



## Vitamin D Increases CTLA-4 Gene Expression in Patients with Mild to Moderate Ulcerative Colitis

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### ABSTRACT

#### BACKGROUND

Ulcerative colitis (UC) is a chronic inflammatory disorder of the large intestine. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a member of the immunoglobulin superfamily, which binds B7-1 and B7-2 on APCs (antigen-presenting cells), and induces APCs to produce an inhibitory signal to T cells. The aim of this study was to investigate the effect of vitamin D on CTLA-4 gene expression in whole blood samples of patients with UC.

#### METHODS

90 patients with mild to moderate UC were randomized to receive either a single injection of 7.5 mg vitamin D3 or 1 mL normal saline. 90 days following the intervention fold changes in CTLA-4 mRNA expression were determined and statistical comparisons between the two groups were performed.

#### RESULTS

Serum vitamin D increased significantly only in the vitamin D group. CTLA-4 fold changes were significantly higher in the vitamin D group compared with the placebo group (median  $\pm$  IQR:  $1.21 \pm 2.3$  vs.  $1.00 \pm 1.5$ , respectively;  $p = 0.007$ ).

#### CONCLUSION

The results of this study revealed that vitamin D administration in patients with UC enhances the CTLA-4 gene expression.

#### KEYWORDS:

Ulcerative Colitis; Inflammatory Bowel Disease; CTLA-4; Vitamin D; Gene Expression

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### INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic relapsing-remitting inflammatory disorder of the alimentary tract, which includes two main forms, ulcerative colitis (UC) and Crohn's disease (CD). Although to date the etiology of IBD has not been completely understood, a body of evidence suggests that genetic abnormalities coupled with particular environmental factors may lead to IBD. <sup>1-5</sup> In this regard different specific genetic abnormalities have been identified, which are responsible for IBD. One of them is genetic variants of the cytotoxic T-



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lymphocyte-associated protein<sup>4</sup> (CTLA-4) that has been shown to be related to various autoimmune diseases,<sup>6-10</sup> including IBD.<sup>11</sup> A recent meta-analysis suggested that the CTLA-4 + 49A/G variant may be associated with susceptibility to CD, and the CTLA-4 CT60 variant may increase the risk of UC in Asians.<sup>12</sup>

CTLA-4, also known as CD125 (cluster of differentiation 152), is mainly expressed on the surface of T lymphocytes, functioning as an immune checkpoint, which produces an inhibitory signal to T cells.<sup>10</sup> CTLA4 and CD28 have a sequence homology, and both can attach to B7-1 and B7-2 (CD80 and CD86) on the APCs with different affinities. CTLA-4 binds B7-1 and B7-2 with higher affinity, and as a negative regulator in immune system induces an inhibitory signal to T cells, whereas binding of CD28 to B7-1 and B7-2 induces a stimulatory signal.<sup>13-15</sup> The comparatively higher binding affinity of CTLA-4 to B-7 receptors has made it a potential and promising candidate for attenuating immune responses in autoimmune diseases. So, in this regard, previous research has focused on factors that increase the expression of CTLA-4 in inflammatory diseases like rheumatoid arthritis and showed its promising effect in increasing remission in such patients.

Vitamin D has been shown to antagonize the inhibitory effect of inflammatory cytokines on CTLA-4 and enhances CTLA4-induced suppression of inflammation.<sup>16</sup> In addition, calcitriol increased CTLA-4 gene expression in human CD4<sup>+</sup> CD25<sup>-</sup> T-cell cultures.<sup>17</sup> It also increased CTLA-4 expression in T cells of patients with type 1 diabetes mellitus with a CD4 + CD25 high and CD127 low profile.<sup>18</sup> Furthermore, vitamin D enhanced the CTLA-4 expression in both healthy and patients with latent tuberculosis (TB).<sup>19</sup> Therefore, it seems that calcitriol (the active form of vitamin D) has a synergistic effect with CTLA-4 in the suppression of inflammatory responses. In addition, *in vitro* studies revealed that calcitriol enhanced the expression of CTLA-4 by direct effects upon the T cell even in the presence of Th17 polarizing cytokines.<sup>16</sup> However, the possible direct effect of vitamin D on CTLA-4 expression has not yet been clarified *in vivo*.

Considering the anti-inflammatory effects of vitamin D in patients with UC,<sup>20</sup> and its effect on increasing the CTLA-4 expression in *in vitro* studies,<sup>16</sup> we hypothesized that the anti-inflammatory effects of vitamin D in patients with IBD may be partly related to its ability to increase

CTLA-4 expression. Therefore, in the present clinical trial for the first time, we investigated vitamin D effect on CTLA-4 gene expression in whole blood samples of patients with mild to moderate UC.

## MATERIALS AND METHODS

In this two-arm double-blind randomized placebo-controlled trial, following primary assessment for eligibility, 90 patients with UC were randomized to receive either a single injection of 7.5 mg vitamin D3 (vitamin D group) or 1 mL normal saline (placebo group).<sup>20</sup> Investigators and patients were kept masked to group assignments, and allocation/injection staff did not participate in any of the study clinical evaluations or assays. The complete details of the inclusion and exclusion criteria and characteristics of the patients have been discussed before.<sup>20</sup> The study was conducted using the 90 cDNA samples of our previous study, and at the significant level of less than 5%, the precision power higher than 80% was obtained.

The trial was registered at Iranian Registry of Clinical Trials (IRCT) (register number: IRCT2014062318207N1). The study was approved by the Ethics Committee of Golestan University of Medical Sciences. Written informed consent was obtained from all the patients prior to participating in the study.

### Experiments:

Before (maximum 30 minutes) and 3 months after the intervention, fasting blood samples were collected. Total RNA was immediately isolated from whole blood using QIAamp RNA Blood Mini Kit (Qiagen, USA), and treated with DNase 1. The optical density of each RNA isolate was measured at 280, 260, and 230 nm to control for quality and quantity of RNA. Reverse transcription of 1 µg RNA was performed using GeneAll HyperScript<sup>TM</sup> Reverse Transcriptase (GeneAll, South Korea) with oligo-dT and random hexamer primers in 20 µL reaction volume and cDNA of all samples were obtained. The cDNA samples were stored at -80 degrees Celsius until the real-time polymerase chain reaction (PCR) tests. Duplicate real-time quantitative reverse transcriptase PCR (qRT-PCR) were performed by the LightCycler<sup>®</sup> 2.0 Instrument (Roche, Germany) for determination of CTLA-4 relative gene expression. Succinate dehydrogenase complex subunit A (SDHA) gene was used as a reliable

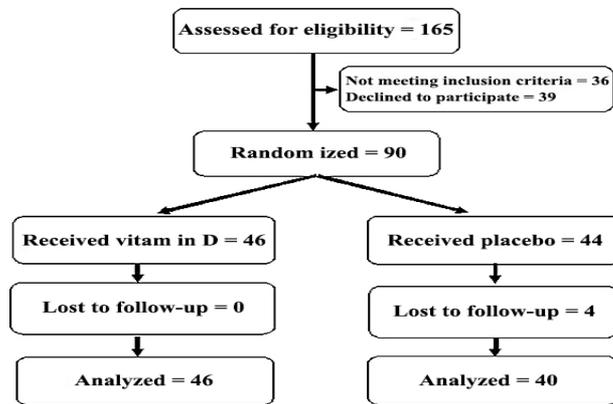


Fig.1: Consort flow diagram of the study.

internal control.<sup>21</sup> PCR was run in a final reaction volume of 20  $\mu$ L, containing 10  $\mu$ L of 2  $\times$  SYBR Green Master Mix (Ampliqon; Denmark), 1  $\mu$ L (10 ng) of forward and reverse primers, and 1  $\mu$ L of cDNA (equivalent to about 50 ng RNA) and PCR-grade water. Reaction mixtures either without reverse transcriptase or template (NTC) were included in each experiment for quality control. The standard curves for both CTLA-4 and SDHA of four serial dilutions of cDNA (50 ng to 50 pg) were plotted. All PCR efficiencies were above 95%.

The CTLA-4 (NM\_001037631) and SDHA (NM\_001294332) primers were designed by Primer3 software. Online medical databases were also searched for obtained primers to find out if they have been used before. Primers for CTLA-4 were as follow: forward: 5'-CTACCTGGGCATAGGCAACG-3', reverse: 5'-CCCCGAACTAACTGCTGCAA-3', and for SDHA: forward: 5'-TGGGAACAAGAGGGCATCTG-3', reverse: 5'-CCACCACTGCATCAAATTCATG-3'.<sup>22</sup>

The amplification profile was as follows: 10 minutes initial denaturation at 95°C followed by 40 cycles of 15 seconds denaturation (95°C), 20 seconds annealing (56°C), and 20 seconds extensions (72°C). At the end of each run, PCR machine was set for 15 seconds at 95°C, 60 seconds at 60°C, and the final temperature at 95°C with 0.1°C/s slope, to plot the melting curve.

Fold changes in CTLA-4 mRNA expression normalized to SDHA and relative to baseline values were determined for each patient using the  $2^{-\Delta\Delta Cq}$  method<sup>23</sup> by a Microsoft Excel file (Microsoft Office 2016) formulated specifically for this study, on which  $\Delta\Delta Cq = (Cq_{CTLA-4} \text{ After} - Cq_{SDHA} \text{ After}) - (Cq_{CTLA-4} \text{ Before} - Cq_{SDHA} \text{ Before})$ .

### Statistical analysis:

Statistical analyses were done by SPSS software version 25.0 (IBM SPSS Inc., Chicago, Illinois, United States). The normality of distribution was checked using Kolmogorov-Smirnov test. Skewed distribution data were expressed as medians  $\pm$  interquartile ranges. Comparisons between the two groups were performed using independent t test for variables with normal distribution and Mann-Whitney U test for variables with skewed distribution. Spearman's rho test was used for the correlation analyses. Statistically significant level was set at  $p$  value  $< 0.05$ .

### RESULTS

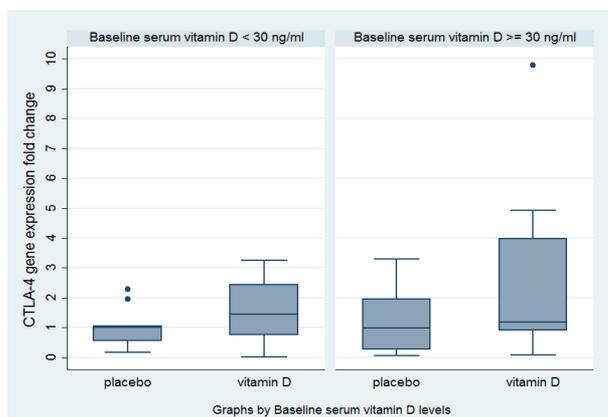
86 patients (40 in the placebo group, and 46 in the vitamin D group) completed the study. Four in the placebo group refused to complete the study. The CONSORT flow diagram of the trial is presented in figure 1.

At baseline, mean serum levels of 25(OH) D<sub>3</sub> of vitamin D and placebo groups were  $33.3 \pm 7.0$  and  $32.9 \pm 9.6$  ng/mL, respectively ( $p = 0.82$ ) and 90 days after the intervention, serum vitamin D levels were higher compared with the baseline levels only in the vitamin D group, which were also significantly higher than the placebo group ( $40.8 \pm 5.2$  and  $33.9 \pm 10.6$ , respectively,  $p < 0.001$ ).<sup>20,24</sup> The baseline values of serum calcium, parathormone, hs-CRP, erythrocyte sedimentation rate (ESR), dietary vitamin D, calcium intake, calorie intake and macronutrient composition, age, disease duration, systolic and diastolic blood pressure, body mass index (BMI), heart rate, and body temperature were not statistically different between the two groups. Drug regimen and sex proportion were also comparable.<sup>20,24</sup>

According to the result of Mann-Whitney U test, CTLA-4 gene expression fold changes were significantly higher in the vitamin D group compared with the placebo group (median  $\pm$  IQR:  $1.21 \pm 2.3$  vs.  $1.00 \pm 1.5$ , respectively;  $p = 0.007$ ).

We also did a subgroup analysis regarding the baseline vitamin D levels (lower, equal, or higher than 30 ng/mL). Gene expression fold change was only significantly higher in the vitamin D group only in whom with baseline vitamin D levels equal to and higher than 30 ng/mL ( $p = 0.029$ , figure 2).

There was a negative but not significant correlation



**Fig.2:** fold change in CTLA-4 (Cytotoxic T lymphocyte associated 4) gene expression normalized to SDHA (Succinate dehydrogenase complex subunit A) and calibrated for baselines regarding baseline vitamin D levels (median  $\pm$  IQR).

between CTLA-4 gene expression fold change and hs-CRP levels (Spearman's  $\rho = -0.2$ ;  $p = 0.056$ ). In addition, there was a significant negative correlation between CTLA-4 gene expression fold change and ESR levels (Spearman's  $\rho = -0.31$ ;  $p = 0.004$ ).

## DISCUSSION

We have previously shown that vitamin D supplementation in patients with UC could have a beneficial anti-inflammatory effect,<sup>20</sup> however, its underlying mechanisms remained unclear. One proposed mechanism, through which this may occur, is its effect on immune related gene expressions such as CTLA-4. Previously, an in vitro study showed that calcitriol enhanced the expression of CTLA-4 by direct effects on the T cell even in the presence of Th17 polarizing cytokines.<sup>16</sup> This effect has also been shown by other studies.<sup>17-19</sup> To the best of our knowledge, there is no clinical trial evidence for this effect. The results of our study for the first time revealed that vitamin D administration in patients with mild to moderate UC enhances the CTLA-4 gene expression. A significant negative correlation between CTLA-4 gene expression and ESR levels (as a clinical inflammatory marker) was also found.

Altering the expression of CTLA-4 gene is an important pathway by which T-reg cells and other CTLA-4 expressing immune cells evoke immune tolerance.<sup>25</sup> The expression of CTLA-4 by effector T cells has been shown to affect the T cell response duration,<sup>26,27</sup> which may be a crucial part of T cell energy after the activation

of immune system. These findings highlight the importance of suppressing immune responses in the treatment of autoimmune diseases such as IBD, in which induction of CTLA-4, for example, might decrease the inflammation in such pathological situations.

It is now demonstrated that peripheral monocytes, leukocytes, and activated CD4 + and CD8 + T cells possess vitamin D receptor (VDR).<sup>28</sup> Furthermore, 1, 25(OH)<sub>2</sub>D<sub>3</sub> was shown to decrease the proliferation of Th1 and Th17 cells while stimulation of T regulatory cell,<sup>29</sup> was supported by the evidence of the link between vitamin D deficiency with the severity of IBD,<sup>30</sup> and supported by the idea that vitamin D administration could produce beneficial anti-inflammatory impacts in patients with UC.<sup>20</sup> It has been proposed that the principal way by which vitamin D diminishes T cell responses is via down-regulation of co-stimulatory molecules on APCs.<sup>31</sup> Accordingly, in vitro studies have shown that calcitriol induces CTLA-4 through direct effects on the T cells. Moreover, the suppressive effect of calcitriol on T cells is further boosted through the removal of CD80 and CD86 by CTLA-4-mediated transendocytosis.<sup>16</sup> All these findings suggest that low levels of vitamin D followed by decreased CTLA-4 expression could lead to exaggerated inflammatory reactions in pathological inflammatory situations,<sup>16</sup> the hypothesis that has now been shown in our study.

The results of this study also showed that vitamin D supplementation had significant effect on CTLA-4 gene expression in whom with baseline sufficient vitamin D levels, by which it can be concluded that for achieving an efficient effect on CTLA-4 gene expression, final vitamin D levels higher than the current common normal level cut-offs might be needed. However, in this regard low statistical power of subgroup analysis must be considered.

In conclusion in the current study, we showed that vitamin D supplementation in patients with UC upregulates CTLA-4 gene expression as a protective mechanism against the severity of the disease. As we have noted before<sup>20,24</sup> there were some limitations in this study. First of all, we did not screen patients for baseline vitamin D deficiency. Second, only patients with mild to moderate UC were included. Third, we did not assess the dose-response effect of vitamin D. Therefore, larger studies with multiple doses of vitamin D and longer observations

periods are encouraged. Conducting a similar study in vitamin D deficient patients with UC in relapse phase using different doses of vitamin D is suggested.

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#### Authors' contributions:

Amrollah Sharifi: Literature search; the conception and design of the study; carry out the study, acquisition, statistical analysis, and interpretation of the data; drafting the manuscript; final approval of the version to be submitted; agreement to be accountable for all aspects of the work.

Homayoon Vahedi: Design of the study; interpretation of data; revising the manuscript critically for important intellectual content; final approval of the version to be submitted; clinical supervision; agreement to be accountable for all aspects of the work.

Mohammad Reza Honarvar: Acquisition of data, revising the manuscript critically for important intellectual content, final approval of the version to be submitted; agreement to be accountable for all aspects of the work.

Behnam Alipoor: Acquisition of data; interpretation of data; revising the manuscript critically for important intellectual content; final approval of the version to be submitted; agreement to be accountable for all aspects of the work.

Zeinab Nikniaz: Acquisition of data; interpretation of data; revising the manuscript critically for important intellectual content; final approval of the version to be submitted; agreement to be accountable for all aspects of the work.

Hossein Rafiei: Acquisition of data; interpretation of data; revising the manuscript critically for important intellectual content; final approval of the version to be submitted; agreement to be accountable for all aspects of the work.

Mohammad Javad Hosseinzadeh-Attar: Design of the study; interpretation of data; revising the manuscript critically for important intellectual content; final approval of the version to be submitted; agreement to be accountable for all aspects of the work.

#### ETHICAL APPROVAL

There is nothing to be declared.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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