



Evaluation of CD3⁺ T Cell Percentage, Function and its Relationship with Serum Vitamin D Levels in Women with Recurrent Spontaneous Abortion and Recurrent Implantation Failure

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ABSTRACT

Background: Women afflicted with recurrent spontaneous abortion (RSA) and repeated implantation failure (RIF) may have immune abnormalities. The role of vitamin D has been demonstrated in the function of the immune system.

Objective: To assess the percentage and function of CD3⁺ T cells and their relationship with the level of the serum vitamin D or 1,25-dihydroxy vitamin D₃ (the active form of the vitamin) in women with RSA and RIF.

Methods: In this case-control study, peripheral blood was obtained from the patient and the healthy control groups. The ratio of CD3⁺ T cell and activated CD3⁺ CD69⁺ T cell was investigated using flow cytometry. The serum levels of Interferon- γ (IFN- γ) and vitamin D were measured by ELISA.

Results: The mean proportion of CD3⁺ T cells in women with RSA increased significantly compared with the healthy control group ($P < 0.04$). However, no significant difference was observed in RIF women compared with the control group. There was no significant difference in the ratio of activated CD3⁺ CD69⁺ T cells between the patient and the healthy control groups. Serum IFN- γ levels in women with RSA showed a significant increase compared to the control group ($P < 0.031$); however, no significant difference was observed between women with RIF and the control group. Serum levels of vitamin D showed a significant reduction in both RSA ($P < 0.01$) and RIF ($P < 0.04$) groups in comparison with the control.

Conclusion: An increase in the percentage and inflammatory function of T cells was associated with RSA. Decreased vitamin D levels may contribute to immune dysfunction and pregnancy loss.

Keywords: Interferon- γ , Recurrent Implantation Failure, Recurrent Spontaneous Abortion, T-Cells, Vitamin D

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Cite this article as:

Mousavi Salehi A, Ghafourian M,
Amari A, Zargar M. Evaluation of
CD3⁺ T Cell Percentage, Function
and its Relationship with Serum
Vitamin D Levels in Women with
Recurrent Spontaneous Abortion
and Recurrent Implantation Failure.
Iran J Immunol. 2022; 19(4):369-
377,
doi: 10.22034/iji.2022.91464.2083.

Received: 2021-06-02

Revised: 2022-01-03

Accepted: 2022-01-04

INTRODUCTION

Egg cell implantation in the endometrium is a complex process involving various regulatory immune mechanisms. Recurrent spontaneous abortion (RSA) is one of the most frequent pregnancy problems, affecting nearly 1-5 percent of couples seeking pregnancy (1). *In vitro* fertilization (IVF) treatment is also used for couples who suffer from infertility (2). Various studies have indicated that women with a record of RSA and repeated implantation failure (RIF) may have immunological abnormalities in PB and placenta (3, 4). There is some evidence of T cell presence in the space between the mother and the fetus and their effect on the development and survival of the fetus (5), which has been observed during recent investigations on the etiology of RSA. The etiology of RSA is unknown in 40% to 50% of cases, which may be attributed to immunological problems (6, 7), among which T cell disorders are of special importance. Various studies have reported that T cell in the PB of women with RSA and RIF show completely abnormal behavior (8). Normal pregnancy is an immunological process in which the fetus is sheltered from the mother's immune system by various mechanisms. Therefore, immunological mechanisms play a significant role in preventing fetus rejection as well, as in pregnancy success. The interaction of cells at the maternal-embryonic interface causes changes in the type and level of cytokines expressed. Modifications in the pattern of T-cell cytokines may be linked to immunological tolerance or immune stimulation (7). The T cell population undergoes favorable changes during the implantation in pregnant women; these cells are divided into subcategories according to the expression of surface markers. CD3 is a marker that is expressed on all T cell. It is the most important marker for identifying these cells. The CD4 molecule is expressed on TH cells mediating cellular immunity. The increasing number and function of TH cells increases the function of the maternal

immune system and can have detrimental effects on the fetus. The two main categories of CD4⁺ T cell are TH1 and TH2 cells, each of which has a distinct cytokine pattern and function (9). TH1 cells are involved in cellular immunity through releasing IL2, TNF α , and IFN- γ whereas TH2 cells secrete IL4, IL5, IL6, IL9, IL10, and IL13 and contribute to humoral immunity (7, 9). Several studies have stated that the function of TH1 cells is harmful to pregnancy, while the TH2 cells' activities are beneficial to pregnancy (9). The ratio of these two cells has also been shown to change dramatically in the PB of women with pregnancy loss (10). Changes in the balance of TH1/TH2 cells, increase in TH17 and TH1 cells, and decrease in TH2 and Treg cells can be critical factors in the development of RSA and RIF (11). Meanwhile, some studies have examined the total CD3⁺ T cell population in RSA and RIF women (12, 13). In other studies, the percentage and function of these cells showed no significant change in the patient group compared with the healthy individuals (14). Some of the previous investigations indicated that the percentage of peripheral CD3⁺ T cell in RSA women was not different from the normal group (15), while K.M. Yang et al. showed that the ratio of CD3⁺CD4⁺ T cell and CD3⁺CD8⁺ T cell was remarkably higher in women with RSA relative to the normal groups (16). According to Ghafourian M's research, the proportion of CD3⁺CD8⁺ T cell in RSA women was significantly higher than in the control group [14]. Vitamin D was found to play an essential role in modulating immune system responses, effectively preventing the proliferation of inflammatory cells (17). Vitamin D has an inhibitory effect on the adaptive immune system and affects both innate and adaptive immune responses. It, on the other hand, suppresses TH1 cytokines (such as IFN- γ) and promotes TH2 responses by downregulating IFN- γ and upregulating IL-4 (18). The active form of vitamin D (calcitriol) may inhibit CD3⁺ T-cell proliferation. It has also been reported to be able to shift immune responses from

TH1 to TH2. However (19), there have been contradictory results regarding the impact of vitamin D on immune cells in PB of women with RSA and RIF. Multiple people with RSA have vitamin D deficiency (VDD) in the clinical context. As a result, their cellular immune systems have been compromised, with greater peripheral NK numbers, NK cytotoxicity, and higher Th1/Th2 ratios (18). The present research intended to examine the percentage and function of CD3⁺ T cell in the PB of RSA and RIF patients in addition to its relationship with the serum levels of vitamin D in the two groups of patients with RSA and RIF.

MATERIALS AND METHODS

Study Population

A total of 54 women volunteers participated in this case-control study, including three groups as follows: 21 women with RSA, 18 with RIF, and 15 healthy individuals. In the healthy group, women had at least one case of successful pregnancy with no record of miscarriage. A gynecologist selected the patient group from among the patients referred to medical centers that were affiliated with Ahvaz Jundishapur University of Medical Sciences. Each patient had a history of at least two to three consecutive spontaneous abortions (SA) or implantation failures (IFs). Women with a history of autoimmune diseases, infectious diseases, diabetes, chromosomal abnormalities, or immunodeficiency were excluded from the study. This research received an ethics code from the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (Ethics No: IR.AJUMS.MEDICINE.REC.1398.038), and all participants signed the informed consent form. Blood samples were collected from all women at the secretory phase of their menstrual cycle as defined by self-reporting, since we see an increase in the number and function of leukocytes in the endometrium and peripheral blood in

the secretory phase of the menstrual cycle (20). We tried to sample all participants in this specific phase so that all have the same conditions. 6 ml of the PB taken from each participant was divided into two parts: 2 ml was transferred to a heparin tube for flow cytometry and 4 ml was poured into a blood clotting tube for serum preparation and ELISA tests for vitamin D and IFN- γ measurements.

ELISA

Serum from all the participants was collected and kept at -70°C to measure cytokine and vitamin D levels. Vitamin D and IFN- γ levels in the serum were quantified using an IFN- γ ELISA kit (Diaclon Co. France) and 25-OH Vitamin D ELISA Kit (Euroimmun Co. Germany) following the manufacturer's instructions and in the OD 450nm. All measurements were carried out in duplicate to minimize the effect of technical error and intra-assay variation.

Antibodies and the Flow Cytometric Analysis

The lymphocyte subsets of PB were detected by the flow cytometry (FACS Calibur; Becton-Dickinson, San Jose, CA, USA) and stained using fluorescein isothiocyanate (FITC)-conjugated anti-CD3, allophycocyanin (APC)-conjugated anti-CD69 antibodies to identify CD3⁺ T cell in addition to the activated T cell. T cell were subsequently classified as CD3⁺ and activated CD3⁺CD69⁺ T cell based on distinct phenotypic features of these cells. All antibodies were purchased from the Biologend Thermo Fisher Scientific (USA). First, 5 μl of each antibody was added to a micro tube, followed by 100 μl of PB incubated for 20 min. in a dark place at room temperature. Next, erythrocyte lysis buffer was added and the supernatant was discarded using a centrifuge. Afterward, the sediment was washed three times using PBS buffer, and by adding 500 μl of PBS, the cells were analyzed by the flow cytometry (14). Fluorescence minus one (FMO) control was utilized for the CD69 antibody, and the gating technique

is shown in Figure 1. The quadrant gates of Q1 were set for CD69⁺ lymphocytes, Q2 CD3⁺CD69⁺ activated T cell, Q3 CD3⁺T cell, and Q4 CD3⁻CD69⁻ lymphocytes, as shown in Figure 1C. A total of 10⁵ cells were analyzed in fluorescence-activated cell sorting (FACS) Calibur flow cytometer (Becton Dickinson) by FlowJo software 10.0 (TreeStar, Ashland, OR, U.S.A).

Statistical Analysis

Data were analyzed as the mean ratio of CD3⁺ T cell and CD3⁺CD69⁺ for activated T cell. Statistical analysis was done using Statistical Package for the Social Sciences for Windows (SPSS 22). Graphs were plotted by Graph Pad Prism software 5.0. We expressed categorical data as numbers and percentages and numerical data as the mean (SEM). The nonparametric Kruskal-Wallis test determined the statistical difference between the two groups. The Spearman correlation test was performed for the percentage of T cell and the serum levels of vitamin D among all groups participating in the study. P<0.05 was considered statistically significant.

RESULTS

Clinical Characteristics of Women with RSA, RIF, and the Normal Group

This study recruited 54 women, including 21 RSA patients, 18 RIF patients, and

15 healthy individuals with no history of miscarriage or pregnancy problems. The groups showed no significant difference in terms of age or body mass index (BMI). The average age, the mean year of marriage, and the mean abortion, implantation failure, or live birth in each group are listed in Table 1.

Comparison of the Mean Proportion of CD3⁺T Cell in the Patient Groups with the Control Group

No significant differences were observed between the mean percentages of CD3⁺ T cell in patients with RIF relative to the control group. Furthermore, the mean percentage of these cells shows a significant increase in the group of patients with RSA compared with the control group (P<0.04) Figure 2a).

Comparison of the Ratio of Activated CD3⁺ CD69⁺ T Cell between the Patient and the Control Group

No significant differences were observed between the mean rates of activated T cell in the patient groups and the control group. Additionally, the difference in the proportion of these cells between the two groups of patients was not statistically significant (Figure 2d).

Comparison of the Serum IFN- γ Concentrations in the Patient Groups and the Healthy Control

We did not observe any significant difference between the serum levels of IFN- γ

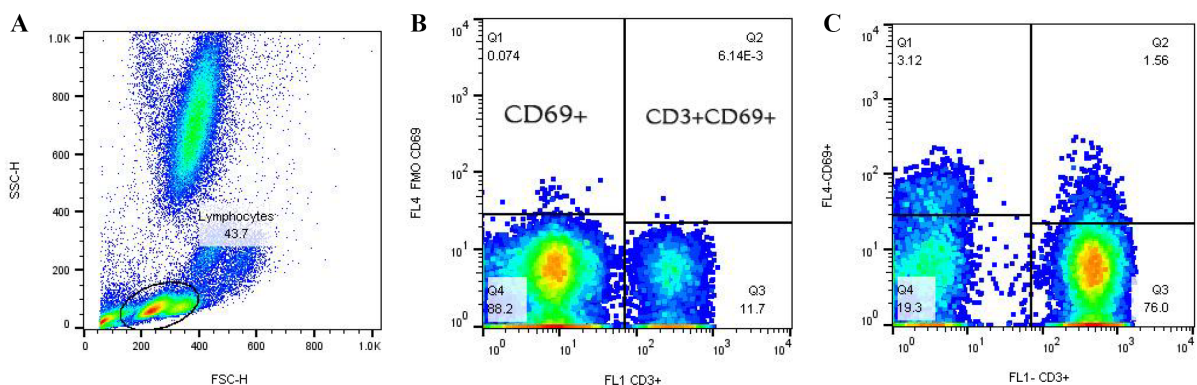


Figure 1. A) Lymphocyte gates in the total cell population to reach CD3⁺ cells; B) A fluorescence minus one (FMO) control gating for CD69, by which we could detect the CD69⁺ cells region; C) A test sample showing cell populations. Specific region Q1 CD69⁺, region Q2: CD3⁺ CD69⁺, region Q3: CD3⁺, and region Q4: CD3⁻CD69⁻.

Table 1. Clinical characteristics of women with RSA, RIF and normal group

	RSA	RIF	Normal group
	N=21	N=18	N=15
Age	32.8±1.13	36.72±0.96	32.17±0.97
BMI	27.14±0.51	27.42±0.74	25.35±0.77
Marriage (years)	9.36±0.9	12.11±1.39	7.94±1.17
Frequency of miscarriage	3.1±0.25	n.a	n.a
IVF failure	n.a	2.72±0.2	n.a

RSA: Recurrent spontaneous abortion; RIF: Repeated implantation failure; IVF: In vitro fertilization; BMI: Body mass index; Data are presented as the mean±SEM. n.a: not assessed

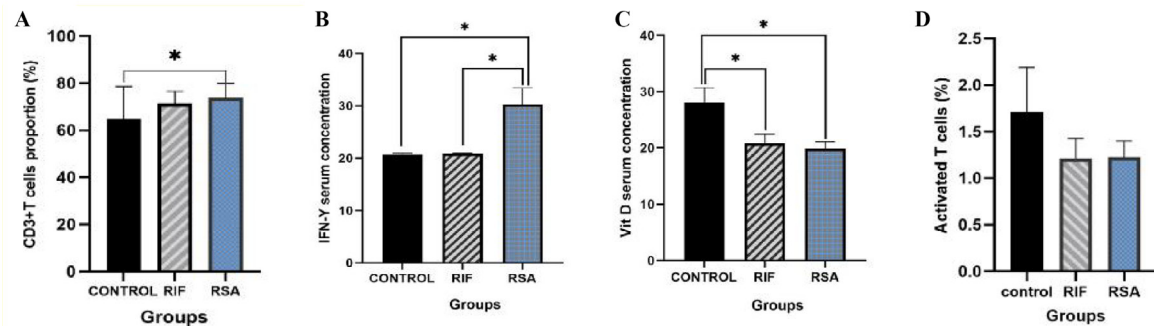


Figure 2. A) Comparison of the average percentage of CD3⁺ T-cells in the three groups. B) Comparison of mean serum level of IFN-γ between the patient and control groups. C) Comparison of the mean serum level of vitamin D between the patient and control groups. D) Comparison of the average percentage of activated CD3⁺ T-cells in the three groups. RSA: Recurrent spontaneous abortion; RIF: Repeated implantation failure; *The difference was statistically significant (P<0.05).

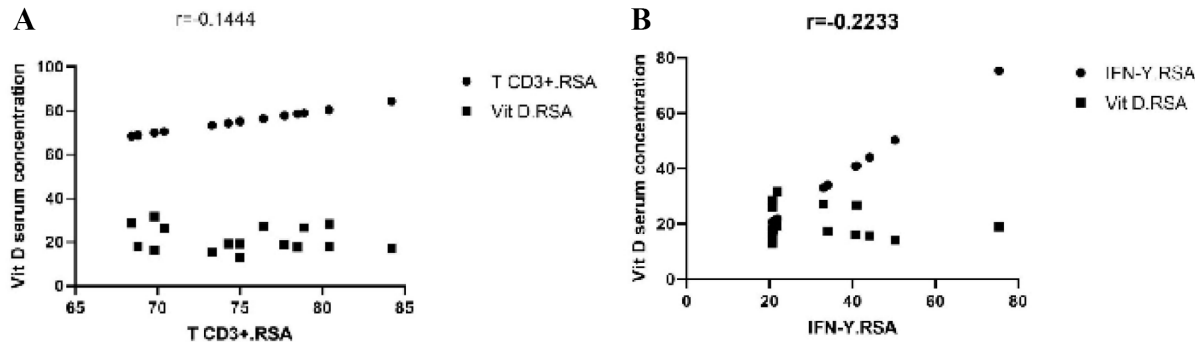


Figure 3. A) Negative correlation between CD3⁺ T-cells and serum levels of vitamin D in RSA group; B) Negative correlation between IFN-γ concentration and serum levels of vitamin D in RSA group. RSA: Recurrent spontaneous abortion

in patients with RIF and the control group. However, this concentration significantly increased in the patients with RSA in comparison with the control group and the group of patients with RIF (P<0.031 and P<0.04, respectively) (Figure 2b).

Comparison of Vitamin D Serum Levels between the Patient Groups and the Healthy Control

Serum vitamin D values in women with

RSA were remarkably lower compared with the healthy control group (P<0.01; Figure 2c). Also, in women with RIF, vitamin D levels significantly reduced compared with the healthy control group (P<0.04). But the levels of vitamin D between the two groups of patients with RIF and RSA were not significantly different (Figure 2c).

Evaluation of the Correlation between the Ratio of CD3⁺ T Cell and the Serum Levels of

Vitamin D between the Study Groups

The Spearman's correlation test was performed for the ratio of CD3⁺T cell and the serum level of vitamin D among all the groups participating in the study. There was a negative correlation between CD3⁺T-cell percentage and the serum vitamin D levels in RSA patients. However, no significant difference was seen between the RIF group and the control group (Figure 3a).

Evaluation of Correlation between IFN- γ Concentration and the Serum Levels of Vitamin D between the Study Groups

The Spearman's correlation test was performed for IFN- γ concentration and the serum levels of vitamin D among all the groups participating in the study (Table 2). There was a negative correlation between IFN- γ concentration and the serum vitamin D level in RSA patients. Nevertheless, there was no significant difference between the RIF group and the control group (Figure 3b).

DISCUSSION

The importance of T cell in fighting pathogens and cancer cells, in addition to boosting and regulating immune system responses, has been documented. Undoubtedly, any change in the behavior of these cells can cause significant changes in the function of the immune system, manifested in various disorders, including T-cell-associated autoimmune diseases, infectious diseases such as HIV, and acute inflammatory diseases (12). The presence of maternal T lymphocytes at the maternal-embryonic interface plays a

major function in the embryo's development and survival (7). The present study examined the percentage and inflammatory function of T cell in patients with RSA and RIF. It was observed that the rate of CD3⁺T cell in patients with RSA increased considerably compared with the control group. There are several mechanisms for the abrogation of T-cell function, which can lead to allograft failure. The researchers also believe that T cell can transform the immune response of a mother to her fetus from aggressive to tolerant and unresponsive (21). CD3⁺ T cell were not significantly different in the endometrium of women with the URSA before and during pregnancy compared with the control group. Nonetheless, during pregnancy, the ratio of these cells in patients' PB decreased compared with the healthy group (22). However, other studies have shown that an increase in the proportion of T cell before and after pregnancy is accompanied by a higher risk of RSA. For example, in the study by Du et al. the authors reported that the higher percentage of CD3⁺ T cell by more than 67.84% increased the risk of miscarriage and RSA (23). These findings are similar to the observations of our study. Previous investigations have shown that cytokines released by T cell can directly influence both the growth and function of trophoblast cells and that they can invade trophoblast cells through the activation of macrophages (7). One of these essential cytokines in inflammatory responses of T cell is IFN- γ . In the current study, the levels of this cytokine in the serum of women with RSA showed a remarkable increase relative to the control group, which may be associated with the increased inflammatory function

Table 2. Mean percentage of CD3⁺T cell, activated T cells and serum levels of IFN- γ and Vitamin D in both patient and control groups

Characteristics	RSA	RIF	Control
T cells (%)	73.79±1.50	71.42±1.2	64.86±3.5
Activated T (%)	1.22±0.17	1.21±0.2	1.71±0.47
IFN- γ (pg/ml)	30.22±3.16	20.88±0.10	20.73±0.18
Vitamin D (ng/ml)	19.88±1.2	20.83±1.6	28.01±2.67

RSA: Recurrent spontaneous abortion; RIF: Repeated implantation failure; Data are presented as the mean±S.E.M

of T cell, and the pathological responses of these cells in RSA patients maintain a balance between cytotoxic T cell (TC) and TH cells, in addition to the equilibrium between TH subclasses, including TH1 and TH2, is critical to preserving maternal tolerance to the fetus and pregnancy health. Keiji Kuroda et al. reported that Th1 cell levels and the ratio of Th1/Th2 were considerably higher in women with ≥ 2 pregnancy loss compared with fertile and infertile women (10). In the present research, we measured the proportion of CD3+ T cell in women with RIF. We also measured the levels of IFN- γ in serum as well as the surface expression of CD69 in CD3+ T cell, and no significant difference was seen between these variables in RIF patients compared with the control group. The results of studies related to T cell function in RIF's patients are also variable and somewhat different. Kwang Moon Yang et al. (16) reported that the proportion of TCD3+CD69+ T cell in PB of women with RIF significantly increased compared with the control group. On the other hand, Chunyu Huang et al. have mentioned the decrease in the percentage of TC cells, the increase in the ratio of TH to TC cells, along with the decrease in inflammatory responses as one of the reasons for the RIF (13). The endometrial immunomodulation by hCG-activated PBMC helps patients with unexplained RIF to achieve implantation. The immunomodulation of the endometrium by PBMCs can be an immunological therapeutic approach as well as other treatment modes (24). Similar to this study, based on our research findings, it seems that inflammatory condition is a prerequisite to increase the chance of successful embryo implantation in early pregnancy and that the lack of proper inflammatory function at the time of implantation can be one reason for the RIF. Vitamin D's potential as an anti-proliferative and immunomodulatory agent has also been established. Vitamin D may influence the course and outcome of pregnancy due to its immunomodulatory properties (25). Various studies have presented considerable

evidence to discuss vitamin D deficiency and its relationship with pregnancy problems and disorders, including RSA and the RIF (18, 26). In this research, we observed that the serum levels of vitamin D in the PB of both groups of women with RSA and the RIF significantly decreased compared with the control group. Chen, Xian et al. (27) reported that most patients with RSA have a shortage or deficiency in vitamin D. On the other hand, in these patients, the proportion of TH and NK cells and the production of inflammatory factors were significantly high. The mentioned study also indicated that after taking vitamin D supplements in patients, the percentage of NK and B cells and production of cytokines such as TNF decreased over time. Many immune cells, including T- and B-cells, express vitamin D receptors (28). Abdollahi, et al. reported that vitamin D increased the ratio of Treg cells and decreased TH17 cells in the PBMC cultures of women with the RSA (29). Sharmin Ferdous et al. showed that the serum vitamin D was at a considerably low level in women with early spontaneous loss of pregnancy compared with pregnant women with preterm live pregnancy, and maternal serum vitamin D deficiency was remarkably associated with early spontaneous pregnancy loss (30). The presence of uncontrolled TH1 responses is one of the possible causes of RSA. The shift of responses from TH1 to TH2 and regulation of immune response in these patients is one of the critical functions of vitamin D (31). Vitamin D has also been reported to suppress the expression of genes involved in TNF- α production in cells. By reducing the polarization of perforin at the surface of TC and NK cells, vitamin D prevents the lethal activity of these cells (32). Other investigations have reported that vitamin D can reduce IFN- γ -producing CD3+ CD4+ cells. By decreasing the production of this inflammatory cytokine, vitamin D plays a main role in regulating the immune system's inflammatory responses (33). Despite all the evidence and studies, the exact position and function of vitamin D in

women with RSA and the RIF have not yet been determined. Vitamin D receptor gene polymorphism is another factor that may play a role in pregnancy disorders. Hubert Wolski et al. indicated that significant differences in genotype distributions and allele frequencies between RSA women and the control groups were observed in *VDR BsmI* polymorphism (25). Studies in this field present significant contradictions that may be due to the different functions of vitamin D in other cells of the immune system under different conditions.

CONCLUSION

In this study, we found that the proportion of CD3⁺ T cell and inflammatory cytokine of IFN- γ increased in women with RSA. However, in women with RIF, no increase in the percentage and function of these cells was observed compared with the control group. Also, the serum vitamin D in the two groups of women with RSA and RIF significantly decreased compared with the control group. It seems that the study of the function and percentage of T cell can be useful in predicting and treating pregnancy disorders and abortion. Adequate amounts of vitamin D can also help regulate immune system responses and pregnancy-related problems. To draw definitive conclusions about these data, additional comprehensive investigations are required in the future.

Conflict of Interest: None declared.

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