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# Vitamin D3 induces the expression of membrane progesterone receptors (mPRs) on naive CD4$^+$ T lymphocyte cells in women of reproductive age

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ABSTRACT

*Objective:* Vitamin D3 and progesterone (P4) both belong to steroid hormones. These hormones have effects on the function of each other in different ways. The immunomodulatory activity of vitamin D3 and P4 and their role in inducing maternal tolerance for fetus have been shown in various studies. The purpose of this study was to evaluate the effect of vitamin D3 on the expression of membrane progesterone receptors (mPRs) on CD4$^+$ T cells.
*Materials and methods:* Naive CD4$^+$ T cells were isolated from peripheral blood of 38 healthy women of childbearing age. After stimulating by anti-CD3 and anti-CD28 monoclonal antibodies (mAb), these cells were exposed to either various concentrations of vitamin D3 or no exposure at all in a culture medium at 37 °C for 3 days. In the final stage, the mean fluorescence intensity (MFI) of mPRα and mPRβ were evaluated using polyclonal and monoclonal antibodies and several gating strategies on CD4$^+$ T cells.
*Results:* Vitamin D3 significantly increased the expression of mPR α and mPR β on the surface of CD4$^+$ T cells ($p \leq 0.05$).
*Conclusion:* The present study demonstrated the potential effect of vitamin D3 on increasing the expression of P4 receptors on CD4$^+$ T cells. This study shows a new aspect of correlation between vitamin D3 and P4 that may influence P4 performance. Therefore, our findings suggest that the appropriate level of this vitamin may affect the optimum P4 immunomodulatory activity during pregnancy.

## 1. Introduction

Vitamin D3 is a member of the steroid hormones family [1]. The active form of this vitamin is 1 and 25 dihydroxycholecalciferol. In the skin, first 7-dehydrocholesterol is converted to vitamin D3 upon UVB radiation. Further activation of this vitamin is accomplished in the liver and kidney by the enzymes 25-hydroxylase and 1-hydroxylase, respectively [2]. Mineral homeostasis and skeletal health by renal retention, bone resorption, and intestinal absorption are the classical roles of this vitamin [3].

In recent years, non-classical functions for vitamin D3 have been reported. One of the most important of which is the regulation of the immune system which is rather important during pregnancy [3,4]. The fetus is considered as a semi-allograft to the mother, but the mother's immune system does not reject the fetus. However, a defect in the maternal immunological tolerance for fetus during pregnancy can contribute to spontaneous abortion [5,6]. Recent studies have identified the role of vitamin D3 deficiency in incidence of abortion, partly due to its role in the immune system regulation and the creation of maternal tolerance for the fetus [4].

Many studies have revealed the regulatory functions of vitamin D3 on various components of the immune system [3,7,8]. It is likely that vitamin D3 could otherwise affect the immune system, which relates to the association between vitamin D3 and progesterone, another regulatory hormone in the immune system.

Progesterone (P4) is a female steroid hormone which has several functions by binding to specific receptors in target cells. Some of these functions are embryo implantation and preservation of pregnancy [9].

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The hormone is secreted by the corpus luteum in the luteal phase of the menstrual cycle [10] and also by the placenta during pregnancy. By pregnancy progression, the concentration of P4 increases in the peripheral blood of the mother and in the placenta. This hormone maintains pregnancy by reducing uterus contractions. Another important function of this hormone, along its classical roles, is the regulation of the immune system in protecting the mother's tolerance for the fetus [11] which is rather similar to vitamin D3's regulating activity.

One of the important immune cell types which is affected by vitamin D3 and P4 in pregnancy is T cell. The regulatory activity of vitamin D3 and P4 on T cells is accomplished through binding to specific receptors [1]. Vitamin D3 performs its function by binding to a specific receptor in the nucleus of T cells [12]. The Progesterone signaling is performed by both genomic and non-genomic pathways. The genomic pathway is related to nuclear receptors and the non-genomic pathway is related to membrane receptors. P4 promotes quick and non-genomic responses through the membrane receptors [13]. Membrane progesterone receptors (mPRα,β,γ) and progesterone receptor membrane component 1,2 (PGRMC1and PGRMC2) are among the membrane receptors.

Recent studies indicate that T cells are not capable of expressing nuclear receptors, but they express membrane receptors such as mPRα, mPRβ, PGRMC1and PGRMC2 [11,14]. Therefore, progesterone may affect immune cells through these receptors. P4 inhibits the production of primary proliferative cytokines for T cells through the non-genomic pathway by blocking the K + and consequently inhibiting the rapid signaling via $Ca^{2+}$ secretion [15].

Steroid hormones such as vitamin D3, progesterone and mineralocorticoids play important roles in the regulation of various biological processes. These hormones can influence each other in different ways, even though they have their own functions through receptor or receptors. The steroid hormones in the same family can affect the hormonal performance of each other [1]. Some studies indicated that the production and function of progesterone is influenced by vitamin D3, although the mechanism has not been exactly determined [16,17]. Considering the importance of vitamin D3 and progesterone in preserving pregnancy and confirming the effect of their interactions on different aspects of function, this study aimed to investigate the effect of vitamin D3 on the expression of mPRα and mPRβ, two progesterone membrane receptors, in order to show the probability of the association between vitamin D3 and progesterone in the immune system. Any possible effect of vitamin D3 on progesterone receptors of $CD4^+$ T cells may affect the regulatory function of this important hormone during pregnancy. Furthermore, this study demonstrates a reason for addressing whether vitamin D3 is able to help maintain pregnancy, by preserving the integrity of the fetus through the effects on the progesterone regulatory activity.

## 2. Materials and methods

### 2.1. Subjects

This study was conducted in Isfahan University of Medical Sciences from October 2017 to October 2018. Thirty eight healthy women aged 20–35 years participated in the study considering the following inclusion criteria: women with regular menstrual cycles, lack of pregnancy and breastfeeding at the time of sampling, no history of abortion, cysts, myomas, and any recent infections in the past, non-use of hormonal contraceptives. It is notable that participants with low serum levels of vitamin D3 (< 20 ng/mL) entered this study. In order to avoid any possible interaction of P4 on expression of progesterone membrane receptors, blood samples were taken from all subjects in the follicular phase of the menstrual cycle. A consent form was received from all participants in this study.

### 2.2. Blood collection and naïve $CD4^+$ T lymphocyte cells isolation

A fresh blood sample (20 mL) was collected from all women in tubes containing anticoagulant heparin sodium (0.2 mL). Peripheral blood mononuclear cells (PBMC) were isolated using the Ficoll-Hypaque (Bio Sera) gradient centrifugation method. In the next step Naïve $CD4^+$ T lymphocyte cells were isolated and prepared by negative selection using EasySep human naive $CD4^+$ T cell isolation kit (STEM CELL).

### 2.3. Cell culture

The naïve T cell culture composed of RPMI 1640 medium with 2 g/mL sodium bicarbonate, 16 mM HEPES, 10% fetal bovine serum and 1% penicillin/streptomycin. $5 \times 10^4$ cells per 200 μL were cultured in 96 well plates. Cells were stimulated with anti-CD3 mAb and anti-CD28 mAb (0.1 μg/mL; Mabtech). Then, in the presence or absence of different concentrations of 1α, 25-dihydroxy vitamin D3 (sigma) (1–200 nM), the cells were incubated at 37 °C incubator with 5% $CO_2$ for 3 days.

### 2.4. Flow cytometry analysis of mPR α and β receptors on naïve $CD4^+$ T cells

In order to evaluate the α progesterone receptor we used mPR alfa Rabbit Anti-Human (Invitrogen) antibodies and to detect the β progesterone receptor we used the Rabbit Anti-Membrane progestin receptor beta (Invitrogen) antibodies. The cells were incubated in two separate tubes containing polyclonal antibodies at 37 °C for 1 h. Then, cells were washed with Phosphate-buffered saline (PBS) at 300 × g for 5 min. Cells were incubated with anti-human CD4 antibody conjugated to phycoerythrin and cyanine dye (PE Cy5) (Biolegend, clone RPA-T4) and also with mouse anti-rabbit IgG-CFL 488 (SANTA CRUZ BIOTECHNOLOGY) as secondary antibody for 30 min in the dark. Finally, flow cytometric analysis was performed on FACS Calibur Flow cytometer (BD Bioscience) by accumulating up to 20,000 events per tube.

The Flowjo (7.6) software was used to analyze the data obtained from the flow cytometric method. Expression changes were performed for each of the mPRα and mPRβ receptors in several steps. First, the lymphocyte cells were gated based on the Forward-scattered/Side-scattered (FSC/SSC). In this cell population, $CD4^+$ T cells were identified and gated as Ssc/fl3, which in fact constituted > 93% of the population. In the final stage, among the $CD4^+$ T cells, the subset of $mPR\alpha^+$ or $mPR\beta^+$ cells were gated and analyzed for mean fluorescence intensity (MFI).

### 2.5. Statistical analysis

Data analysis was performed using spss software (version 20). The quantitative results are reported as mean ± standard error (SE). The results of untreated and treated samples in various concentrations of vitamin D3 in both $mPR\alpha^+$ and $mPR\beta^+$ groups were compared using a repeated measure ANOVA with a Bonferroni post-hoc test. $p$ value < 0.05 was considered as a significant level.

## 3. Results

Naïve T cells were isolated from peripheral blood of 38 healthy women with a mean age of 27.2 ± 7.1. After stimulation with anti-CD3 mAb and anti-CD28 mAb for 3 days, the cells were treated either with various concentrations of vitamin D3 or not treated at all. The cells were examined for expression of mPRα and mPRβ separately by flow cytometry. Fig. 1 shows our flow cytometric gating strategy for the analysis of $mPR\alpha^+$ and $mPR\beta^+$ populations in $CD4^+$ T cells.

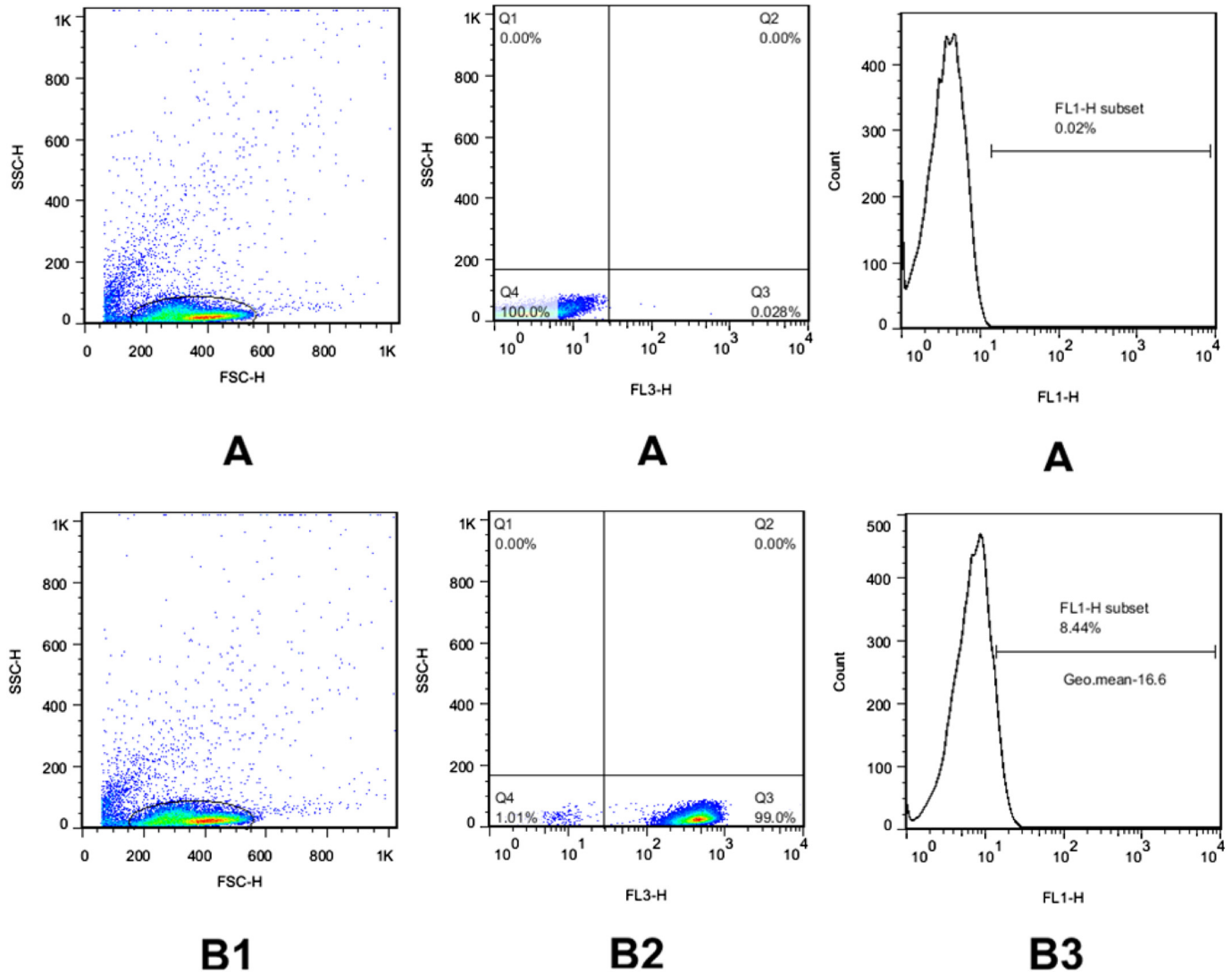



**Fig. 1.** Flow cytometric gating strategy for analysis of mPR α and β expression on naïve $CD4^+$ T cells. (A): isotype control. (B1): naïve T lymphocyte cell population. (B2): CD4-positive cell population (cells stained with PE-Cy 5-labeled anti-CD4 (FL3)) (B3): mPR α or β–positive cell population (cells staining with anti mPR α or β as a primary polyclonal antibody and then with PE-Cy 5-labeled anti-CD4 and mouse anti-rabbit IgG-CFL 488 as a secondary antibody (FL1)).

### *3.1. The expression level of mPRα on $CD4^+$ T cells*

The results showed that vitamin D3 significantly increased the expression of mPRα receptor on the $CD4^+$ T cells compared to the untreated samples ($p = 0.02$, $p = 0.000$ for concentrations of 100 and 200 nM of vitamin D3 as compared to the untreated sample). Furthermore, by increasing the concentration of this vitamin to 100 nM and 200 nM, the expression of this receptor amplified in a dose-dependent manner ($p = 0.02$, for concentration of 10 nM, compared to 100 nM) and ($p = 0.02$, $p = 0.000$, $p = 0.000$ for scales 1, 10 and 100 nM, in comparison to 200 nM) (Fig. 2).

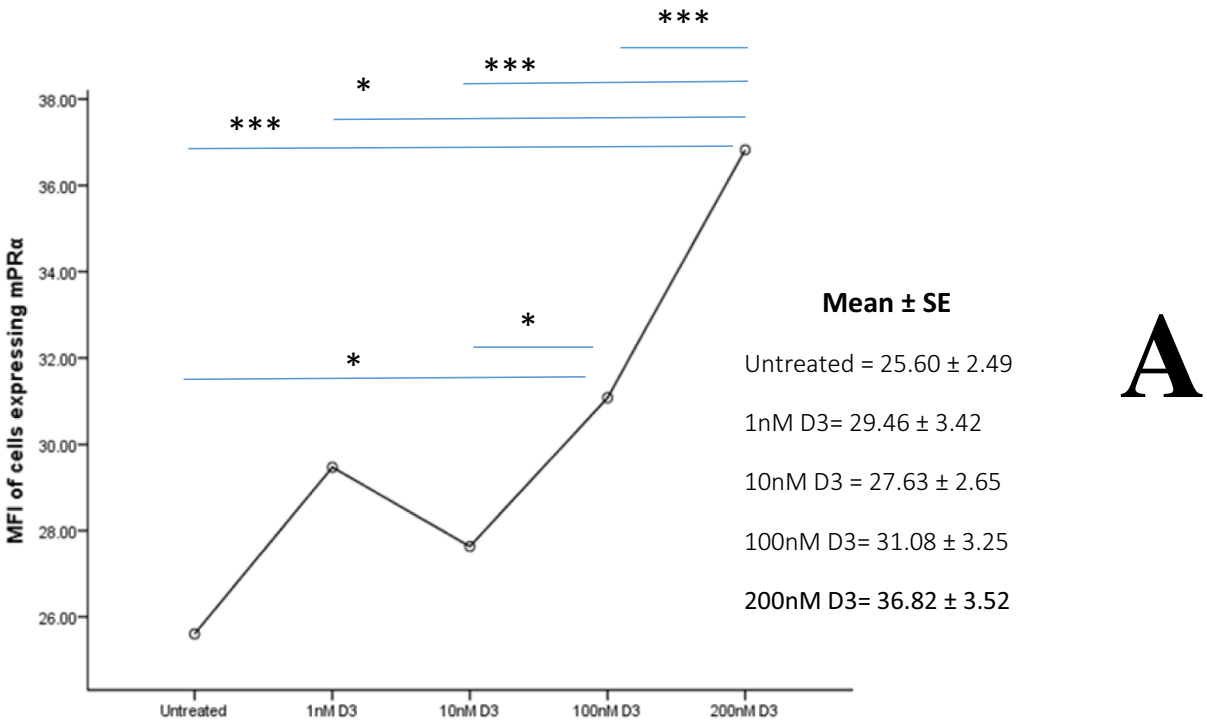
### *3.2. The expression level of mPRβ on $CD4^+$ T cells*

The results showed that the expression of mPRβ on $CD4^+$ T cells increased after vitamin D3 treatment compared to untreated samples ($p = 0.04$, $p = 0.000$, $p = 0.000$ for concentrations of 10, 100 and 200 nM of vitamin D3 compared to untreated samples). The comparison of 100 and 200 nM vitamin D3 concentrations with other groups showed a significant increase in the expression of mPRβ in a dose-dependent manner ($p = 0.000$, $p = 0.000$, for concentration of 1 nM and 10 nM compared to 100 nM) and ($p = 0.000$, $p = 0.000$, $p = 0.000$ for concentrations of 1, 10 and 100, compared with 200 nM) (Fig. 3).

## 4. Discussion

In the present study we showed that vitamin D3 induces the expression of membrane progesterone receptors.

Vitamin D3 and progesterone are both known as immune regulatory steroid hormones [18]. A successful pregnancy requires inhibition of inflammation caused by T cells. Failure to inhibit the production of inflammatory cytokines by T cells can be a factor in the incidence of abortion. Accordingly, in women who encounter frequent abortion, reduction of Treg cells and increased Th1 and Th17 cells which indicate inflammatory response to the embryonic *alloantigens* has been seen [1,19]. The immunoregulatory functions of vitamin D3 and P4 include shift of Th1 to Th2 phenotype, increased Treg cell production, inhibition of the inflammatory phenotype of Th17 cells, which reduces the production of inflammatory cytokines such as IL-17 and also an increase in the production of anti-inflammatory cytokines such as IL-10 [20–22]. The P4 hormone incretion during pregnancy is very effective in this relation [1]. On the other hand, vitamin D3 is one of the important factors in the normal functioning of the female reproductive system and pregnancy [18]. Vitamin D3 deficiency can be linked to



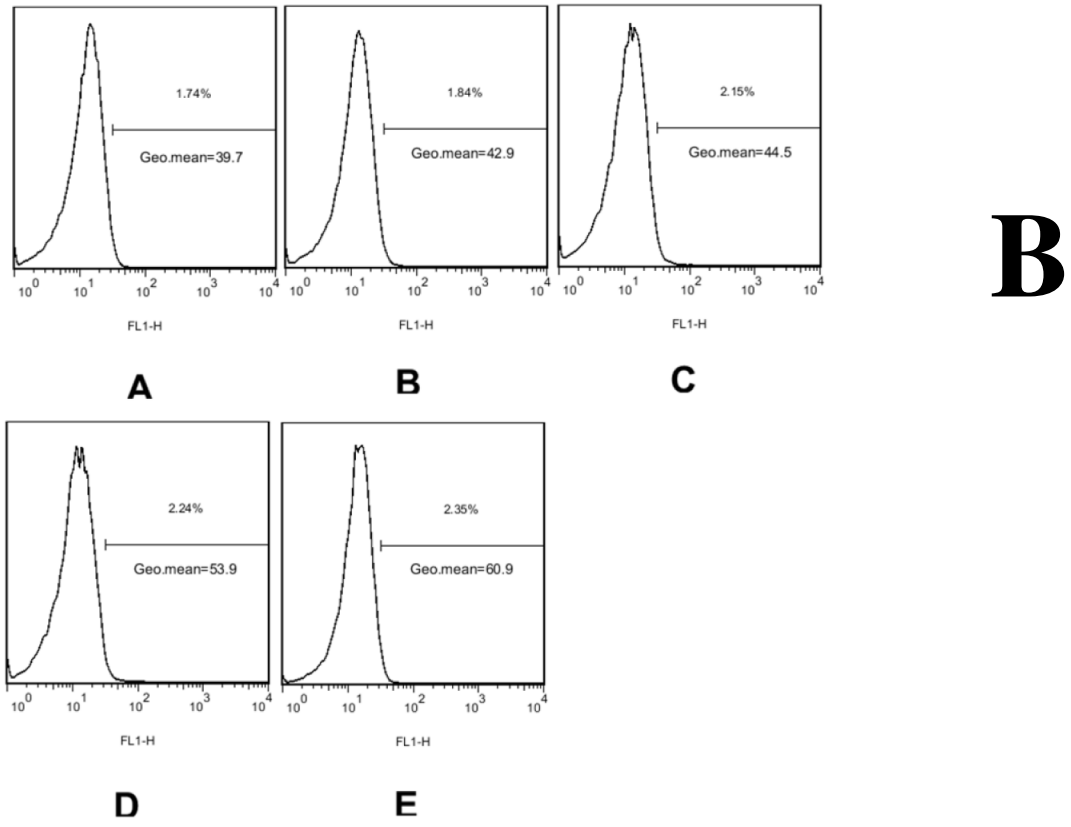
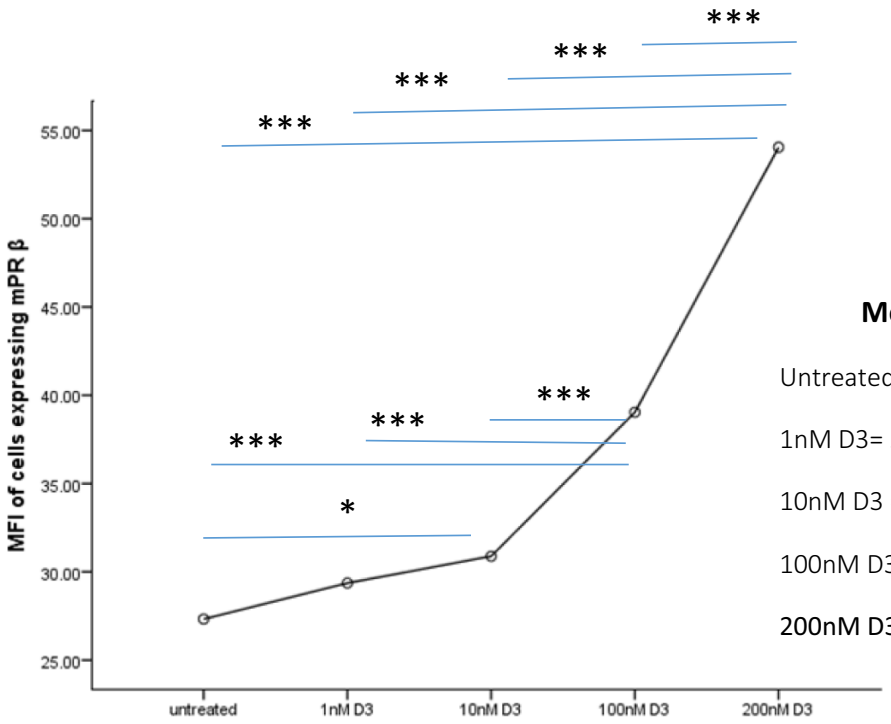




**Fig. 2.** (**A**) Chart representative of the Mean fluorescence intensity (MFI) variation of mPR α in different concentrations of vitamin D3. Data are presented as mean and standard error (SE). *p* values significance is defined as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. (**B**) Flow cytometric analysis of mPR α expression on $CD4^+$ T cells in the absence or presence of vitamin D3 (1, 10, 100 and 200 nM). (A) MFI and percent of mPR α in $mPR\alpha^+$ cell population after cell stimulation with anti-CD3/CD28 mAb. MFI of mPR α in $mPR\alpha^+$ cell population after cell stimulation with anti- CD3/CD28 mAb and vitamin D3 in (B) 1 nM, (C) 10 nM, (D) 100 nM and (E) 200 nM concentrations.




**Mean ± SE**

Untreated = 27.32±2.44

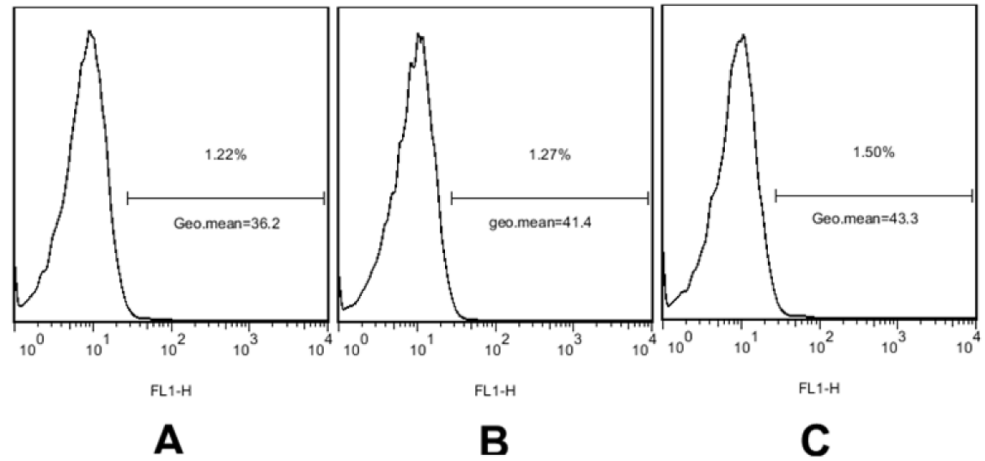
1nM D3= 29.36±2.95

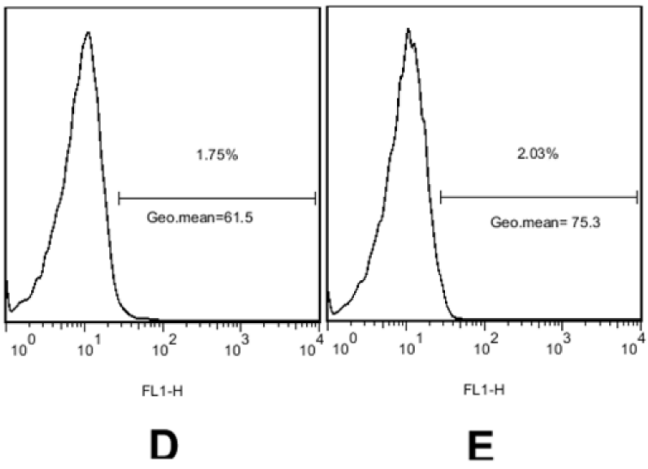
10nM D3 = 30.88±3.16

100nM D3= 39.03±3.57

200nM D3= 54.04±3.53

**A**




**B**

*(caption on next page)*

**Fig. 3.** (**A**) Chart representative of the Mean fluorescence intensity (MFI) variation of mPR β in different concentrations of vitamin D3. Data are presented as mean and standard error (SE). *p* values significance is defined as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. (**B**) Flow cytometric analysis of mPR β expression on $CD4^+$ T cells in the absence or presence of vitamin D3 (1, 10, 100 and 200 nM). (A) MFI of mPR β in $mPR\beta^+$ cell population after cell stimulation with anti-CD3/CD28 mAb. MFI of mPR α in $mPR\beta^+$ cell population after cell stimulation with anti- CD3/CD28 mAb and vitamin D3 in (B) 1 nM, (C) 10 nM, (D) 100 nM and (E) 200 nM concentrations.

some pregnancy difficulties such as defect in maternal immunity against fetus, as well as abortion and preeclampsia [23,24].

Despite the fact that each of the steroid hormones has their own specific activities, their influence on the performance of each other in different ways is the point that has been addressed in various studies. In 2014, Thanagamani et al. examined the effect of progesterone on expression of Vitamin D Receptor (VDR). They showed that P4 in a dose-dependent manner increases the expression of VDR on naive human $CD4^+$ T cells. The outstanding point in this study was that the presence of P4 significantly increased the effect of vitamin D3 in inducing the differentiation of Treg cells and the inhibition of Th1 and Th17 cells [25]. Kathryn B. et al. showed that estradiol increases the expression of progesterone receptor on human breast cancer cell line in a dose-dependent manner [26]. On the other hand Swami and coworkers showed that Vitamin D3 has antiproliferative effects on human breast cancer cells and this effect is mediated through down-regulation of estrogen receptors by vitamin D3 [27].

Parikh et al. showed that vitamin D3 stimulates the production of progesterone, estradiol and estrone from human ovarian cells in the culture medium [16]. Both vitamin D3 and progesterone play a role in protecting the central nervous system (CNS); previous studies have shown that vitamin D3 deficiency prevents the protective effect of progesterone on CNS [28]. These studies showed that P4 performance and production can be related to vitamin D3 levels although the exact mechanism is not clear. We investigated the effect of vitamin D3 on the expression of a group of progesterone membrane receptors expressed on the surface of naive $CD4^+$ T cells. Our results showed that vitamin D3 in a dose-dependent manner increases the expression of mPRα and mPRβ receptors on the surface of T cells. It is notable that the effective dosage of vitamin D3 for mPRβ (10 nM) was lower than the dosage required for mPRα (100 nM). However, Al-Hendy et al. in year 2014 showed that vitamin D3 reduces the expression of nuclear P4 receptors in human uterine fibroids [29]. The incompatibility of the results could be due to the different receptors and the cells studied.

One of the important points about progesterone membrane receptors is their role and importance during pregnancy. Areia et al. indicated that in a normal pregnancy, the percentage of Treg $mPR\alpha^+$ cells increased from the second trimester to the third trimester. The evaluation of these cells on the day of delivery showed a significant decrease compared to the third trimester of pregnancy. It is suggested that expression of mPRα on Treg cells may play an important role in the progesterone immunomodulatory property during pregnancy [30]. Szekeres-Bartho and colleagues revealed that in subjects with a history of abortion, the progesterone receptor expression in T lymphocytes was significantly reduced in comparison to subjects with normal pregnancies [31]. Although the exact type of progesterone receptor has not been identified in this study but the importance of the up-regulation of progesterone receptors during pregnancy has been shown.

Participants in our study were healthy non-pregnant women with low serum levels of vitamin D3. Sampling was performed in the follicular phase of the menstrual cycle to minimize the progesterone interaction effect on the expression of receptors. So the results of our study cannot precisely be extended to the pregnancy duration. During pregnancy, the cells are exposed to various regulatory factors including high concentrations of progesterone and estrogen which could affect the expression of receptors on T cells. In this initial study we showed the possible effect of vitamin D3 on the activity of P4 as another steroid hormone. Further studies are needed to assess the effect of vitamin D3 on the expression of receptors in the presence of P4 and to evaluate the effect of vitamin D3 on the immunoregulatory activity of P4.

Regarding the interaction of steroid hormones on each other, this study may further illustrate the importance of vitamin D3 in P4 performance through effect on P4 receptors expression. Vitamin D3 is likely to influence the sensitivity of T cells to P4 by increasing the expression of progesterone receptors. Considering the essential role of P4 in pregnancy maintenance, the results of this study indicate that more importance should be given to vitamin D3 levels before and during pregnancy.

## Conflict of interest

The authors report no conflict of interest.

## Acknowledgments

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