ levels at baseline (Standardized Beta = -0.32 , $R = 0.32$, $p = 0.031$).

may support the beneficial effects of vitamin D that were reported on the clinical and cognitive outcomes of MS [46–49].

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.clim.2017.05.017>.

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Declaration of conflicting interests

The authors have no conflict of interest to declare.

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