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Modulation of CD28 and p38 Expression by Treatment with Vitamin D in Patients with Rheumatoid Arthritis

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Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease. Vitamin D has been postulated to have a role in autoimmune diseases. The aim of this study was to answer, "whether treatment with vitamin D can improve RA patient outcome". Forty female patients with rheumatoid arthritis with the age of 49.38±13.11 years, and forty healthy females as control with age of 45.14±12.63 years were included in the study. Patients were given a dose of 50,000 IU/week of vitamin D for two months. Blood samples were withdrawn from patients before and after treatment to study the effect of vitamin D on disease activity and its impact on plasma levels of TNF-α. Vitamin impact on the expression of activation and regulatory proteins CD28, CD25, CD120 and TNF receptor I (GITR) on helper CD4 and cytotoxic T cells (CD8) were assessed using flow cytometry. A highly significant difference was detected in total vitamin D in the RA group after treatment with vitamin D (P< 0.0001). Additionally, a significant difference was detected in vitamin D3 in the RA group after treatment with vitamin D compared to the control group (P< 0.0129). The results showed that untreated RA patients had a significant increase in serum TNF- α compared to healthy subjects. After treatment with vitamin D the TNF- α value was significantly decreased (P<0.0001). A significant increase was detected in P38 MAPK in leukocytes of RA patients that were treated with Phorbol 12-myristate 13-acetate (PMA). Administration of 1, 25(OH) vitamin D3 (1,25 D) significantly (P<0.0291) decreased the levels of p38 MAPK in an activated sample. A significant decrease was also detected in regulatory cytotoxic T cells ($CD8^+$ $CD28^-$) in RA group after treatment with vitamin D (P < 0.0337). On the other hand, there was a significant increase in $CD8^+$ $CD28^+$ in RA group after treatment with vitamin D (P<0.05). A significant decrease was detected in $GITR^+$ $CD8^+$ in RA group after treatment with vitamin D (P< 0.0085). A significant increase was detected in $CD4^+$ $CD25^+$ in RA group after treatment with vitamin D (P<0.05). A significant decrease was detected in $CD4^+$ $CD120^+$ in RA group after treatment with vitamin D (P<0.05). A significant decrease was detected in the diagnostic marker for RA after treatment with vitamin D (DAS-28 (P-value = 0.000), CRP (P<0.0008), ESR (P<0.0001), Anti-MCV (P< 0.0001), and rheumatoid factor (P< 0.02620)). In conclusion, the treatment of RA patients with vitamin D showed an improvement in the biochemical and immunological parameters that led to an improvement in physical movement. Vitamin D levels' improvement may be attributed to the modulation of P38 MAPK.

Key words: Rheumatoid arthritis, vitamin D, TNF-alpha cytokines

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Introduction

Vitamin D (Vit D) is a fat-soluble vitamin and can be obtained from food, sun exposure, and supplements (Ali *et al.*, 2018). It is a hormone synthesized in human skin under the stimulation of ultraviolet radiation. It has an important role in bone metabolism (Ishikawa *et al.*, 2017). Vitamin D has also a main contribution in keeping serum calcium and skeletal homeostasis, but is also plays a key role in the regulation of other necessary cellular processes including cellular differentiation, proliferation, apoptosis, and angiogenesis (Zidan *et al*., 2018). It is an important contributor to increase muscle mass, strength and quality of life (Mady *et al*., 2018). Cells of the immune system have vitamin D receptors that trigger dendritic cells, suggesting that vitamin D might have immunoregulatory properties (Mosaad, 2014). Vitamin D deficiency is involved in several health issues, such as immune-mediated diseases like autoimmune disorders (Ishikawa *et al.*,

2017). Rheumatoid arthritis (RA) is a chronic inflammatory joint disease, and often symmetrically influence the small joints of the hands (McInnes and Schett, 2017). Studies showed that vitamin D intake can decrease the risk of RA. The medical enhancement was highly related to the immunomodulating prospective in vitamin D-treated RA patients (Lin *et al.*, 2016).

To maintain systemic tolerance, the immunoregulatory networks must be balanced. In rheumatoid arthritis and other autoimmune diseases, this will occur in the homeostatic balance of inflammatory mediators, immune organizers, and immune effectors (Dai *et al.*, 2013).

Sufficient data have indicated that treatment with 1alpha, 25-(OH) (2) D (3) may considerably suppress TNF-α production (Ishikawa *et al.*, 2017). TNF-α prevents telomerase activity in T cells via a p38 MAPK passage (Di Mitri *et al.* 2011). Vitamin D up-regulates MKP5 (novel calcitriol-responsive gene) expression, leading to a decrease in the production of pro-inflammatory cytokines by interfering with the signaling of pleiotropic inflammatory cytokines, like TNFα, supporting a role for vitamin D in the protection and early therapy of autoimmune disorders (Liu *et al.*, 2018). Senescence in T cells via a p38 MAPK pathway cells is regulated by active cell signaling ways. The reduced expression of CD28 in T cells compromises immunocompetence in RA patients (Parish *et al.*, 2009).

Therefore, the aim of this study was to study the effect of 50,000 IU/week of vitamin D treatment in the homeostatic balance of inflammatory mediators, immune organizers, and immune effect in rheumatoid arthritis (RA) patients.

Materials and Methods

Patients

The investigation included patients from the rheumatology outpatient clinic at the King Abdulaziz University Hospital, Saudi Arabia. This research comprised of 40 female patients with rheumatoid arthritis aged 49.38±13.11 years and 40 healthy females as control with the age of 45.14±12.63 years. Patients were given a dose of 50,000 IU/week of vitamin D for two months. Blood samples were taken from patients pre- and post-treatment. The diagnosis of RA was carried out at the Department of internal medicine at King Abdulaziz University Hospital according to the American College of Rheumatology (ACR) criteria for the diagnosis of RA (Arnett *et al.*, 1988). Disease activity was determined utilizing DAS28-CRP/ESR based on previously established techniques (Prevoo *et al.*, 1995). Based on DAS-28, patients were classified as active RA (ARA) and inactive RA (IRA). The investigation was verified by the ethical committee and the procedures followed were in line with the ethical standards of Faculty of Medicine at King Abdulaziz University ethics committee.

Blood Separation for Serum and Buffy Coat Collection

Six ml of blood was withdrawn from participants in Ethylenediamine tetra-acetic acid (EDTA) tubes and separated within 12 hours by centrifugation at 1500 rpm, 4 ℃, acceleration = 9, deceleration = 9, for 5 minutes. Plasma were collected and kept in 1 ml Eppendorf tubes and stored at -80 ℃ until analyzed. For the flow cytometric analysis, the remaining blood was collected and treated for buffy coat collection (lymphocytes).

Biochemical analyses

Total vitamin D was measured using the ADVIA Centaur and ADVIA Centaur XP system, Cat. No. 10699201 (100 tests). Erythrocyte sedimentation rate (ESR) was measured using the Wintergreen technique. C-reactive protein (CRP) was measured by nephelometry.

Immunostaining

Immunostaining was performed by 1) ELISA (Enzyme-Linked Immunosorbent Assay) kits, using BioTek (ELx50/8) Microplate Strip Washer and BioTek (ELx800) multichannel absorbance microplate reader, and 2) Flow Cytometry technique (Gallios Flow Cytometer) using Navios software.

Enzyme-Linked Immunosorbent Assay

For manual Immunostaining, the following antibodies were used: 1) Human 25 Hydroxy Vitamin D (25OHVD) (MyBioSource.com, Cat. No. MBS722906) was used for assessment of vitamin D3, and 2) Human TNF-alpha (Biolegend ELIZA MAX DELUXE Sets, Cat. No. 430206) for both patients and healthy controls. All samples were measured in duplicate.

Flow cytometric analysis

Lymphocytes samples (Buffy Coat) that have been frozen in the freezing media, were thawed in room temperature and transferred into tubes containing phosphate buffered saline (PBS) and washed by centrifugation (1500 rpm, acceleration=9, deceleration=9 (BREAK)) for 10 minutes. Lymphocytes were then transferred into three tubes to be stained with appropriate antibodies. The first tube was blank (no antibody), the second tube was stained with single antibody and the third one was mixed with the required antibody.

The antibodies used for this study are:

- PE/Cy7 anti-human CD8 Antibody (Biolegend) (yellow Laser, Excitation max. 496,565 (nm)).
- FITC anti-human CD28 antibody (Biolegend) (Blue Laser, Excitation max. 493 (nm)).
- APC anti-human CD357 (GITR) Antibody (Biolegend) (red Laser, Excitation max. 652 (nm)).
- PE anti-human CD4 Antibody (Biolegend) (yellow Laser, Excitation max. 496,565 (nm)).
- Alexa fluor ® 647 MS Anti-P38MNFR: BD (red Laser, Excitation max. 650 (nm)).
- Alexa fluor ® 647 Rt anti-HU MNFR: BD BD (red Laser, Excitation max. 650 (nm)).

- APC/Cy7 anti-HU MNFR: BD BD (red Laser, Excitation max. 650 (nm)).

The tubes kept in dark for 30 minutes, and read by using Gallios Flow Cytometer with Navios Software, and then the resulted graphs were saved for later analysis by statistical programs.

Phosphorylation/Translocation assay

After buffy coat collection, the lymphocytes were added to 6-well plates with culture media RPMI-1640. the first well left as a blank, 2 microliter from SB 239063 was added to the second well as P38 inhibitor, 5 microliter from phorbol 12-myristate 13-acetate (PMA) was added to the third well as P38 activator, the fourth well had 5 microliter from PMA and 16.5 microliter of vitamin D (PMA should be added one hour before vitamin D) , the fifth well had P38 inhibitor (16.5 microliter from 3 mM vitamin D) and in the last well 5.5 microliter from 1 mM vitamin D were added. Then, the plate was incubated for 24 h in CO_2 at 37 ℃. P38 MAPK (Phospho-Thr180/Tyr182) cell-based phosphorylation/translocation assay kit (Cayman chemical company Cat. No. 10010374) was used. It provided a highly specific phospho-p38 MAPK (phospho-Thr180 and Tyr182) primary antibody together with a DyLight™ conjugated secondary antibody in a ready-to-use format. Then samples were read by using Gallios Flow Cytometer with Navios Software and resulted graphs were saved for later analysis by statistical programs.

Statistical analysis

Data are expressed as mean ± SM. Analyses were carried out with the Statistical Package for the Social Science (SPSS for Windows, version 20, Chicago, IL). To determine treatment effect and compare differences among group means, data were analyzed by One-way ANOVA. Statistical significance was accepted at P<0.05.

Results

Figure (1) shows the percentage of $CD4^+P38^+$ in cells of RA patients and untreated control cells. A significant increase was detected in P38 MAPK in leukocytes of RA Patients that were treated with Phorbol 12-myristate 13-acetate (PMA). Administration of 1, 25(OH) vitamin D3 (1,25D) significantly (P<0.0291) decreased the levels of p38 MAPK in the activated samples.

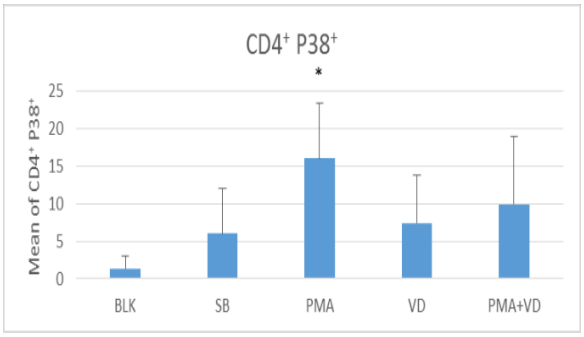

Figure 1: Mean percentage of $CD4^+$ $P38^+$ in cells of RA Patients and untreated control cells.

*significant different between PMA and blank, vitamin D, vitamin D PMA and SB.

The results in Figure (2) showed the mean percentage of $GITR^+$ $CD8^+$ in ARA, IRA, and healthy control. A significant decrease was detected in $GITR^+$ $CD8^+$ in RA group after treatment with vitamin D (P <0.0085).

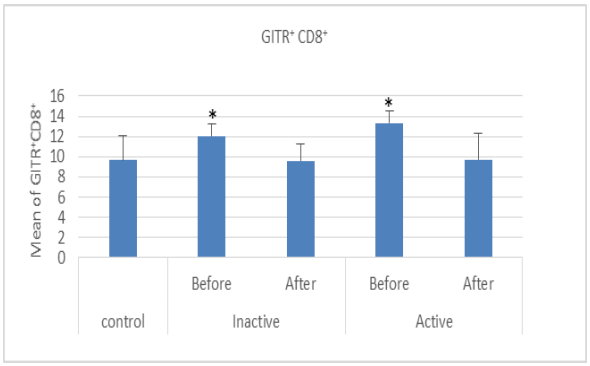

Figure 2: Mean percentage of $GITR^+$ $CD8^+$ in ARA, IRA, and healthy control.

*Significant difference was detected in $GITR^+$ $CD8^+$ between ARA and IRA patients before treatment and control. Significant difference was detected before and after treatment with vitamin D in ARA and IRA. n=9 for control, n=24 for ARA, and n=13 for IRA.

Figure (3) shows the mean percentage of $CD4^+$ $CD120^+$ in ARA, IRA, and healthy control. A significant difference was detected in $CD4^+$ $CD120^+$ in ARA and IRA patients before and after treatment with vitamin D as compared to the healthy control. The P-value was P < 0.05.

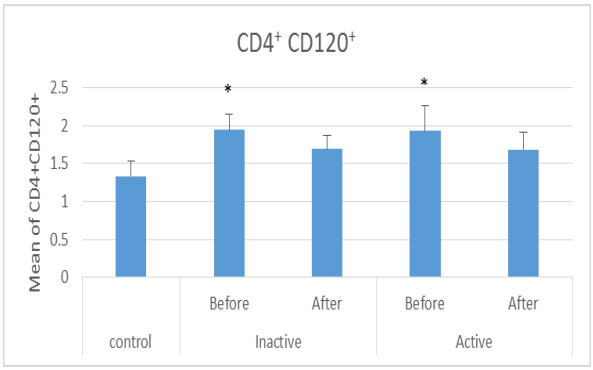

Figure 3: Mean percentage of $CD4^+$ $CD120^+$ in ARA, IRA, and healthy control.

*Significant difference was detected in $CD4^+$ $CD120^+$ between ARA and IRA patients and before and after treatment with vitamin as compared to the healthy control. n=9 for control, n=24 for ARA, and n=13 for IRA.

Figure (4) illustrates the mean percentage of $CD4^+$ $CD25^+$ in ARA, IRA, and healthy control. No considerable variation was revealed in CD4+ CD25+ among IRA and IRA patients and control. On the other hand, a significant difference was detected in $CD4^+$ $CD25^+$ between before and after treatment with vitamin D in ARA and IRA patients. The P-value was P<0.05.

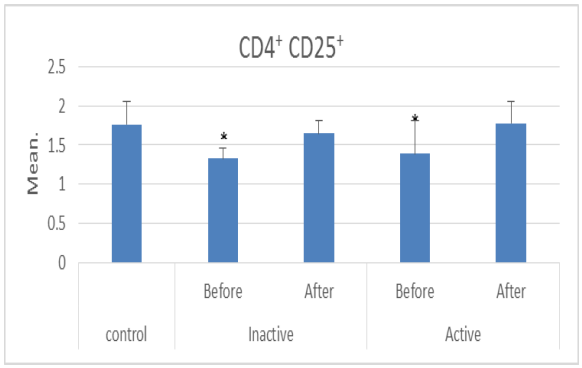

Figure 4: Mean percentage of $CD4^+$ $CD25^+$in ARA, IRA, and healthy control.

*Significant difference was detected in $CD4^+$ $CD25^+$ between before and after treatment with vitamin D in ARA and IRA patients. n=9 for control, n=24 for ARA, and n=13 for IRA.

The results in Figure (5) revealed that the mean percentages of the plasma concentrations of TNF-α were significantly increased in patients with active RA and inactive RA compared to that of healthy subjects. After treatment with vitamin D, the TNF-α level significantly decreased. The P-value was < 0.0001.

Based on the results in Table (1) regarding the variation in clinical and biochemical markers of patient groups and the healthy group (control), a considerable decrease was observed in the diagnostic marker for RA after treatment with vitamin D (DAS-28 (P-value<0.000), CRP (P <0.0008), ESR (P < 0.0001), anti-MCV (P<0.0001), and rheumatoid factor (P < 0.02620)). A significant difference was detected in total vitamin D in RA group after treatment with vitamin D (P<0.0001). A significant difference was detected in vitamin D3 in RA group after treatment with vitamin D as compared to control (P < 0.0129).

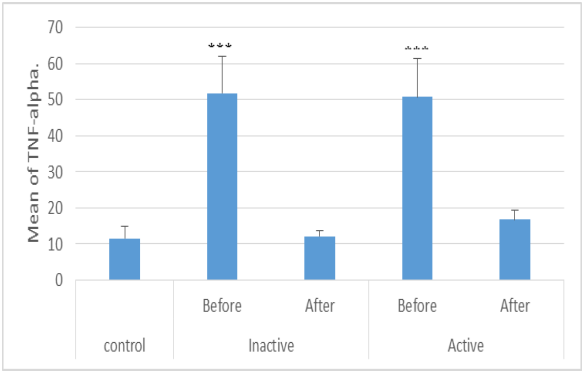

Figure 5: Mean percentages of the plasma concentrations of TNF-α**.**

*Significant difference was detected in TNF-α between before and after treatment with vitamin D in ARA, IRA patients and control. n=9 for control, n=24 for ARA, and n=13 for I.

Table 1: Variations between clinical and biochemical markers of patient groups compared with healthy group as a control.

Clinical and biochemical markers	Control	Inactive		Active	
		Before	After	Before	After
RF	11.75±5.793	*99.32±12.962	**79.10±17.020	*176.80±4.385	**101.63±12.303
Anti-MCV		*75.93±2.323	**61.56±7.326	*77.00±2.864	**64.25±11.033
DAS-28		4.2005±0.118	3.9358±0.134	5.9275±0.243	5.5767±0.139
ESR	25.273±2.831	*35.615±5.303	**28.3500±3.362	*44.200±6.443	**38.5556±5.210
CRP	5.027±0.654	*12.7500±2.081	**7.500±1.48404	*14.546±3.279	**8.800±2.3084
Vitamin D total	51.187±2.923	39.500*±3.276	65.300**±5.442	37.00*±3.795	72.000**±6.466
Vitamin D3	12.20±1.496	11.50±1.781	11.75±1.566	8.22±0.846	8.75±1.750

Value are presented as mean ± SE, n = 40 for control, n = 24 for ARA, n = 13 for IRA, *significant different.

The results presented in Table (2) represent the mean percentages of $CD28^- CD8^+$ and $CD28^+ CD8^+$ in patients with ARA and IRA. A significant decrease was also detected in regulatory cytotoxic T cells ($CD8^+ CD28^-$) in RA group after treatment with vitamin D ($P < 0.0337$). On the other hand, there was a significant increase in ($CD8^+ CD28^+$) in RA group after treatment with vitamin D ($P < 0.05$).

Table 2: Mean percentages of $CD28^- CD8^+$ and $CD28^+ CD8^+$ in patients with ARA and IRA.

	Control	Inactive		Active	
		Before	After	Before	After
CD28-CD8+	14.40±1.42	*18.57±1.547	16.19±1.246	*19.25±1.750	14.29±1.769
CD28+ CD8+	25.57±6.200	26.15±4.712	24.00±5.475	**7.71±1.017	*38.63±9.320

Value are presented as mean ± SE, n=9 for control, n=24 for ARA, n=13 for IRA,*significant different.

Discussion

The adequate levels of vitamin D (25(OH) D3) are essential for the ideal role of the body, as observed by the outcomes of 25(OH) D3 shortage. Epidemiological research in Saudi Arabia reported that 25 (OH) D3 insufficiencies are common in 28.3 % of males and 44.6 % of females (Hussain *et al*., 2014).

25 (OH) D3 deficiency has been related to diseases like osteoporosis, thyroid, Crohn's disease, cancer, blood pressure diseases, type 1 diabetes, and rheumatic autoimmune disorders like systemic lupus erythematosus and rheumatoid arthritis (RA). Growing research have shown that 25 (OH) D3 insufficiencies are common in patients with RA (Haga, 2013).

Rheumatoid arthritis (RA) is a systemic inflammatory disease differentiated by chronic and erosive polyarthritis may be due to the abnormal growth of synovial tissue and it could be an irreversible joint inability (Lin *et al.,* 2016). In the present study, RA patients were found to be deficient in vitamin D. However, vitamin D level was increased significantly after treating RA patients with 50,000 IU/week vitamin D for two months.

Rheumatoid arthritis is suggested to be regulated in part by the p38 pathway. The p38 MAPK signaling pathway explains how cells regulate a wide variety of external signals and respond suitably by producing many biological effects (Liu *et al.,* 2018).

It has been suggested that vitamin D is a P38 inhibitor by inhibiting the production of proinflammatory cytokines in monocytes (Ding *et al.,* 2013). In agreement with these data, our results showed significant decrease in p38 after treating lymphocytes with vitamin D *ex-vivo* even when PMA were added with vitamin D.

P38 is also a critical regulator of TNF-α production (Zhang *et al.,* 2012). Individuals with RA have high levels of TNF-α in the synovial fluid and it plays an important role in inflammation and joint destruction that are distinguish feature of RA (Kuo *et al.,* 2010; Ishikawa *et al.,* 2017; Liu *et al*., 2018). Our findings agree with previous studies which reported that treatment with vitamin D could significantly suppress TNF-a production.

More research in the past have observed that cytokine TNF-α can encourage loss of CD28 expression and T cell discrimination and prevent telomerase activity in suppressor CD8 T-lymphocytes (Parish, Wu *et al.,* 2009). Peripheral blood CD8 CD28- regulatory T cells (Treg) contribute to the immunoregulatory network in health and in RA (Ceeraz *et al.,* 2013). Also, it has been shown that there is an increase in the $CD8^+ CD28^-$ in patients. Our study also showed that $CD8^+CD28^-$ are significantly higher in RA, and that their percentages decreased significantly after vitamin D exposure. Our study also agree with Dai *et al* (2013) study that the $CD8^+CD28^+$ are decreased in ARA patients, and after treatment with vitamin D, $CD8^+CD28^+$ levels are increased.

$CD4^+CD25^+$ regulatory T cells (Treg) play an important role in the maintenance of immunological tolerance. In RA, the degree of immune regulation is affected by the balance between activated regulatory $CD4^+CD25^+$ T cells and inflammatory cells. $CD4^+CD25^+$ Tregs were decreased in the peripheral blood of patients with early rheumatoid arthritis (Minami *et al.,* 2006). Our study showed that $CD4^+CD25^+$ percentages were lower in RA, and after vitamin D consumption CD4+CD25+ percentages increased which helped to regulate immune response in RA patients.

GITR ligand (GITRL) is one of the TNF superfamilies that perform various roles in the cells of immune system. When GITRL binds to its receptor, GITR inhibits the suppressive functions of $CD4^+CD25^+$ Treg cells, and inducse macrophages activation. $CD8^+$ T cells are key players in the immunity against viruses and tumors, and GITR has been demonstrated to be an important molecule for these cells to induce an immune response (Ronchitti *et al.,* 2012). Our study revealed that $CD8^+$ $GITR^+$ levels are significantly higher in RA, and after vitamin D consumption $CD8^+$ $GITR^+$ levels are decreased in RA patients.

CD120b which belongs to the TNF receptor superfamily can interact with ligand TNF-α. TNFRII has been shown to mediate MAPK activation. The important role of CD120b is influencing apoptosis of numerous cells (Chan and Lenardo, 2000).

In RA, the increase of CD120b expression specifically on $CD4^+$ T cells led to an accelerated onset of disease and to more-severe signs of inflammation (Bryl *et al.,* 2005). Our finding showed that $CD120b^+$ $CD4^+$ is significantly higher in RA compared to healthy

control. CD120b$^+$ CD4$^+$ levels decreased in RA patients after vitamin D consumption.

There are many studies linking vitamin D supplementation with improvement of muscle strength and body balance (Muir and Montero-Odasso 2011; Halfon *et al.*, 2015). In addition, there was an association between insufficient vitamin D level and chronic pain, skeletal muscle weakness, and mood disorders (Raczkiewicz, 2015). Our study agrees with Raczkiewicz (2015) that showed an improvement in the movement of the patients and they were able to walk easily with less pain.

Several studies have assessed the link between vitamin D levels and disease activity in RA (Sen and Ranganathan, 2013). Our study coincides with Sen and Ranganathan, as we found a significant decrease in DAS-28, CRP, ESR, Anti-MCV, and rheumatoid factor, the diagnostic markers for RA, after treatment with vitamin D.

In conclusion, treatment of RA patients with vitamin D showed an improvement in biochemical parameters that led to improvement in physical movement. Vitamin D levels normalization after treatment with a dose of 50.000 IU/week of vitamin D3 led to improvement in the modulation of P38 MAPK and immunophenotyping. Further studies on larger patient populations are warranted to validate the usefulness of vitamin D in the management plans of RA.

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Declaration of interest

The authors declare no conflict of interest associated with this work.

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