Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Serum levels of vitamin D and immune system function in patients with COVID-19 admitted to intensive care unit

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ABSTRACT

Objective: Vitamin D is believed to affect the functionality of the immune system for the prevention of coronavirus disease. To investigate the role of this vitamin against the Coronavirus, this study analyzed the serum levels of vitamin D, the transcription pattern of inflammatory cytokines, and the frequency of total lymphocytes, $TCD4^+$, $TCD8^+$, and NK cells in 50 COVID-19-affected subjects in comparison to 50 healthy participants.
Materials and methods: This study diagnosed and evaluated 100 patients. Frequency of lymphocytes was determined using flow cytometry. Cytokine expression levels were measured using Real-Time PCR. Serum levels of vitamin D and cytokines levels in cultured cell supernatant were measured by ELISA.
Results: Patients with COVID-19 exhibited decreased serum levels of vitamin D versus the healthy participants ($p = 0.0024$). The total number of lymphocytes, $TCD4^+$, $TCD8^+$, and NK cells was significantly reduced in patients with COVID-19 ($p < 0.0001$). Considerable upregulation of IL-12, IFN-γ, and TNF-α was seen in COVID-19 patients compared to the control group, whereas IFN-α was downregulated in COVID-19 patients. ELISA results also had increased levels of IL-12, TNF-α, and IFN-γ ($p = 0.0014$, 0.0012, and $p < 0.0001$, respectively), and decreased level of IFN-α ($p = 0.0021$) in patients with COVID-19 compared to the control group.
Conclusion: These findings suggest a probable association among vitamin D concentrations, immune system function, and risk of COVID-19 infection. As a result, it is recommended that vitamin D be considered as a candidate for handling and controlling COVID-19 because of its ability to target the cytokine storm and its antiviral effects.

1. Introduction

In late December 2019, a number of pneumonia cases with undetermined aetiology were recognized in Wuhan City, China (Bogoch et al., 2020). Scientists promptly detected a new variant of coronavirus (SARS-CoV-2) among the verified pneumonia-infected subjects that had >95% homology with the bat coronavirus (Jiang et al., 2020; Wu et al., 2020). The most important complications during hospitalization of COVID-19 patients were Acute Respiratory Distress Syndrome (ARDS), arrhythmia, and shock (Soltani-Zangbar et al., 2021; Wang et al., 2020). The binding of human pathogenic coronaviruses with host cell receptors, angiotensin-converting enzyme 2 (ACE2), expressed via the epithelial cells of the nasal mucosa, lung, intestine, kidney, heart, and blood vessels is an important contributing factor in the pathogenesis of infection (Fan et al., 2021; Soltani-Zangbar et al., 2021; Song et al., 2018). After receptor binding and modifications in the conformation of the spike (S) protein, SARS-CoV-2 releases RNA into the host cell (Chen et al., 2020). The cytokine storm has been considered an important

Abbreviations: ACE2, Angiotensin-converting enzyme 2; ARDS, Acute respiratory distress syndrome; ELISA, Enzyme linked immunosorbent assay; IFN-γ, Interferon gamma; PBMCs, Peripheral blood mononuclear cells; TNF-α, Tumor necrosis factor-alpha.

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contributor to ARDS and it causes mortality for the grave SARS-CoV-2 infection cases (Vaninov, 2020). The cytokine storm represents an excessive systemic inflammatory reaction where pro-inflammatory cytokines including interleukin (IL)-1β, IL-6, IL-12, IL-17, interferon (IFN)-γ, tumor necrosis factor-alpha (TNF-α), and chemokines are generated fast in large amounts in order to provide protection against SARS-CoV infection (Channappanavar and Perlman, 2017; Mahmoodpoor et al., 2021). T-cells have the most important part in the clearance of viruses because $CD8^+$ cytotoxic T cells (CTLs) secrete perforin, granzymes, and IFN-γ to eliminate them from the host (Etemadi et al., 2021). Previous reports point to a considerable decrease in the count of T-cells $CD4^+$ and $CD8^+$ existing in the peripheral blood of SARS-CoV-2-induced subjects. However, their status was hyperactivated (Xu et al., 2020b).

A number of studies have confirmed the role of vitamins in decreasing the risk of pneumonia. Vitamin D, a fat-soluble secosteroid, is essential to ensuring normal immune functioning against pathogens and shielding against autoimmune diseases (Fraser et al., 2020). The three main steps in vitamin D metabolism, 25-hydroxylation, 1α-hydroxylation, and 24-hydroxylation, are completed by cytochrome P450 mixed-function oxidases (CYPs) (Bikle, 2014). Following the absorption of vitamin D from the diet or its intake into the skin through sunlight, it is metabolized in the liver into 25-hydroxyvitamin D [25(OH)D] by CYP2R1 (the most important 25-hydroxylase) and CYP27A1 (the only mitochondrial 25-hydroxylase) (Bikle, 2014). It remains biologically inactive and must be converted in the kidneys by CYP27B1 (main 1α-hydroxylase) into its biologically active form, 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] (Holick and Disorders, 2017). All genomic actions of vitamin D are mediated by the Vitamin D Receptor (VDR). The VDR is present in a wide variety of tissues. After binding to 1,25(OH)2D3, the VDR heterodimerizes with Retinoid X Receptor (RXR) and is translocated to the nucleus where it binds to the VDR Responsive Element (VDRE) in target genes to influence gene expression (Holick and Disorders, 2017). An alternative pathway of vitamin D activation by CYP11A1 leads to the production of more than 10 metabolites including 20-hydroxyvitamin D3 [20(OH)D3], 22(OH)D3, 20,23(OH)2D3, 20,22 (OH)2D3, 17,20,23(OH)3D3, …, which are generated by placenta, adrenal glands, and epidermal keratinocytes (Slominski et al., 2012). This novel pathway of D3 metabolism was initiated by CYP11A1 and modified by CYP27B1 activity and it showed the product profiles that were tissue- and cell-type specific (Slominski et al., 2014a). It was discovered that CYP11A1 hydroxylated the side chain of vitamin D3 without its cleavage. CYP11A1 displayed significant flexibility towards substrate specificity acting on a range of naturally moving steroid molecules (other than cholesterol) including cholesterol precursors, hydroxycholesterols, plant sterols, ergosterol, lumisterol, and vitamins D3 and D2 (Slominski et al., 2015b). Products of the novel CYP11A1-initiated secosteroidal pathways exhibit anti-proliferative, pro-differentiation, anti-fibrotic and anti-cancer activities and anti-inflammatory effects, which are comparable to, or greater than, those of 1,25(OH)2D3 (Slominski et al., 2015a). CYP11A1 is expressed in CD4 and CD8 human T lymphocytes, B cells, and monocytes. CYP11A1 converts cholesterol into pregnenolone, a precursor of all steroids (Slominski et al., 2020d). Peripheral T-cells produce steroids, particularly pregnenolone. The regulation of local glucocorticoid biosynthesis and CYP11A1 activity could be targeted in immune cells or their target organs (Slominski et al., 2020d). Local production of corticosteroids in the skin, initiated by CYP11A1, regulates the protective barrier and skin immune functions (Slominski et al., 2021b).

Nuclear receptors include VDR, Retinoid-related Orphan Receptors (ROR)α and γ, and arylhydrocarbon receptor (AhR), with each particular compound potentially displaying different affinities (Slominski et al., 2020a). Recent evidence has revealed that in addition to acting as biased agonists to VDR, CYP11A1-derived D3-derivatives can, together with lumisterol hydroxyderivatives, act as inverse agonists to ROR α and γ (Slominski et al., 2014b) and as agonists to the AhR (Slominski et al., 2018; Slominski et al., 2021a). 20(OH) D3, 20, 23(OH) 2D3, and 1, 25 (OH) 2D3 somewhat act as antagonists or inverse agonists to the RORα and RORγ receptors. In addition, they inhibit the ROR-responsive element (RORE)-mediated activation of a reporter in keratinocytes and melanoma cells as well as IL-17 production by immune cells (Slominski et al., 2014b). The top canonical nuclear receptor pathway induced by 20, 23(OH) 2D3 was AhR signaling, followed by VDR/RXR. 20, 23(OH) 2D3 stimulated CYP1A1 and CYP1B1 gene expression, thus affecting downstream AhR signaling. A similar stimulation was observed for 20(OH)D3 and 17,20,23(OH)3D3 (Slominski et al., 2018). Additionally, 1, 25(OH) 2D3, 1, 20(OH) 2D3, 25(OH) D3, 20(OH) D3, and lumisterol (L3) derivatives such as 20(OH) L3 and 20, 22(OH) 2L3 exhibited different, yet overlapping, interactions with Liver X receptors (LXR). The majority of metabolites functioned as LXRα/β agonists. While 1,20,25(OH)3D3, 1,25(OH)2D3, 1,20(OH)2D3, and 25(OH)D3 acted as inverse agonists to LXRα, they functioned as agonists to LXRβ (Slominski et al., 2021a).

The outcome of published clinical trials confirmed the potential role of vitamin D supplementation in fending off acute respiratory infection through the modulation of the innate immune response and enhancement of antibody generation post-vaccination (Xu et al., 2020a; Zheng et al., 2020). Past epidemiological studies demonstrated the link of lower levels of vitamin D to the greater risk of developing ARDS, heart failure, and sepsis as well as to the danger of contracting serious COVID-19 and even mortality due to COVID-19 (Molloy et al., 2020). One of the latest related reviews has confirmed the probable role of vitamin D in reducing the risk of contracting COVID-19 infections and death rate (Grant et al., 2020). Vitamin D maintains cell junctions, increases cellular immunity upon reducing the cytokine storm with effect on IFN-γ and TNF-α, and regulates adaptive immunity by the inhibition of Th1 cell responses (Laviano et al., 2020). Given the wide-ranging differences in baseline vitamin D levels in the public and considering that beneficial effects are mainly based on serum vitamin D concentrations, measurement of serum vitamin D would provide a more precise insight into COVID-19. Accordingly, this study aims to examine 25-hydroxyvitamin D [25(OH) D] levels, i.e., the major circulating form of vitamin D, and the rate of immune response in patients with COVID-19.

2. Material & methods

2.1. Study design

Fifty Covid-19-infected patients (detected through clinical diagnosis and radiologic results) aged over 18 years were selected among the patients hospitalized in Intensive Care Unit (ICU). Patients who had malnutrition, systemic disease, diarrhea, pregnancy, vascular graft, and history of rheumatic fever were excluded from the study. All the patients gave their consent to peripheral blood sampling prior to entry to the study. The control group included 50 healthy age-matched people who did not have COVID-19. Participants' demographics and medical background were obtained. The current study was approved by the Research Ethics Committee of Tabriz University of Medical Science (No: IR.TBZMED.REC.1398.1313).

2.2. Isolation of PBMCs

All blood samples at approximately 8 mL were obtained from those infected by COVID-19 and the subjects in the healthy control group under sterile conditions. Of 8 mL, 3 mL was employed for serum preparation and kept at −70° for further vitamin D measurement. Peripheral Blood Mononuclear Cells (PBMCs) were collected from heparinized blood samples through standard Ficoll (lymphosep) 1.077 g/mL (Biosera, UK) gradient centrifugation. They were then washed twice with RPMI 1640 medium (Sigma-Aldrich, Schnelldorf, and Germany). Then, 5×10^6 of cells were cultured in 5 mL complete medium with 10% Fetal Bovine Serum (FBS). After 48 h, the cultured cells and supernatant were applied to gene expression analysis and cytokine level evaluation,

respectively (Ahmadi et al., 2017).

2.3. Vitamin D assay

In line with the manufacturer's protocol by ELISA kits (MyBioSource, CA), 25-hydroxyvitamin D [25(OH) D] (ng/mL) level was measured.

2.4. Flow cytometry analysis

Following the isolation of PBMCs, the cells were washed twice with PBS (Sigma-Aldrich, Germany). In the sample gating, SSC-H and FSC-H were comparatively employed to gate all lymphocytes through forward and side scatter. They were analyzed even further for their $CD4^+$, $CD8^+$, $CD56^+$, and $CD16^+$ expression.

This study conducted peripheral blood immunophenotyping assay in accordance with flow cytometry to identify Natural Killer (NK) cells. For PBMC staining, triple-color immunofluorescence analyses of the lymphocyte markers were performed through the use of anti-CD3, anti-CD16, and anti-CD56 antibodies labelled by fluorescein isothiocyanate (FITC) (BD Biosciences), phycoerythrin (PE) (BD Biosciences), and allophycocyanin (APC) (BD Biosciences) fluorochromes, respectively. The NK population comprised $CD3^-$ $CD56^+$ $CD16^+$ cells in the peripheral blood. Anti-CD4 and anti-CD8 fluorescent conjugated antibodies were used for evaluating CD4+ and CD8+ T lymphocytes after lymphocytes gating, respectively. The isotype controls included FITC, PE, and APC mouse IgG2a. Flow cytometry was performed using FACSCalibur (BD Biosciences, San Jose, CA, USA), and FlowJo software was then used for data analysis.

2.5. Real-time PCR

Total RNA was derived from cultured PBMCs via RNX-PLUS Solution (SinaClon, Tehran, Iran) and it was quantified by spectrophotometric measurement (Nano Drop; Agilent Technologies, USA). Next, Revert Aid Reverse Transcriptase kit (Thermo Fisher, Waltham, MA, USA) was applied to complementary DNA (cDNA) synthesis. Real-time PCR was performed to measure the expression levels of IL-12, IFN-α, -IFN-γ, and TNF-α. The Corbett research RG-6000 real-time rotary analyzer PCR machine (Corbett Research, Bosch Institute) was used. We employed the $2^{-\Delta\Delta CT}$ method to measure gene expression in association with the β-actin housekeeping control to keep the expression folds of the target gene normalized.

2.6. Enzyme Linked Immunosorbent Assay (ELISA)

The concentrations of cytokines IL-12, IFN-α, -IFN-γ, and TNF-α (pg/mL) were measured in PBMCs cultured supernatant using ELISA kits (Mybiosource, San Diego, USA) based on the manufacturer's instructions. For the sake of higher accuracy, the whole samples were investigated in duplicate. The absorbance rates were read at 450 nm by a micro plate ELISA reader system (BP-800, Biohit, Finland). Softmax software of the reader along with standard calibration lines was employed to calculate the concentration of the samples.

2.7. Statistical analysis

Statistical analysis was carried out by SPSS PC Statistics (version 24.0; SPSS Inc.). Descriptive statistics for continuous data were expressed as the mean ± SD. Unpaired *t*-test was employed to comparatively study the statistical differences in factors between the healthy control group and the patients with COVID-19. The GraphPad Prism (Ver. 8.00) for Windows (GraphPad Software, La Jolla, CA, USA, www.graph pad.com) was utilized for graph drawing. *P*-values < 0.05 were reportedly significant in terms of statistics.

3. Results

3.1. Study flow

A total of 100 participants (50 with COVID-19 and 50 healthy subjects) were evaluated in this study. The mean age of the participants was 51.8 ± 16.42 and 48.2 ± 15.64 years in patients with COVID-19 and the healthy control group, respectively. There was a significant difference in systolic blood pressure, fasting blood sugar, triglyceride, LDL-cholesterol, and albumin levels between study groups ($p < 0.05$). Table 1 shows the demographic and clinical characteristics of the patients.

3.2. Vitamin D concentration

COVID-19 patients experienced significantly lower serum levels of 25(OH) D than the healthy control group [23.10 ± 10.89 vs. 32.06 ± 17.22, $p = 0.0024$] (Fig. 1).

3.3. Lymphocytes frequency

This paper used flow cytometry to determine the level of total lymphocytes, T $CD4^+$, T $CD8^+$, and NK cells in patients with COVID-19 and controls. Fig. 2A shows the percentage of T $CD4^+$ and T $CD8^+$ escalated in patients with COVID-19 in comparison to the control group. The frequency of total lymphocytes, T $CD4^+$, T $CD8^+$, and NK cells in patients with COVID-19 was significantly lower than that in the healthy control group ($p < 0.0001$, $p < 0.0001$, $p = 0.0003$, and $p < 0.0001$, respectively) (Fig. 2B).

3.4. Expression levels of cytokines gene

The mRNA expression levels of IL-12, IFN-α, -IFN-γ, and TNF-α in the PBMCs were measured and compared in the healthy control group and patients with COVID-19 using real-time PCR. As shown in Fig. 3, the mRNA expression levels of IL-12 ($p < 0.0001$), TNF-α ($p = 0.0028$), and IFN-γ ($p < 0.0001$) increased considerably in patients with COVID-19 compared to healthy controls, whereas IFN-α ($p < 0.0001$) was remarkably reduced in COVID-19 patients (Fig. 3).

Table 1
Clinical and biological characteristics of the COVID-19 patients and healthy individuals.

Target (mean ± SD)	Healthy control (mean ± SD)	COVID-19 patients
N = 50	*N* = 50	
Age	48.2 ± 15.64	51.8 ± 16.42
(M-F)	(24–26)	(22–28)
BMI (kg/m^2)	26.54 ± 3.88	27.56 ± 4.92
Systolic blood pressure (mm Hg)	110.4 ± 12.1	124.7 ± 18.42*
Diastolic blood pressure (mm Hg)	72.44 ± 8.46	74.32 ± 10.18
Fasting blood sugar (mg/dL)	105.5 ± 22.92	128.4 ± 37.66*
Triglyceride (mg/dL)	128.2 ± 36.94	176.3 ± 60.34*
Cholesterol (mg/dL)	174.3 ± 40.28	188.2 ± 35.22
Vitamin D3 (ng/mL)	32.06 ± 17.22	23.10 ± 10.89*
HDL-cholesterol (mg/dL)	48.32 ± 5.02	45.24 ± 6.89
LDL-cholesterol (mg/dL)	116.8 ± 28.48	137.9 ± 29.12*
Albumin (g/dL)	3.521 ± 0.2252	3.168 ± 0.2663*
Creatinine (mg/dL)	1.284 ± 0.729	1.211 ± 0.823
GFR	76.22 ± 26.34	71.14 ± 20.32
Clinically positive tests subjects	0	50

BMI: body mass index; HDL: high density lipoprotein; LDL: low density lipoprotein; GFR: glomerular filtration rate. Data are presented as mean ± standard division.

* $P < 0.05$ was considered as statistically significant.

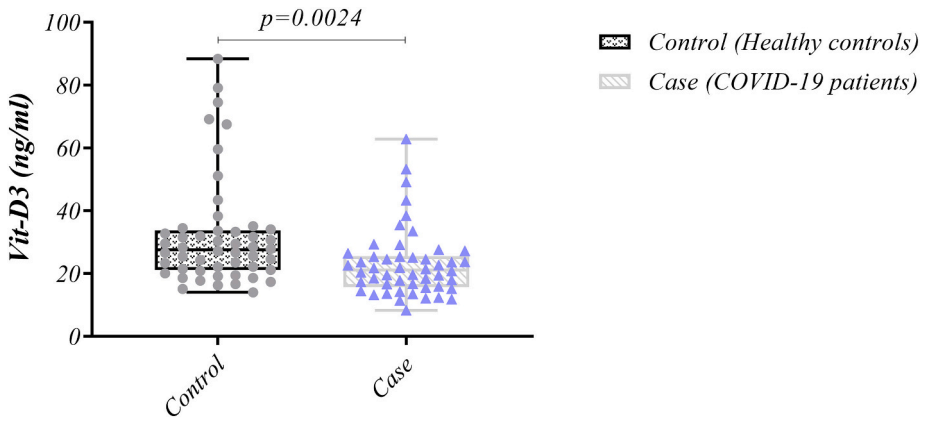

Fig. 1. The serum levels of Vitamin D. The levels of Vitamin D was evaluated using ELISA technique. $p < 0.05$ was considered as statistically significant (Healthy control group, $n = 50$, patients with COVID-19, n = 50).

3.5. Cytokines levels

Secretion levels of IL-12, IFN-α, -IFN-γ, and TNF-α in cell supernatant were checked out by ELISA. The levels of IL-12 ($p = 0.0014$), TNF-α ($p = 0.0012$), and IFN-γ ($p < 0.0001$) were significantly higher in patients with COVID-19 than in healthy controls. It is necessary to note that our study determined the decreased concentration levels of IFN-α ($p = 0.0021$) in COVID-19 patients (Fig. 4). Molecular and cellular changes in patients with COVID-19 are shown in Table 2.

3.6. Correlational analysis of vitamin D and cytokine levels

A significant negative correlation was found between 25(OH) D levels and TNF-α levels ($R = -0.413$, p = 0.0028), IL-12 levels ($R = -0.430$, $p = 0.0018$), and IFN-γ levels ($R = -0.466$, $p = 0.0006$). There is a significant positive correlation between 25(OH) D levels and IFN-α levels ($R = 0.345$, $p = 0.0139$). Table 3 shows correlational analysis of vitamin D deficiency and cytokine levels in COVID-19 patients.

4. Discussion

One can find the major reasons for COVID-19-induced morbidity and mortality in patients with acute respiratory disease syndrome and inflammation-related disorders (Teymoori-Rad et al., 2020). According to previous findings on COVID-19 patients, vitamin D deficiency is clear in patients with acute respiratory tract infections. Vitamin D enjoys special properties that reinforce the immune system through the modulation of both adaptive and innate immune systems as well as cytokines and regulation of cell signaling pathways (Laird et al., 2020). Active forms of vitamin D including the classical 1,25(OH)2D3 and 25(OH)D3 and novel CYP11A1-derived hydroxyderivatives including 20(OH)D3 and 20,23(OH)2D3 stimulate effective anti-inflammatory activities such as inhibition of IL-1, IL-6, IL-17, TNFα, and IFNγ production through downregulation of NF-κB and inverse agonism to RORγ and induce anti-oxidative responses through activation of NRF-2 and p53-dependent pathways (Slominski et al., 2020c). A range of vitamin D3-related compounds including 7-dehydrocholesterol (7-DHC) and lumisterol (L3) hydroxyderivaties display anti-SARS-CoV-2 activities and have the ability to reduce the viral load in blood stream (Qayyum et al., 2021). 25 (OH)L3, 24(OH)L3, and 20(OH)7DHC metabolites inhibited RNA-dependent RNA polymerase (RdRP) by 50%–60% (Qayyum et al., 2021). 1,25(OH) 2D3 downregulated IFN signaling, TNSFF noncanonical and TRAF-activated NFκβ pathways, downregulated inflammasomes, and upregulated IL-4, IL-10, and IL-13 signaling (Slominski et al., 2020a). Low vitamin D level was found to be linked to the increased level of inflammatory cytokines, considerably high risk of pneumonia, and viral upper respiratory tract infections (Weir et al., 2020). Vitamin D also modulates T-cell immunity, decreases the production of Th1 cells, and induces Th2 responses (Bae and Kim, 2020). Therefore, it can suppress the progression of inflammation by attenuating the generation of inflammatory cytokines and increasing anti-inflammatory cytokines (Panfili et al., 2021). SARS-CoV-2 would cause downregulation of ACE2 expression, leading to exacerbated inflammatory reaction as well as cytokine storm and lethal ARDS (Ahn et al., 2020). Remarkably, vitamin D upregulates the levels of ACE2 in the lungs and alleviates inflammatory responses through its anti-inflammatory properties (Aygun, 2020). Therefore, vitamin D could reduce cytokine storm syndrome in patients with severe COVID-19 infection and inhibit multiple organ damages (Aygun, 2020). Vitamin D3 and its hydroxyderivatives are advantageous to bind the active site of TMPRSS2 to the binding site(s) between ACE2 and SARS-CoV2-RBD, showing that vitamin D3 and its hydroxyderivatives can serve as TMPRSS2 inhibitor and inhibit ACE2 binding of SARS-CoV-2 RBD to prevent SARS-CoV-2 entry (Song et al., 2021).

This study evaluated serum levels of Vitamin D, frequency of lymphocytes, and levels of inflammatory cytokines in patients with COVID-19. Our results exhibited that the serum levels of vitamin D were significantly reduced in patients with COVID-19 compared to the healthy control group. Moreover, the frequency of total lymphocytes, T $CD4^+$, T $CD8^+$, and NK cells was reduced in the case of patients with COVID-19. Indeed, there was a relationship between vitamin D deficiency and COVID-19. Our results illustrated that the mRNA expression and levels of IL-12, IFN-α, TNF-α, and IFN-γ significantly increased in patients with COVID-19. It can be suggested that decreased levels of vitamin D in these patients led to increased levels of proinflammatory cytokines. This can suggest the potential role of vitamin D for use as an adjunctive therapy for COVID-19 patients.

A limited case study in Indonesia targeted 10 COVID-19 patients (Pinzon and Pradana, 2020). All patients in this study suffered low vitamin D levels. It was found that vitamin D deficiency might serve as a risk factor in viral infection (Pinzon and Pradana, 2020). Done in the U. S., a retrospective study employed many cases and found that vitamin D might be associated with the reduced risks of contracting both COVID-19 and mortality (Li et al., 2020). Eleven research findings that had investigated 360,972 cases of COVID-19 patients in the form of a meta-analysis revealed that 37.7% and 32.2% of the patients suffered vitamin D deficiency and vitamin D insufficiency, respectively. Also, patients with low levels of vitamin D were susceptible to significantly high risk of contracting COVID-19 (Ghasemian et al., 2021). There was only a 5% mortality rate for vitamin-D-sufficient COVID-19 patients, while patients with severe deficiency of vitamin D faced a 50% mortality rate following 10-day hospitalization (Carpagnano et al., 2021). The results of cross-sectional study in the Asia pacific population demonstrated that vitamin D levels inversely correlated with the number of COVID-19 cases (Yadav et al., 2021). In a cohort study of >190,000 patients

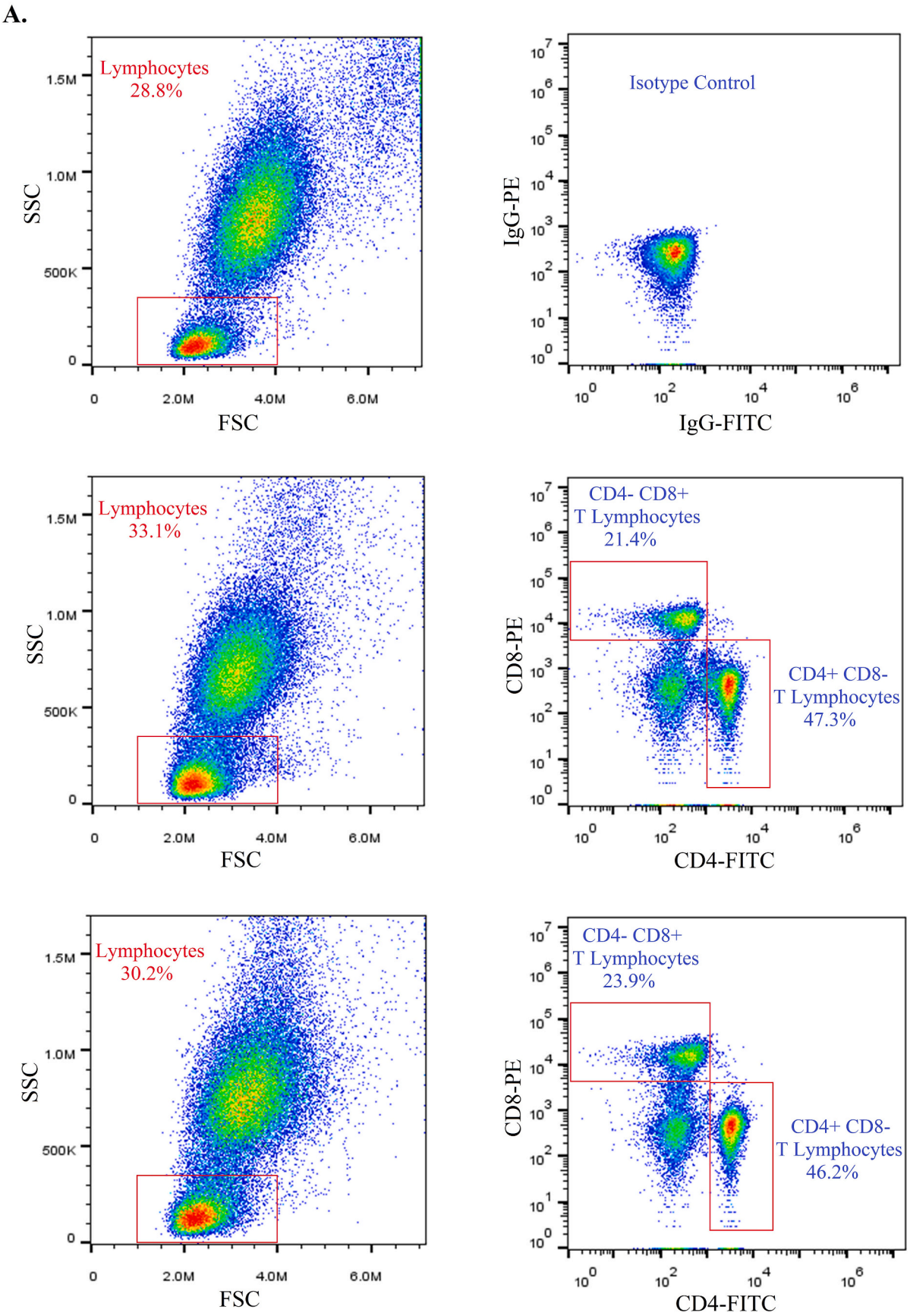

Fig. 2. The frequency of total lymphocytes, T $CD4^+$, T $CD8^+$ and NK cells in in patients with COVID-19 and control group. A) At the first cells were gated based on the side scatter and forward scatter (SSC-H vs. FSC-H). B) The gated cells were analyzed based on CD4, CD8, CD56 and CD16 expression. $p < 0.05$ was considered as statistically significant (Healthy control group, n = 50, patients with COVID-19, n = 50).

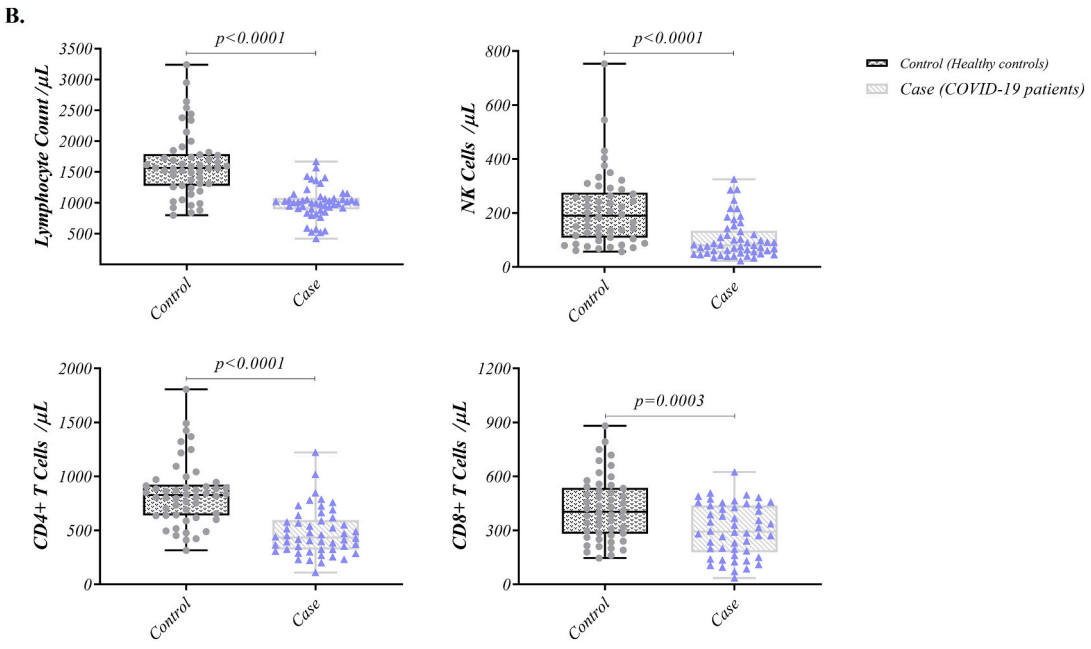

Fig. 2. (*continued*).

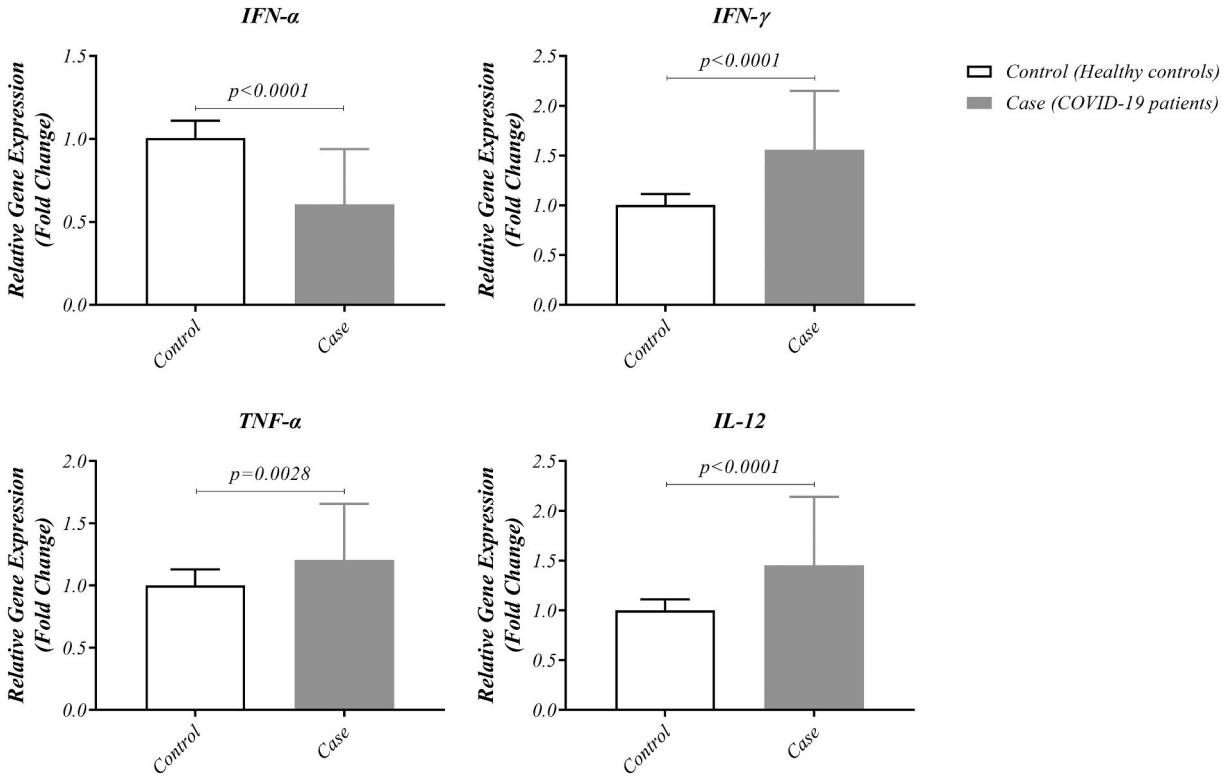

Fig. 3. The mRNA expression of IL-12, IFN-α, TNF-α, and IFN-γ in patients with COVID-19 and control group. The expression levels of IL-12, IFN-α, TNF-α, and IFN-γ were evaluated using quantitative real-time PCR. $p < 0.05$ was considered as statistically significant (Healthy control group, n = 50, patients with COVID-19, n = 50).

from the USA, an obvious inverse relationship between circulating 25 (OH)D levels and SARS-CoV-2 positivity was detected (Kaufman et al., 2020). Patients who had a circulating level of 25(OH) D < 20 ng/mL had a 54% higher positivity rate than those who had a blood level of 30–34 ng/mL in multivariable analysis (Kaufman et al., 2020). Patients who are vitamin D deficient or insufficient, i.e., 25(OH)D 30 ng/mL, could be treated with an appropriate amount of vitamin D as soon as it is feasible to do so (Slominski et al., 2020b). Those people that are subjected to the greater risk of vitamin D deficiency under the current COVID-19 pandemic must seriously consider taking vitamin D supplements to optimize the serum 25-hydroxyvitamin D level (75–125 nmol/L) (Ali, 2020).

5. Conclusion

A possible link among vitamin D concentrations, immune system

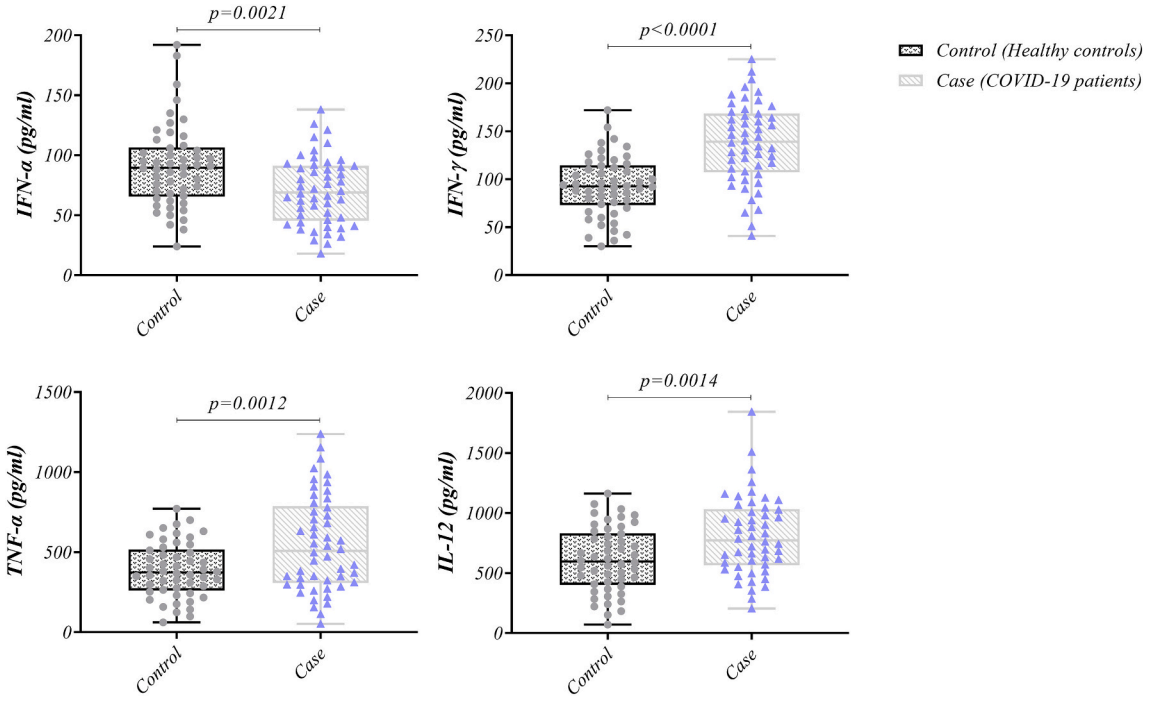

Fig. 4. The cytokines secretion levels in cell supernatant of patients with COVID-19 and control group. The secretion levels of IL-12, IFN-α, TNF-α, and IFN-γ were evaluated using ELISA technique. $p < 0.05$ was considered as statistically significant (Healthy control group, n = 50, patients with COVID-19, n = 50).

Table 2
Molecular and cellular changes in patients with COVID-19 vs healthy individuals.

Target	Healthy control (Mean ± SD)	COVID-19 patients (Mean ± SD)	*p* value
	N = 50	N = 50	
Flow cytometry			
Total lymphocytes (/μl)	1622 ± 530.1	997.4 ± 257.1	<0.0001
CD4+ T lymphocytes (/μl)	839.0 ± 298.4	480.7 ± 218.2	<0.0001
CD8+ T lymphocytes (/μl)	420.8 ± 175.5	301.3 ± 143.7	0.0003
NK cells (/μl)	211.1 ± 134.5	104.8 ± 72.54	<0.0001
Real-time PCR (fold change)			
IFN-γ	1.003 ± 0.1099	1.559 ± 0.5894	<0.0001
IFN-α	1.006 ± 0.1033	0.6060 ± 0.3325	<0.0001
IL-12	1.000 ± 0.1109	1.453 ± 0.6876	<0.0001
TNF-α	1.000 ± 0.1305	1.205 ± 0.4531	0.0028
ELISA (cell supernatant)			
IFN-γ (pg/mL)	92.74 ± 31.25	137.5 ± 42.32	<0.0001
IFN-α (pg/mL)	90.52 ± 34.68	70.48 ± 28.30	0.0021
IL-12 (pg/mL)	610.0 ± 269.0	807.9 ± 328.0	0.0014
TNF-α (pg/mL)	388.8 ± 170.4	551.0 ± 297.6	0.0012

NK Cells: Natural Killer cells, IFN-γ: Interferon Gamma, IFN-α: Interferon alpha, IL-12: Interleukin 12, TNF-α: Tumor Necrosis Factor alpha.

Table 3
Correlation of vitamin-D3 levels with evaluated cytokines in COVID-19 patients.

Target	Vit-D3 vs. TNF-α	Vit-D3 vs. IL-12	Vit-D3 vs. IFN-γ	Vit-D3 vs. IFN-α
r	−0.4136	−0.4308	−0.4669	0.3458
95% CI	−0.6257 to −0.1446	−0.6383 to −0.1650	−0.6643 to −0.2086	0.06624 to 0.5750
P (two-tailed)	0.0028	0.0018	0.0006	0.0139

function, and risk of COVID-19 infection was observed in this study. Vitamin D deficiency was strongly accompanied by the increased risk of COVID-19. Given the crucial role of vitamin D in the fight against the cytokine storm in COVID-19 patients, its supplementation can be considered as adjunctive therapy for COVID-19 patients, and it will possibly facilitate boosting the immune system, inhibiting virus spread, and attenuating the disease progression into severe stages. Large-scope controlled researches are required to prove the impact of vitamin D on COVID-19.

CRediT authorship contribution statement

Mohammad Sadegh Soltani-Zangbar: wrote the article and contributed to acquisition of data and data analysis. Sanam Dolati: wrote the article and participated in study design and supervised the study. Ata Mahmoodpoor and Mehdi Yousefi: helped in sample collection and performed the monitoring of patients. Ali Shamekh and Sepehr Valizadeh contributed to edited final version. Sarvin Sanaie: supervised the study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declarations

Availability of data and materials

The datasets used and/or analyzed during the current study are

available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The current study was approved by the Research Ethics Committee of Tabriz University of Medical Science (No: IR.TBZMED.REC.1398.1313).

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