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Effects of Vitamin D receptor gene polymorphisms on the prognosis of COVID-19

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Running Title: Vitamin D gene polymorphisms and COVID-19

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cen.14664.

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Acknowledgments

This study did not receive specific grants from funding agencies in the public, commercial, or nonprofit sectors.

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Conflict of interest: No potential conflict of interest.

Abstract

Purpose: Vitamin D deficiency has emerged as another potential risk factor for coronavirus disease (COVID-19) due to the immunomodulatory effects of 25 hydroxyvitamin D [25 (OH)D]. Vitamin D receptor (VDR) gene polymorphisms such as Fok I, Bsm I, Apa I, and Taq I are also associated with different courses of viral infections. This study aimed to evaluate the association between the VDR gene polymorphism at Fok I, Taq I, Bsm I, and Apa I genotypes and the prognosis of COVID-19 in respect to vitamin D deficiency.

Methods: Two-hundred ninety-seven patients with COVID-19 were enrolled. Serum 25 (OH)D levels were measured. Four variant regions of the VDR gene, FokI, BsmI, ApaI, and TaqI were determined.

Results: Eighty-three percent of subjects had vitamin D deficiency, and 40.7% of the whole group had severe deficiency. Median 25 (OH)D level was 11.97 ng/mL. Vitamin D levels were not related to inflammatory markers, disease severity, admission to intensive care unit (ICU), and mortality. While disease severity was related to Fok I Ff genotype, it was Taq TT genotype for ICU admission. Moreover, the ApaI aa genotype was common among the patients who were died. None of the deceased subjects had the Fok I FF genotype.

Conclusion: 25 (OH)D levels were not related to the severity and mortality of COVID-19. VDR gene polymorphisms are independently associated with the severity of COVID-19 and the survival of patients.

Keywords: COVID-19; vitamin D deficiency; VDR polymorphism; Fok I; Apa I

Introduction

Coronavirus Disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) led to a global pandemic after the first case had been detected in December 2019. COVID-19 mainly affects the respiratory tract, and clinical severity ranges from asymptomatic to fatal outcomes¹⁻³. Several factors, including age, obesity, diabetes, hypertension, and ethnicity, are blamed for the increased risk of COVID-19. Also, vitamin D deficiency has emerged as another potential risk factor for COVID-19⁴.

25 hydroxyvitamin D [25 (OH)D] plays a role in many biological processes, such as bone metabolism, immunomodulation, cell proliferation, differentiation, and regulation⁵⁻⁷. Also, it has anti-inflammatory, antifibrotic, and antioxidant effects. Due to the immunomodulatory effects of 25 (OH)D, its deficiency is blamed for a higher risk for SARS-CoV-2 infection. Serum concentrations of 25 (OH)D were inversely

associated with proinflammatory cytokines such as increased IL-6 and C-reactive protein (CRP) levels and increased risk of pneumonia or acute respiratory distress syndrome (ARDS). Lower 25 (OH)D concentrations are associated with a higher risk for infections, especially in the respiratory tract^{8,9}. Chronic vitamin D deficiency can activate the renin-angiotensin system and leads to fibrotic changes that can cause lung injury by inducing proinflammatory cytokine production in human monocytes/macrophages¹⁰.

Increased frequency of COVID-19 at high latitudes and worse prognosis of these cases made clinicians to think that 25 (OH)D levels may affect the risk and the prognosis of COVID-19¹¹. The reports published in the early periods of the pandemic have reported a higher prevalence of vitamin D deficiency in COVID-19 cases with a higher risk of invasive mechanical ventilation (IMV) and mortality¹². It was thought that 25 (OH)D might protect against COVID-19. On the contrary, in recent reports, 25 (OH)D levels were not associated with disease severity or lethality¹³⁻¹⁵.

Calcitriol, the active form of vitamin D, binds to the vitamin D receptor (VDR) and modulates its responses. Vitamin D-VDR signaling regulates the expression of a wide range of physiological functions. Hereby, VDR gene polymorphisms cause a dysfunctional receptor that affects VDR activity. Both innate and adaptive immune responses can vary according to different polymorphisms of VDR, such as Cdx, A1012G, FokI, BsmI, ApaI, and TaqI. Indeed VDR polymorphisms have been previously found to be associated with bacterial infections such as tuberculosis¹⁶ and severe respiratory syncytial virus (RSV) bronchiolitis in respect to vitamin D deficiency¹⁷. Moreover, different VDR polymorphisms such as FokI, BsmI, ApaI, and TaqI could affect the course of RSV infection in several studies, respectively¹⁷⁻¹⁹.

This study aimed to evaluate an association between the VDR gene polymorphism at FokI, TaqI, BsmI, and ApaI genotypes and the prognosis of COVID-19 in respect to vitamin D deficiency.

Material and Methods:

Selection of patients

Two-hundred ninety-seven patients with reverse-transcription polymerase chain reaction (RT-PCR)-confirmed COVID-19 who were admitted to Marmara University Education & Research (E&R) Hospital between April and October 2020 were enrolled.

Data of subjects registered in Marmara University Medical Genetics Biobank with no known additional disease were used as the control group. There were 150 individuals with FokI, BsmI, and ApaI genotype results and 55 individuals with BsmI genotype results.

The study protocol was approved by the Local Ethics Committee of Marmara University School of Medicine (09.2020.533) and the Turkish Ministry of Health. We have conducted the study according to the International Conference on Harmonization Guidelines for Good Clinical Practice and the Declaration of Helsinki.

Data collection

Clinical presentations and demographic parameters (age and sex), medical histories, symptoms at admission, medications, duration of hospitalization, thorax computed tomography (CT) findings, oxygenation, and vital signs were recorded. The laboratory data [lymphocyte counts and percentages, neutrophil counts, thrombocyte counts, alanine aminotransferase (ALT), creatinine, high-sensitive C-reactive protein (hs-CRP), lactate dehydrogenase (LDH), ferritin, d-dimer, procalcitonin, and albumin values] were obtained from the laboratory information system. In addition, blood

samples were taken within 48 hours of admission to the hospital, have centrifuged, and serum aliquots were stored at -20°C. Serum 25 (OH)D levels were measured from these samples.

Evaluation of World Health Organization (WHO) clinical severity scores

According to WHO criteria, the severity of COVID-19 patients was classified into 1-10. After that, patients were grouped into three according to the WHO clinical progression scale; 1. mild ambulatory disease (1–3), 2. hospitalized: moderate disease (4–5), 3. hospitalized: severe disease and dead (6–10) ²⁰.

The patients' requirement for noninvasive mechanical ventilation (NIMV) or reservoir mask, their requirement for admission to intensive care unit (ICU), mortality, and WHO clinical progression scales were also reviewed.

Biochemical measurements and genetic assessment

Complete blood counts were measured with Unicel DxH800 Coulter Cell Analyzer (Beckman Coulter, USA) from K2EDTA samples. Serum LDH, creatinine, and ALT parameters were analyzed with AU 680 (Beckman Coulter, USA) spectrophotometrically. Ferritin levels were measured with a two-site immunoenzymatic assay in Access Analyzer (Beckman Coulter, USA). The D-dimer parameter was quantitated with an immuno-turbidimetric assay in 3.2% sodium citrated venous plasma (STA Compact, Diagnostica Stago, France). hs-CRP levels were measured nephelometrically (BN Prospec, Dade Behring, Germany). Serum 25 (OH)D levels were measured by paramagnetic particle, chemiluminescent immunoassays (DxI800, Beckman Coulter, USA). The electrochemiluminescence immunoassay method used procalcitonin (Cobas e-411 analyzer, Roche Diagnostics, Mannheim, Germany) measurement.

Four variant regions of VDR, FokI, BsmI, ApaI and TaqI were determined using the Restriction Fragment Length Polymorphism (RFLP) technique. 5 mL of peripheral blood is used for DNA isolation using the ZeeSan Lab-Aid® 824s Blood DNA isolation kit. The resulting genomic DNA samples were tested for concentration and purity at NanoDrop Spectrophotometer. For PCR reaction, 100 ng genomic DNA is added to 10x PCR Buffer, 2 mM MgCl₂, 0.5 mM dNTP, 0.25 µl of primers, and 0.2 µl Taq polymerase with a total of 25 microliters. The primer sequences for the four variant regions of VDR are as follows; FokI forward: 5'-TGGCCCTGGCACTGACTC-3', FokI reverse: 5'-GGCACGTTCCGGTCAAAGTC-3'; BsmI forward: 5'-GTGCCCCTCACTGCCCTTA-3', BsmI reverse: 5'-CCTCAATAACAGGAATGTTGAGCCCA-3'; TaqI forward: 5'-GGGCCAGGCAGTGGTATCAC-3', TaqI reverse: 5'-AGGTCGGCATGCTCCTGGATCA-3'; ApaI forward: 5'-CAGAGCATGGACAGGGAGCAAG-3', ApaI reverse: 5'-GCAACTCCTCATGGCTGAGGTCTC-3'. For each locus, reactions were run as 35 cycles of 95°C for 3-minute initial denaturation, 95°C 30 seconds for denaturation, 56°C 30 seconds for annealing, 72°C 1 minute for elongation, and a final extension of 5 min at 72°C. Annealing temperatures for ApaI were 56°C, BsmI and FokI were 55.9°C, and TaqI was 55°C. Following amplification, PCR amplicons were electrophoresed at 99 V for 32 min on 1.5% agarose gel.

After confirmation of amplicons, enzyme digestion was performed with 1 µl of restriction enzyme, 1µl of Tango Buffer, 9 µl of distilled water and 5 µl of PCR product were incubated overnight at 37°C. At the end of the digestion period, the resulting products were analyzed on 3% agarose gel after electrophoresis for 36 minutes at 99 V.

Allelic frequency was searched from a patient population admitted with different complaints other than VDR pathologies to Medical Genetics Department as the control group. The allelic frequency was detected from the NGS Clinical Exome Sequencing (CES) data. VDR rs2228570 (FokI), rs7975232 (ApaI), and rs731236 (TaqI) regions were examined with Integrated Genome Browser (IGV). Only rs1544410 (BsmI) allelic frequency was screened from literature because its NGS sequence data was not covered in the IGV data of our controls.

The presence of a restriction site was designated by a lowercase letter and its absence by an uppercase letter: “f” and “F” were used for the Fok I site; “a” and “A” were used for the Apa I site; “t” and “T” were used for the Taq I site; and “b” and “B” were used for the Bsm I site.

Endocrinological evaluation

Vitamin D deficiency was defined as a circulating 25 (OH)D level of less than 20 ng/mL (50 nmol/L), insufficiency as 20–29 ng/mL, sufficiency as ≥ 30 ng/mL (75nmol/L) according to the Endocrine Society Clinical Practice Guideline ²¹. Severe vitamin D deficiency is defined as 25 (OH)D levels less than 10 ng/mL ²².

Statistical analysis

The comparison of the continuous variables among independent groups was executed with Mann-Whitney U and Kruskal-Wallis tests. Consequent measurements were analyzed with the Wilcoxon test. The crosstables of categorical variables were analyzed with Chi-square and Fisher's exact tests. The correlation between numerical variables was tested with Spearman's correlation test. The uni- and multivariate binary logistic regression analyses were performed. According to the posthoc power analysis, power (1-Beta) was calculated as 0.99 for a total sample size of 268 patients with an

alfa error of 0.05. $p < 0.05$ was considered statistically significant. All analyses were executed by using Stata 15.1 software (StataCorp, Texas 77845 USA).

Results:

Baseline characteristics, vitamin D status, and distribution of four VDR polymorphisms in patients

Baseline demographic data, clinical characteristics, vitamin D status, and four VDR polymorphisms are shown in Table 1. Male preponderance was observed over female [n=170 (57.3%) vs n=127 (42.7%)]. The median age was 59 years (IQR: 21, min-max: 20-96). The most frequent comorbidity was hypertension (43.7%), followed by type 2 diabetes (DM), coronary heart disease (CAD), and chronic obstructive pulmonary disease (COPD). The median length of hospitalization was 11 days (IQR: 9, min-max: 2-116). Vitamin D deficiency was present in 83% of patients (n=206). Severe vitamin D deficiency (<10 ng/mL) was detected in 40.7% (n=101) of the samples. Median 25 (OH)D level was 11.97 ng/mL (IQR: 9.69, min-max:0.21-151.3).

Clinical severity scores, admission to intensive care unit, and mortality

Mild, moderate, and severe diseases were present in 36 (12.1%), 165 (55.5%), and 96 (32.3%) patients, respectively. Of the 297 patients, 51 (17.7%) have required ICU admission, and 16 patients (5.4%) have died (Table 1).

Age, duration of hospitalization, neutrophil count, ferritin, LDH, d-dimer, hs-CRP, and procalcitonin were higher, and lymphocyte count, lymphocyte percentage was lower in the severe group ($p < 0.001$, for all) (Table 2). However, no difference was noticed between vitamin D levels in these three groups ($p = 0.700$).

Similar to disease severity, older age ($p = 0.009$), duration of hospitalization, increased neutrophil count, ferritin, LDH, d-dimer, hs-CRP, procalcitonin levels ($p < 0.001$ for all), and decreased lymphocyte count, lymphocyte percentage ($p < 0.001$) were

detected in patients who have required admission to ICU (Table 3). hs-CRP levels were higher in subjects who died than subjects admitted to ICU but survived ($p<0.001$). Vitamin D levels were similar in both groups ($p=0.065$).

Sixteen subjects who have died were older ($p=0.012$), and their length of hospitalization was longer ($p<0.001$). D-dimer ($p=0.010$), neutrophil count, ferritin, LDH, d-dimer, hs-CRP, and procalcitonin levels ($p<0.001$ for all) were significantly higher in the death group together with low lymphocyte count and percent ($p<0.001$) (Table 4). Vitamin D levels were also similar in these two groups ($p=0.902$).

General characteristics and laboratory parameters of patients according to the vitamin D status

Patients were divided into two groups as deficient and sufficient groups. No difference was observed among these groups in laboratory and clinical parameters (Table 5).

This result was not changed when we classified subjects as vitamin D levels above and below 10 ng/mL. The severity scores, the frequency of admission to ICU, and the mortality were not different in deficient and sufficient groups or subjects with severe deficiency when the vitamin D cut-off limit was considered as 10 ng/mL.

Vitamin D receptor gene polymorphisms and their relationship with laboratory parameters, disease severity scores, admission to intensive care unit, and mortality

No differences were observed between clinical parameters and inflammatory markers for each allele of four polymorphisms. Vitamin D levels were also similar between each genotype of Fok I, Taq I, Apa I, and Bsm I ($p>0.05$).

Among them Ff genotype for Fok I was more common in the moderate and severe group compared to the mild group (46.6% and 47.1% vs. 22.8%) ($p=0.001$) (Table 6). The severe group consists of 45.9% ff genotype, 47.1% Ff genotype, and the remaining 7% was FF genotype. When comparing each haplotype among themselves,

the FF genotype was more common than the Ff genotype in the mild group ($p < 0.001$). Moreover, the FF genotype was more frequent than the ff genotype in the mild group ($p = 0.026$). There was no statistically significant difference between ff and Ff genotypes in the COVID-19 group ($p = 0.143$).

However, the presence of the Fok I genotype was not statistically different between surviving and non-surviving subjects; the FF genotype was seen in none of the cases of the death group [0% vs. 8.1% ($n = 21$)].

When we focused on Taq I polymorphism, the most frequent genotype was Tt (48.7%), and the tt genotype was the least frequent (6.3%) in COVID-19 patients. Most of the subjects (62.8%) who have been admitted to ICU had TT genotype for TaqI polymorphism, while the Tt genotype was more common in the ICU admission negative group (52.7%) ($p = 0.008$). When we assessed each haplotype in pairs for the ICU admission, there was a statistically significant difference between Tt and TT genotypes ($p = 0.004$). However, the comparison of tt to TT genotypes and tt to Tt genotypes was not statistically significant ($p = 0.924$ vs. $p = 0.070$). There was no difference in polymorphisms between the group discharged after the intensive care unit and the death group ($p > 0.05$).

In respect to Apa I polymorphism, Aa (68.1%) was the most frequent genotype, while AA (2.9%) was the least one in COVID-19 patients. ApaI aa genotype (81.2%) was common in the death group ($p = 0.001$). None of the deceased patients had the AA genotype. LDH levels were higher in the aa genotype group than in the AA group ($p = 0.038$). When we compared the aa genotype to Aa genotype, it was the only one that was statistically significant for mortality ($p < 0.001$). However, there was no difference between Aa and AA genotypes ($p = 0.768$) and aa and AA genotypes ($p = 0.313$) for mortality.

Comparison of vitamin D receptor polymorphisms with the control group

The distribution of FokI genotypes was similar in the control and the COVID-19 group. The Apa I AA genotype was more common in the control group than the COVID-19 group (28.6% vs. 2.9%, $p < 0.001$). Furthermore, while Taq I Tt genotype was more frequent in the COVID-19 group (48.7 % vs. 32.6%), it was the tt genotype in the control group (20% vs. 6.3%) ($p < 0.001$). As Bsm I Bb genotype was more frequent in the COVID-19 group (66.1% vs. 41.8%), it was the bb genotype in the control group (52.7% vs. 18.8%) ($p < 0.001$).

The most common haplotypes were AaFfTtBb (n=39), AaffTtBb (n=29), AaffTTBb (n=29), AaFfTTBb (n=28), aaffTtBb (n=17), respectively. There was no statistically significant difference between laboratory and clinical parameters among each haplotype.

Uni- and multivariate logistic regression analyses

Univariate analysis showed that disease severity was associated with age (OR: 1.031, CI: 1.015-1.047, $p < 0.001$, R^2 :2.7%), lymphocyte count (OR= 0.998, CI: 0.997-0.998, $p < 0.001$, R^2 :6.7%), ferritin (OR= 1.001, CI: 1.000-1.002, $p < 0.001$, R^2 :7.9%), LDH (OR= 1.003, CI: 1.002-1.005, $p < 0.001$, R^2 :8.5%), hs-CRP levels (OR= 1.016, CI: 1.012-1.019, $p < 0.001$, R^2 :18.1%), procalcitonin (OR= 2.902, CI: 1.651-5.099, $p < 0.001$, R^2 :8.7%), and also Fok I Ff genotype (OR= 3.172, CI: 1.182-8.511, $p = 0.022$, R^2 :1.1%).

In univariate analysis, mortality was associated with the Apa I aa genotype (OR=11.828, CI: 2.493-56.104, $p = 0.002$, R^2 : 14.6%). Moreover, ICU admission was associated with the TaqI TT genotype (OR=2.854, CI: 0.851-10.755, $p = 0.005$, R^2 : 3.8%).

In multivariate logistic regression analysis, hs-CRP (OR=1.016), ApaI aa genotype (OR=14.581) were found to be related to mortality ($R^2=37.1\%$).

Discussion:

In this cross-sectional study, vitamin D deficiency was present in 83% of the subjects, and 40.7% of the whole group had severe deficiency. Median 25 (OH)D level was 11.97 ng/mL. There was no relationship between disease severity, admission to ICU, mortality, and inflammatory markers with 25 (OH)D levels. While disease severity was related to Fok I Ff genotype, it was Taq TT genotype for ICU admission. Moreover, the ApaI aa genotype was common in the death group.

Vitamin D acts as an immunoregulatory hormone associated with monocyte activation, stimulation of cell-mediated immunity, and suppression of lymphocyte proliferation, antibody production, and cytokine synthesis such as IL-6^{4,8,23}. It modulates the adaptive immune response, suppressing the T helper 1 (Th1) response and promoting cytokines production by Th2 cells²⁴.

Merzon et al.'s study with 7807 individuals showed that the mean 25 (OH)D level was lower in subjects with the COVID-19²⁵. Also, in a retrospective observational study with 186 positive cases and 2717 negative controls, COVID-19 patients had lower 25 (OH)D levels²⁶. In the study of Hernández et al., which included 216 COVID-19 patients and 197 controls, vitamin D deficiency was statistically higher in the COVID-19 group (82.2% vs. 47.2%)²⁷. A significant negative correlation was detected between mean 25 (OH)D levels and the number of COVID-19 cases²³. On the other side, in the UK biobank studies, there was no link between the risk of COVID-19 and 25 (OH)D levels^{28,29}. In Hastie et al.'s study²⁸, which obtained 25 (OH)D concentrations from the UK biobank, the PCR positivity was assessed for a limited duration (between March and April 2020). However, it was not considered that those

subjects could have SARS-CoV-2 infection later²⁸. Moreover, in other studies with a control group, the information of COVID-19 was present for the study period such as one or two months²⁵⁻²⁷. We have not included a control group for 25 (OH)D levels in our study. To prove that low 25 (OH)D levels predispose to SARS-CoV-2 infection, controls should be exposed to COVID-19 at the same period and need to match groups with each other such as age, sex, comorbidities. Therefore, we thought it would be more appropriate to evaluate the relationship between 25 OH(D) status and disease severity, admission to ICU, and mortality. The prevalence of vitamin D deficiency was high in Turkey, reported as 63.5% among adults³⁰. However, our study group's deficiency rate was higher (83%) than the previously published frequency. Hypovitaminosis D (25 (OH)D level < 30 ng/mL) prevalence was reported as 62%³¹, 74.1%³², 81%³³, 82.2%²⁷ among COVID-19 patients. While the severe deficiency rate was 24% in Carpagnano et al.'s study³³, it was 40.7% in our study. Vitamin D deficiency was associated with a higher risk of invasive mechanical ventilation and death in several studies^{12,26,34}. In the study of Carpagnano et al., which included 42 subjects from the respiratory ICU, patients with severe vitamin D deficiency had significantly increased mortality risk³³. The severity of hypovitaminosis D was related to the prognosis of COVID-19 in nursing-home residents³⁵. In a meta-analysis, individuals with poor prognosis had lower serum levels of 25 (OH)D than those with a good prognosis³⁶. Contrary to previous reports^{32,36}, there was no relationship between 25 (OH)D levels or vitamin deficiency and disease severity or mortality^{13-15,23,26,27,37}. Our study did not find a relationship between 25 (OH)D levels and disease severity, admission to ICU, or mortality. Furthermore, 25 (OH)D levels can be reduced as a result of infection or inflammation such that the association does not necessarily imply causality.

Hernández et al. revealed that 25 (OH)D levels were inversely correlated with serum ferritin and d-dimer levels ²⁷. In Carpagnano et al.'s study, IL-6 levels were higher in patients with severe vitamin D deficiency ³³. Daneshkhah et al. reported that high CRP was inversely correlated with 25 (OH)D levels ³⁸. None of the inflammatory markers were related to 25 (OH)D levels in our study.

The VDR, a member of the nuclear receptor family of transcription factors, is expressed on B cells, T cells, macrophages, monocytes, and several tissues like respiratory epithelial cells. The activation of VDR modulates inhibition of Th1 cell proliferation and proinflammatory cytokine production and induction of Th2 cell proliferation and anti-inflammatory cytokine production ³⁹. Moreover, it plays a role in innate and adaptive immune systems ⁴⁰. After calcitriol binds to VDR, this complex induces transcriptional expression of antimicrobial peptides such as cathelicidins and defensins ³⁶. This VDR related response helps immune-mediated defense against several viral infections, such as human immunodeficiency virus, hepatitis B virus (HBV), dengue virus, and RSV ¹⁹. Vitamin D receptor gene polymorphisms impact vitamin D response on tissue level. Enveloped virus infections promote both cell-mediated and humoral immunity. In this context, SARS-CoV-2 as an encapsulated virus may also be associated with VDR polymorphisms considering VDR function.

The FF genotype of Fok I, which is related to a shorter form of VDR, leads to higher transcriptional activity that forms more active complexes of VDR-vitamin D, inhibits the Th1 response, and induces the Th2 cell response ³⁹. The f allele reduces the ability of the vitamin D receptor complex to bind to gene elements responsive to vitamin D ¹⁹. Roth et al. reported that the FokI ff genotype was associated with an adjusted relative odds of acute lower respiratory infections (ALRI) 7 times than FokI FF ¹⁸. A meta-analysis conducted by Laplana et al. ¹⁹ also revealed that enveloped virus

infections, like RSV, were increased in individuals with Fok I f allele. The results of another meta-analysis on RSV-bronchiolitis were similar¹⁷. The f allele is blamed for encoding a less active VDR. Also, the FF genotype is related to a better prognosis in liver cirrhosis⁴¹. We found that the Ff genotype was more common in the moderate and severe group than the mild group, and only 7% of the severe group consisted of the FF genotype. In addition, none of the deceased subjects had the FF genotype. In short, the f allele has affected the prognosis of subjects, and the FF genotype was mainly detected in subjects with a good prognosis, which can be a protective factor against ICU admission. In Abdollahzadeh et al.'s study⁴², Fok I f allele was also positively associated with signs, symptoms, and the severity of COVID-19 affected people.

The Taq I polymorphism is located on exon 9, and it also has functional effects linked to alterations in VDR mRNA stability. Taq I may also alter VDR gene expression, VDR protein structure, and binding specificity for vitamin D, resulting in reduced vitamin D-related signaling pathways activated in target cells⁴³. The study of Roth et al. has shown that Taq I Tt genotype had a greater risk of acute lower respiratory infection than those with tt genotypes¹⁸. In Abdollahzadeh et al.'s study⁴², Taq I was not associated with clinical manifestations and severity of COVID-19. However, in our study, TT showed a poor prognosis for admission to ICU, and Tt was protective. The age differences and the number of patients, and the existence of different etiologies could explain the difference. The TT genotype has been associated with higher VDR expression levels in peripheral blood mononuclear cells (PMBC) than those with the tt genotype^{18,44}. On the other hand, increased VDR expression in PMBC might have triggered cytokine release, contributing to the cytokine storm,

leading to a higher rate of ICU admission. As there were few studies in the literature with Taq I polymorphisms, any deduction may be misleading.

ApaI aa genotype was common in the non-surviving subjects in our study. Also, LDH levels were higher in the aa genotype group. Multivariate analysis showed that Apa I aa genotypes were associated with mortality with an OR of 14.5, explaining 37.1% of the model with hs-CRP. Although there were no studies with Apa I and viral infection affecting the respiratory tract, studies with HBV showed that Aa/aa genotypes of Apa I polymorphism cause Th2 proliferation. Contrary, AA genotypes cause Th1 proliferation and produce anti-inflammatory cytokines resulting in liver disease progression to cirrhosis ⁴⁵. Th2 can also produce IL-6, which is related to COVID-19 prognosis ⁴⁶ and may explain the higher death rate in subjects with aa genotype of Apa I polymorphism in our study. In Abdollahzadeh et al.'s study, ⁴² subjects with severe/critical and mild/moderate disease who had "Aa" genotype compared to "AA +aa" genotypes had a rising severity risk. However, in symptomatic-asymptomatic and mild/moderate-asymptomatic subjects with AA genotype made subjects more prone to possess signs and symptoms versus both "Aa +aa" and "Aa" genotypes. Contrary to our study, none of the deceased subjects had an AA genotype.

Bsm I was not related to disease severity in our study, but Abdollahzadeh et al. ⁴² reported that the b allele was found to be a predisposition factor to COVID-19 severity.

There were several limitations in our study. First, the information on dietary habits was lacking. Secondly, the control group for polymorphisms was used from our biobank. This may not be correct for comparison; as we mentioned before, in order to be a control case, simultaneous exposure to the virus was necessary, and other predisposing factors should be eliminated. Vitamin D levels were not measured in the

control group. We compared vitamin D status with disease severity, mortality, and intensive care hospitalization. Even we do not know serum 25 (OH)D levels of those with polymorphism in control cases; we think that the level of 25 (OH)D in the tissue is more important than serum.

In conclusion, our results supported that serum 25 (OH)D levels were not related to COVID-19 severity and mortality. Additionally, it indicated that VDR polymorphisms are independently associated with the severity of COVID-19 and the survival of patients. More clinical studies with a larger population and tissue vitamin D levels are needed to determine the impact of polymorphisms on COVID-19 and explain the underlying cause.

Acknowledgements: This study did not receive specific grants from funding agencies in the public, commercial, or not-for-profit sector.

Conflict of interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Data Availability: Data available on request from the authors

Author contribution statement: All authors made substantial contributions to conception and design, and acquisition of data, and analysis and interpretation of data; participated in drafting the article or revising it critically for important intellectual content, and final approval of the version to be submitted.

Ethical Approval: All procedures performed in studies involving human participants followed the institutional research committee's ethical standards, the 1964 Helsinki declaration, and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all participants included in the study.

Abbreviations

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CAD, Coronary artery disease

CARD, Chronic obstructive respiratory disease;

CT, Computed tomography;

DM, Type 2 diabetes mellitus;

hs-CRP, high sensitive C reactive protein;

IMV, Invasive mechanical ventilation;

ICU, intensive care unit;

LDH, Lactate dehydrogenase;

NIMV, noninvasive mechanical ventilation;

25(OH)D, 25-hydroxyvitamin D

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Table 1. Baseline characteristics of patients

Parameter	n	%
	297	
Sex		
Female	127	42.7
Male	170	57.3
Symptoms		
Fatigue	205	69.0
Cough	179	60.3
Shortness of breath	124	52.3
Myalgia	118	39.7
Fever	106	35.7
Comorbidities		
Hypertension	130	43.7
Type 2 DM	85	28.6
CAD	44	14.8
COPD or asthma	35	11.8
Malignity	11	3.7
CT findings	265	89.5
Oxygen demand	196	66.0
Nasal prongs	100	33.7
NIMV or reservoir mask	58	19.5
IMV	38	12.8
Mortality	16	5.4

Intensive care unit admission	51	17.7
Vitamin D Status (n=248)		
<10 ng/mL	101	40.7
10-20 ng/mL	105	42.3
20-30 ng/mL	29	11.7
>30 ng/mL	13	5.2
Fok I polymorphism (n=268)		
ff	130	48.5
Ff	117	43.6
FF	21	7.8
Total F allele	159	29.6
Total f allele	377	70.3
Bsm I polymorphism (n=267)		
bb	50	18.7
Bb	177	66.3
BB	40	14.9
Total B allele	257	48.1
Total b allele	277	51.8
Apa I polymorphism (n=273)		
aa	79	28.9
Aa	186	68.1
AA	8	2.9
Total A allele	202	37.0
Total a allele	344	63.0
Taq I polymorphism (n=267)		

tt	17	6.3
Tt	130	48.7
TT	120	44.9
Total T allele	370	69.2
Total t allele	164	30.7

CAD, coronary heart disease; COD, chronic obstructive pulmonary disease; CT, computerized tomography; DM, Diabetes mellitus; IMV, invasive mechanical ventilation; NIMV, Non-invasive mechanical ventilation

Table 2. Comparison of general characteristics of patients allocated into 2 groups as mild-moderate and severe which were defined according to WHO criteria

Parameters	median (min-max)	IQR	p value
Age (years)			
Mild	47.5 (21-69)	19.5	<0.001
Moderate	60.0 (23-94)	20	
Severe	59.5 (20-96)	21	
Duration of hospitalization			
(days)	6 (4-14)	2	<0.001
Mild	9 (2-40)	5	
Moderate	19 (5-116)	13	
Severe			
Lymphocyte count (/μL)			
Mild	1000 (400-2100)	700	<0.001
Moderate	500 (100-2500)	500	
Severe	500 (100-6000)	350	
Lymphocyte percentage (%)			

Mild	19.7 (5.1-37.5)	12.4	<0.001
Moderate	14.9 (0.9-44.4)	11.6	
Severe	6.0 (0.4-50.1)	5.7	
Neutrophil count (/μL)			
Mild	2900 (900-8900)	1900	<0.001
Moderate	3500 (600-14700)	2100	
Severe	4600 (1200-21300)	3500	
Thrombocyte count (x10³/μL)			
Mild	197 (65-340)	76	0.431
Moderate	180 (36-499)	70	
Severe	187 (84-562)	126	
Ferritin (μg/L)			
Mild	156.0 (3.5-1862)	489.8	<0.001
Moderate	284.0 (10.0-2307)	402.0	
Severe	683.5 (10.8-12010)	857.5	
Lactate dehydrogenase (U/L)			
Mild	294 (154-577)	150	<0.001
Moderate	308 (156-1396)	160	
Severe	508 (198-3256)	277	
D-dimer (mg/L)			
Mild	0.56 (0.27-1.78)	0.36	<0.001
Moderate	0.85 (0.10-13.70)	0.9	
Severe	2.65 (0.30-20.00)	5.5	
hs-CRP (mg/L)			
Mild	28.9 (0.9-169.0)	70.7	<0.001

Moderate	56.8 (1.1-254)	110.9	
Severe	157.8 (7.6-472)	140.5	
Procalcitonin (ng/mL)			
Mild	0.07 (0.03-0.73)	0.07	<0.001
Moderate	0.08 (0.02-2.47)	0.07	
Severe	0.12 (0.02-20.5)	0.14	
Creatinine (mg/dL)			
Mild	0.70 (0.43-1.10)	0.3	0.012
Moderate	0.84 (0.29-7.35)	0.33	
Severe	0.84 (0.23-8.34)	0.48	
Alanine aminotransferase			
(U/L)	20 (8-217)	25	0.493
Mild	24 (3-157)	21	
Moderate	24 (7-227)	22.5	
Severe			
Vitamin D level (ng/mL)			
Mild	13.4 (3.1-33.3)	10.0	0.700
Moderate	11.9 (0.4-151.3)	8.98	
Severe	11.7 (0.2-79.5)	11.24	

Percentages are given according to the column.

Mild cases (n=36, 12.1%), moderate cases (n=165, 55.5%), and severe diseases (n=96, 32.3%)

Alanine aminotransferase, (reference range: 10-40 U/L); creatinine, (reference range: 0-1.2 mg/dL); D-dimer, (reference range: 0-0.5 mg/L); ferritin, (reference range: 12-150 µg/L for women; 30-400 µg/L for men); hs-CRP, high sensitive C-reactive protein (reference range: 0-5 mg/L); lactate dehydrogenase, (reference range: 0-248 U/L); lymphocyte count, (reference range: 1.2-3.1 × 10³/µL); lymphocyte percentage, (reference range: 20-50%); procalcitonin, (reference range: 0-0.5 ng/mL); thrombocyte count, (reference range: 150-440 × 10³/µL)

Bold values denote statistically significant p values.

Table 3. Comparison of general characteristics of patients according to admission to the intensive care unit

Parameters	median (min-max)	IQR	p value
Age (years)			
ICU+	62 (20-96)	23	0.009
ICU-	58 (21-94)	19	
Duration of hospitalization (days)			
ICU+	22 (5-116)	23	<0.001
ICU-	9 (2-40)	7	
Lymphocyte count (/μL)			
ICU+	400 (100-1600)	300	<0.001
ICU-	800 (200-7200)	600	
Lymphocyte percentage (%)			
ICU+	5.3 (0.4-23.0)	5.2	<0.001
ICU-	14.2 (0.9-50.1)	12.8	
Neutrophil count(/μL)			
ICU+	5500 (1200-21300)	5500	<0.001
ICU-	3500 (600-18200)	2000	
Thrombocyte count (x10³/μL)			
ICU+	192 (84-562)	132	0.063
ICU-	181 (36-532)	82	
Ferritin (μg/L)			
ICU+	959 (99.8-12010)	958	<0.001
ICU-	298 (3.5-3497)	483	
Lactate dehydrogenase (U/L)			

ICU+	577 (291-3256)	315	<0.001
ICU-	325 (154-1894)	194	
D-dimer (mg/L)			
ICU+	1.45 (0.51-20)	1.42	<0.001
ICU-	0.86 (0.1-20)	0.48	
hs-CRP (mg/L)			
ICU+	191.4 (26.3-472.0)	118.6	<0.001
ICU-	64.7 (0.9-285)	110.1	
Procalcitonin (ng/mL)			
ICU+	0.14 (0.02-20.5)	0.17	<0.001
ICU-	0.08 (0.02-2.47)	0.06	
Creatinine (mg/dL)			
ICU+	0.9 (0.23-3.43)	0.49	0.275
ICU-	0.82 (0.29-8.34)	0.35	
Alanine aminotransferase (U/L)			
ICU+	24 (7-157)	22	0.428
ICU-	23 (3-227)	21	
Vitamin D level (ng/mL)			
ICU+	13.0 (0.8-79.5)	10.3	0.065
ICU-	11.4 (0.1-151.3)	10.1	

Fifty-one (17.7%) patients required admission to intensive care unit

Alanine aminotransferase, (reference range: 10-40 U/L); creatinine, (reference range: 0-1.2 mg/dL); D-dimer, (reference range: 0-0.5 mg/L); ferritin, (reference range: 12-150 µg/L for women; 30-400 µg/L for men); hs-CRP, high sensitive C-reactive protein (reference range: 0-5 mg/L); lactate dehydrogenase, (reference range: 0-248 U/L); lymphocyte count, (reference range: $1.2-3.1 \times 10^3/\mu\text{L}$); lymphocyte percentage, (reference range: 20-50%); procalcitonin, (reference range: 0-0.5 ng/mL); thrombocyte count, (reference range: $150-440 \times 10^3/\mu\text{L}$)

Bold values denote statistically significant p values.

Table 4. Comparison of general characteristics of patients according to the presence of death

Parameters	median (min-max)	IQR	p value
Age (years)			
Exitus	69 (20-96)	27	0.012
Alive	58 (21-94)	20	
Duration of hospitalization (days)			
Exitus	20 (5-55)	11	<0.001
Alive	9 (2-116)	9	
Lymphocyte count (/μL)			
Exitus	350 (100-1300)	350	<0.001
Alive	700 (100-6000)	600	
Lymphocyte percentage (%)			
Exitus	4.45 (1.0-23.0)	5.4	<0.001
Alive	12.9 (0.4-50.1)	12.3	
Neutrophil count (/μL)			
Exitus	5900 (1200-11000)	3200	<0.001
Alive	3700 (600-21300)	2500	
Thrombocyte count (x10³/μL)			
Exitus	195 (84-388)	120	0.231
Alive	87 (36-562)	87	
Ferritin (μg/L)			
Exitus	1011 (112-9196)	937	<0.001
Alive	343 (3. 5-12010)	550	
Lactate dehydrogenase (U/L)			

Exitus	543 (359-3256)	470	<0.001
Alive	344 (154-2549)	205	
D-dimer (mg/L)			
Exitus	5.92 (1.12-17.04)	5.90	0.010
Alive	0.99 (0.1-20)	1.77	
hs-CRP (mg/L)			
Exitus	168 (73.3-289.5)	81.1	<0.001
Alive	79 (0.9-412)	75.5	
Procalcitonin (ng/mL)			
Exitus	0.15 (0.07-20.5)	0.39	<0.001
Alive	0.08 (0.02-2.47)	0.07	
Creatinine (mg/dL)			
Exitus	1 (0.23-3.43)	0.60	0.107
Alive	0.82 (0.28-8.34)	0.36	
Alanine aminotransferase (U/L)			
Exitus	23.5 (7-51)	20	0.443
Alive	23 (3-227)	22	
Vitamin D level (ng/mL)			
Exitus	11.7 (0.8-79.5)	9.04	0.902
Alive	12.0 (0.2-151.3)	9.78	

Sixteen patients (5.4%) have died

Alanine aminotransferase, (reference range: 10-40 U/L); creatinine, (reference range: 0-1.2 mg/dL); D-dimer, (reference range: 0-0.5 mg/L); ferritin, (reference range: 12-150 µg/L for women; 30-400 µg/L for men); hs-CRP, high sensitive C-reactive protein (reference range: 0-5 mg/L); lactate dehydrogenase, (reference range: 0-248 U/L); lymphocyte count, (reference range: $1.2-3.1 \times 10^3/\mu\text{L}$); lymphocyte percentage, (reference range: 20-50%); procalcitonin, (reference range: 0-0.5 ng/mL); thrombocyte count, (reference range: 150-440 $\times 10^3/\mu\text{L}$)

Bold values denote statistically significant p values.

Table 5. Comparison of general characteristics of patients according to Vitamin D deficiency

Parameters	median (min-max)	IQR	p value
Age (years)			
<20 ng/mL	57.5 (20-96)	19	0.276
≥20 ng/mL	60.5 (38-90)	22	
Duration of hospitalization (days)			
<20 ng/mL	11 (2-82)	10	0.571
≥20 ng/mL	11 (3-116)	15	
Lymphocyte count (/μL)			
<20 ng/mL	700 (100-6000)	600	0.124
≥20 ng/mL	600 (200-1800)	600	
Lymphocyte percentage (%)			
<20 ng/mL	12.2 (0.4-50.1)	13.5	0.143
≥20 ng/mL	10.0 (1.8-36.6)	15.1	
Neutrophil count (/μL)			
<20 ng/mL	3700 (600-21300)	2500	0.478
≥20 ng/mL	3850 (1200-18200)	3400	
Thrombocyte count (x10³/μL)			
<20 ng/mL	183 (36-562)	79	0.814
≥20 ng/mL	180 (37-356)	125	
Ferritin (μg/L)			
<20 ng/mL	342 (3.5-12010)	559	0.071
≥20 ng/mL	460.1 (7-2858)	754	
Lactate dehydrogenase (U/L)			
<20 ng/mL	345 (154-3256)	211	0.112
≥20 ng/mL	369 (172-2549)	251	
D-dimer (mg/L)			
<20 ng/mL	1.02 (0.1-20)	1.85	0.095
≥20 ng/mL	1.20 (0.29-20)	2.86	
hs-CRP (mg/L)			
<20 ng/mL	92.4 (0.9-461.7)	122.2	0.937

≥ 20 ng/mL	81.6 (3.1-472)	150.8	
Procalcitonin (ng/mL)			
<20 ng/mL	0.08 (0.02-20.5)	0.06	0.670
≥ 20 ng/mL	0.10 (0.02-2.47)	0.08	
Creatinine (mg/dL)			
<20 ng/mL	0.83 (0.23-8.34)	0.38	0.076
≥ 20 ng/mL	0.91 (0.20-7.35)	0.42	
Alanine aminotransferase (U/L)			
<20 ng/mL	23 (7-227)	20	0.716
≥ 20 ng/mL	24 (3-92)	22	

Alanine aminotransferase, (reference range: 10-40 U/L); creatinine, (reference range: 0-1.2 mg/dL); D-dimer, (reference range: 0-0.5 mg/L); ferritin, (reference range: 12-150 $\mu\text{g/L}$ for women; 30-400 $\mu\text{g/L}$ for men); hs-CRP, high sensitive C-reactive protein (reference range: 0-5 mg/L); lactate dehydrogenase, (reference range: 0-248 U/L); lymphocyte count, (reference range: $1.2-3.1 \times 10^3/\mu\text{L}$); lymphocyte percentage, (reference range: 20-50%); procalcitonin, (reference range: 0-0.5 ng/mL); thrombocyte count, (reference range: $150-440 \times 10^3/\mu\text{L}$)

Bold values denote statistically significant p values.

Table 6. Vitamin D receptor gene polymorphisms and its relationship with disease severity, admission to intensive care unit, and mortality

Parameters	Disease severity			ICU admission		Mortality	
	n (%)			n (%)		n (%)	
	Mild	Moderate	Severe	+	-	+	-
Fok I			γ				
ff	19	72 (48.6)	39	20	110	6	124
Ff	(54.3)	69 (46.6)	(45.9)	(46.5)	(48.9)	(54.5)	(48.2)
FF	8 (22.8)	7 (4.7)	40	20	97	5	112
	8 (22.8)		(47.1)	(46.5)	(43.1)	(45.5)	(43.5)
			6 (7.0)	3 (7.0)	18 (8.0)	0 (0.0)	21 (8.3)
Taq I					η		
tt	2 (5.6)	8 (5.5)	7 (8.2)	4 (9.3)	13 (5.8)	3	15 (5.9)
Tt	17	72 (49.3)	41	12	118	(27.2)	125
TT	(47.2)	66 (45.2)	(48.3)	(27.9)	(52.7)	7	(48.8)

	17		37	27	93	(63.6)	116
	(47.2)		(43.5)	(62.8)	(41.5)	1 (9.1)	(45.3)
Apa I							§
aa	10	41 (27.0)	28	11	68	9	70
Aa	(27.8)	107 (70.4)	(32.9)	(26.2)	(29.5)	(81.8)	(26.7)
AA	24	4 (2.6)	55	30	156	2	184
	(66.7)		(64.7)	(71.4)	(67.5)	(18.2)	(70.2)
	2 (5.5)		2 (2.4)	1 (2.4)	7 (3.0)	0 (0.0)	8 (3.1)
Bsm I							
bb	4 (11.4)	32 (21.0)	15	5 (11.9)	45	3	47
Bb	22	97 (65.5)	(17.8)	30	(20.0)	(27.2)	(18.4)
BB	(62.9)	20 (13.5)	58	(71.4)	147	7	170
	29		(69.1)	7 (16.7)	(65.3)	(63.6)	(66.4)
	(25.7)		11		33	1 (9.1)	39
			(13.1)		(14.7)		(15.2)

% were given for each column.

γ, Fok I allele for disease severity (p=0.001); ¶, Taq I allele for ICU admission (p=0.008); §, Apa I allele for mortality (p=0.001)