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Research Article

Evaluation of Vitamin D binding protein and 25-hydroxy Vitamin D metabolites in COVID-19 patients

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Abstract

Objectives: The immunomodulatory roles of Vitamin D and Vitamin D binding protein (VDBP) are in interest with incidence or outcome of coronavirus disease-2019 (COVID-19). This study aimed to investigate the association between the severity of COVID-19 with VDBP, total 25-hydroxy Vitamin D (25(OH)D), and its metabolites free Vitamin D (VD_{free}) and bioavailable Vitamin D (VD_{bio}).

Methods: Study group consisted of 68 COVID-19 patients and 20 healthy subjects. Patients were subgrouped as asymptotic, mild/moderately pneumonia, or severe pneumonia. Plasma total 25(OH)D was quantitated by liquid chromatography with mass spectrometry and serum VDBP by a polyclonal sandwich enzyme immunoassay. In addition, routinely used laboratory parameters in follow-up were recorded. VD_{free} and VD_{bio} were calculated using total 25(OH)D, VDBP, and albumin levels.

Results: Plasma total 25(OH)D (13.3±5.7 vs. 30.3±13.3 ng/dL), VD_{free} (2.18 [1.52–3.44] vs. 4.34 [3.74–6.48] pg/mL), and VD_{bio} (1.86 [1.09–2.81] vs. 4.28 [3.45–6.34] nmol/L) levels were lower in COVID-19 patients ($p<0.001$). Despite the insignificance of 25(OH)D and metabolites between COVID-19 severity subgroups, serum VDBP was highest in mild/moderately pneumonia (601.8±278.6 ng/mL) and lowest in severe pneumonia (427.9±147.2 ng/mL) ($p<0.001$). In addition, VDBP was positively correlated with lymphocyte counts (B:87.9, r2=0.068, p=0.031) and negatively correlated with D-Dimer levels (B:–0.024, r2=0.081, p=0.032).

Conclusion: COVID-19 patients have lower plasma 25(OH)D levels and lower 25(OH)D metabolites VD_{free}, VD_{bio} which are physiologically active. In addition, serum VDBP concentrations significantly decrease in critically ill patients which needs further studies to be associated in the etiopathogenesis of the disease severity.

Keywords: 25(OH)D, bioavailable Vitamin D, COVID-19, free Vitamin D, Vitamin D binding protein

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The coronavirus disease 2019 (COVID-19), which emerged in the last months of 2019, caused a pandemic affected the whole world. The severity of disease was all related to underlying inflammation in many organ systems, leading to serious clinics such as pneumonia, thrombosis, and cytokine storm [1, 2]. In addition to risk factors predisposing to adverse outcomes such as age, obesity, diabetes mellitus, hypertension, and ethnicity [3–7], Vitamin D inadequacy has also been claimed to be a potential risk factor [8–10]. Previous studies have shown that Vitamin D decreases inflammatory cytokines

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and increases anti-inflammatory cytokines [1, 10]. Therefore, it is thought that adequate Vitamin D levels can prevent the cytokine storm associated with COVID-19 and Vitamin D supplementation to reduce the impact of the pandemic can be a relatively easy and cheaper option of preventive treatments from serious complications [11, 12].

The main carrier protein of 25-hydroxy Vitamin D (25(OH)D) in plasma is Vitamin D binding protein (VDBP). According to the "free hormone hypothesis," only unbound, or "free," hormones are physiologically active, while protein-bound hormones are inactive. Therefore, free Vitamin D is expected to be the biologically active form and its levels are dependent on Vitamin D binding protein levels and isoforms. The term of "bioavailable" 25(OH)D refers to all 25(OH)D not bound to VDBP, in other words 25(OH)D bound to albumin (an alternative to free form) plus free 25(OH)D. Although it can be expressed in many tissues, VDBP is mainly produced in the liver in an estrogen-dependent manner. Therefore, it increases significantly during pregnancy and estrogen therapy. In addition, it decreases in liver diseases, nephrotic syndrome, malnutrition, septic shock, or trauma [13]. However, there is no relationship between Vitamin D itself or any of its metabolites in the regulation of VDBP expression [14, 15]. VDBP also has potential immunoregulatory functions beyond Vitamin D binding as only 1–2% of the sterol binding sites are used [16, 17]. Filamentous actin (F-actin) is detected in the blood of patients with acute respiratory distress syndrome (ARDS), which can lead to the development of microembolism, pulmonary vascular angiopathy, and multi-organ dysfunction syndrome [18]. VDBP is a member of the extracellular actin scavenger system. However, high concentrations and/or prolonged exposure to VDBP-actin complexes can cause endothelial cell damage and death [19]. As a result, VDBP may play a role in the course of COVID-19 through effects such as modulation of innate immunity and inflammatory processes, and actin clearance [16, 17, 20].

Our objective in this study was to evaluate the relation between the COVID-19 severity with serum VDBP levels and 25(OH)D metabolites. We also aimed to investigate their correlations with fibrinogen, D-Dimer, ferritin levels, and lymphocyte counts, which have previously shown to be associated with disease severity and clinical outcomes [21].

Materials and Methods

Study design and data collection

This cross-sectional study included 68 COVID-19 patients admitted to the Infectious Diseases and Pandemic Clinic from March 2021, to April 2021. It also included 20 healthy laboratory professionals without any signs or symptoms of COVID 19 infection as the control group. The diagnosis of COVID-19 was confirmed by polymerized chain reaction (PCR) for ORF1ab/N/RdRP genes (Bio-Speedy® SARS-CoV-2 + VOC202012/01 RT-qPCR). Exclusion criteria were under the age of 18 years and over the age of 80 years, diagnosis of autoimmune, autoinflammatory, chronic infectious disease, and malignancy. The Local Ethics Committee approved this study under the Declaration of Helsinki (decision number: 2021/329) and all participants signed informed consent.

COVID-19 patients were divided into three groups according to the Guidelines on the Diagnosis and Treatment of COVID-19 by the National Health Commission of China [22]: Asymptomatic group (without pneumonia), mild/moderate pneumonia, and severe pneumonia. Respiratory rate <30 times/minute, pulse oxygen saturation (SpO_2) >93%, and partial pressure of oxygen/fraction of inspired oxygen (PaO_2/FiO_2) >300 mm-Hg criteria were determined as mild/moderate pneumonia and followed in the inpatient clinic. Patients with one of the criteria for respiratory rate ≥30 times/minute, SpO_2 <93% at rest, and PaO_2/FiO_2 ≤300 mm-Hg were evaluated as having severe pneumonia and followed in the Intensive Care Unit (ICU).

The following data were recorded for each patient: Demographic characteristics (age and gender), routinely evaluated laboratory parameters on the 1st day of admission (complete blood count [CBC], C-reactive protein [CRP], ferritin, D-dimer, and fibrinogen). CRP levels were performed on Roche Cobas c501 (Roche Diagnostics, Germany), and ferritin levels performed on Cobas e701 (Roche Diagnostics, Germany). D-Dimer and fibrinogen levels were analyzed on STA Compact (Diagnostica Stago, France). CBC analysis was performed on XN 9000 (Sysmex, Germany).

Sample processing for VDBP, total 25(OH)D and Vitamin D metabolites quantitation

Within the first 24 h after admission, blood samples were collected in a serum separator gel tube (BD, Franklin Lakes, NJ, USA) for VDBP analysis and EDTA-containing tube for total 25(OH)D analysis. The tubes were centrifuged at 2000× g for 10 min, serum and plasma samples were separated, and stored at −80°C until analysis day. Samples were processed for total 25(OH)D measurement according to Immuchrom 25-OH Vitamin D3/D2 kit manual's procedures and quantitated by liquid chromatography with tandem mass spectrometry (LC-MS/MS) (AB-Sciex Q-TRAP) which is regarded as a reference method for total 25(OH)D.

VDBP levels were measured by using a sandwich enzyme immunoassay method (Immundiagnostic ELISA kit, Immundiagnostic AG, Germany). The assay is intended for the quantitative determination of free and not actin bound VDBP. It utilizes both polyclonal binding protein antibody and peroxidase-labeled polyclonal binding protein antibody. The inter-assay coefficients of variation were 8.8% and assay sensitivity, was 1.2 ng/mL. The two-level quality control samples were also run in each plate. An absorbance wavelength of 405 nm, against 620 nm as reference was used for all standards and samples.

The bioavailable 25(OH)D levels were calculated by the following equations using the levels of all parameters in mol/L [20].

$$VD_{free}=VD_{total}/[(1+(6\times10^5)\times Albumin)+((7\times10^8)\times VDBP)]$$

$$VD_{bio}= (1+(6\times10^5)\times Albumin)\times VD_{free}$$

Table 1. Comparison of 25(OH)D, VDBP, and Vitamin D metabolites of COVID-19 patients and healthy controls

Parameters	COVID-19 patients (n=68)	Healthy controls (n=20)	p
Age (years)	54.5 (19–79)	50 (19–68)	0.070
Gender (male)	41 (60.3%)	12 (60%)	0.108
VDBP (ng/mL)	503.8±230.3	480.5±197.0	0.682
25(OH)D (ng/dL)	13.3± 5.7	30.3±13.3	<0.001
VD_{free} (pg/mL)	2.18 (1.52–3.44)	4.34 (3.74–6.48)	<0.001
VD_{bio} (nmol/L)	1.86 (1.09–2.81)	4.28 (3.45–6.34)	<0.001
Albumin (g/L)	38.5 (34–41)	43.0 (39–44)	<0.001

Gender was presented as the number (percentage) of male and compared with the Chi-square test. Normally distributed data were presented as mean±SD and compared with independent samples t-test. Non-normally distributed data were presented as median (first-third quartiles) [median (min-max) for age] and compared with the Mann–Whitney U test. 25(OH)D: Total 25(OH) Vitamin D; VDBP: Vitamin D binding protein; VD_{free}: free Vitamin D; VD_{bio}: bioavailable Vitamin D.

Statistical analysis

Shapiro–Wilk's test and histogram analysis were used to determine the normal distribution of study data, and summary statistics are presented as mean±standard deviation (SD) and median (interquartile range, IQR) for normally distributed and non-normally distributed variables, respectively. Categorical variables were expressed as frequency and percentage, and the Chi-square test was used for group comparisons. In the comparison of COVID-19 patients and the healthy group, independent samples t-test and Mann–Whitney U test were performed for normally distributed and non-normally distributed data, respectively. In comparisons between the three subgroups of COVID 19 patients, one-way ANOVA or Kruskal–Wallis tests were used for normally distributed and non-normally distributed data, respectively, and *post hoc* comparisons were made if a significant result was obtained. Correlation analyzes were performed with linear logistic regression analysis. A p value below 0.05 was considered statistically significant. All statistical analyzes were performed on the SPSS 22.0 package program (IBM Corp., Armonk, NY, USA).

Results

In the comparison of whole COVID-19 patients and healthy controls, age and gender distributions were similar. 25(OH)D, VD_{free}, VD_{bio} and albumin levels were higher in healthy controls than in whole COVID-19 patients (p<0.001; Cohen's d effect sizes 1.7, 1.1, and 1.4, respectively), although there was no difference in VDBP levels (Table 1).

Gender distributions were not different in COVID-19 subgroups (asymptomatic, mild/moderate, and severe pneumonia) (p=0.059). The median age was significantly different and higher in mild/moderate (median [min-max], 55 [30–77] years) and severe pneumonia group (median [min-max], 60.5 [30–79] years) than asymptomatic patients (median [min-max], 44 [19–72] years) (p<0.001). In the comparison of routine laboratory parameters between subgroups, CRP, ferritin, WBC, neutrophil, fibrinogen, and D-Dimer levels increased with increasing disease severity, while lymphocyte levels decreased. Although, there was no difference in VDBP levels between COVID-19 patients and healthy controls, VDBP and albumin differed significantly between the asymptomatic, mild/moderate, and severe pneumonia subgroups. Serum VDBP was highest in mild/moderately pneumonia (601.8±278.6 ng/mL) and lowest in severe pneumonia (427.9±147.2 ng/mL). Albumin decreased with increasing disease severity (median [IQR]; 42.5 [40.7–44.2], 36.3 [32.1–40.6], and 33.5 [31.2–35.9] g/L, respectively). Despite the difference in VDBP levels, 25(OH)D and its metabolites levels were similar (Table 2).

In the correlation analysis, there was no correlation between 25(OH)D and VDBP levels (p>0.05). VDBP was positively correlated with absolute lymphocyte count (B: 87.9, r^2=0.068, p=0.031) and negatively correlated with D-Dimer (B: –0.024, r^2=0.081 p=0.032).

Discussion

In this study, we evaluated the total, free, bioavailable 25(OH)D, and VDBP levels in COVID-19, and their relationship with disease severity. The most important findings were that total, free and bioavailable 25(OH)D levels were decreased in COVID-19 patients, and VDBP levels were similar between whole group of COVID-19 patients and healthy controls, but actually showed significant differences between severity subgroups.

In addition, VDBP concentration was positively correlated with lymphocyte counts and negatively correlated with D-Dimer levels. In literature in terms of other laboratory findings, there was an increasing trend for CRP, WBC, D-Dimer, and fibrinogen and a decreasing trend for lymphocytes as the severity of the disease increases, which are related to poor prognosis and clinical outcomes [21]. Besides lack of a correlation between total 25(OH)D and VDBP in this study is also in accordance with literature that declares the independence of serum VDBP from serum 25(OH)D concentration [15].

Although there is no evidence of a direct link between Vitamin D concentrations and the incidence, prognosis, or outcomes of COVID-19, the role of Vitamin D and VDBP is in

Table 2. Comparison of routine laboratory data, 25(OH)D, VDBP, and Vitamin D metabolites between COVID-19 subgroups

	Asymptomatic (n=17)	Mild/moderate pneumonia (n=21)	Severe pneumonia (n=30)	p
Age (years)	44 (19–72)[a]	55 (30–77)[b]	60.5 (30–79)[b]	<0.001
Gender (male/female)	9/8	11/10	21/9	0.059
CRP (mg/L)	3.4 (1.1–10)[a]	42 (24.4–107.3)[b]	123.4 (61.1–225.4)[b]	<0.001
Ferritin (µg/L)	256 (98.5–341)[a]	456 (258–960)[ab]	669 (397.5–1314.5)[b]	0.035
WBC (10^3/µL)	6.6±1.9[a]	7.6±3.2[ab]	9.5±5.1[b]	0.044
Neutrophile (10^3/µL)	3.4 (2.96–4.5)[a]	5.09 (3.35–6.74)[ab]	7.25 (4.15–9.54)[b]	0.020
Lymphocyte (10^3/µL)	1.98 (1.49–2.23)[a]	1.27 (0.76–1.85)[b]	0.83 (0.63–1.15)[b]	<0.001
Fibrinogen (mg/L)	3629±1377[a]	5603±1519[b]	6508±1924b	<0.001
D-Dimer (µg/L)	370 (130–545)[a]	510 (365–691)[a]	1225 (832.5–2680)[b]	<0.001
VDBP (ng/mL)	516.8±249.3[a]	601.8±278.6[a]	427.9±147.2[b]	0.026
25(OH)D (ng/dL)	11.8±3.9	13.5±5.2	14±6.8	0.701
VD_{free} (pg/mL)	2.2 (1.6–2.8)	2.2 (1.6–2.7)	2.8(2.3–3.4)	0.600
VD_{bio} (nmol/L)	2.1 (1.5–2.7)	1.8 (1.3–2.3)	2.1 (1.7–2.5)	0.619
Albumin (g/L)	42.5 (40.7–44.2)[a]	36.3 (32.1–40.6)[b]	33.5 (31.2–35.9)[c]	<0.001

Gender was presented as the male/female number and compared with the Chi-square test. Normally distributed data were presented as mean±SD and compared with one-way ANOVA. Non-normally distributed data were presented as median (first-third quartiles) [median (min-max) for age] and compared with the Kruskal-Wallis Test, *post hoc* comparisons were shown with "a", "b", "c" letters and the groups with the same letter were not significantly different. 25(OH)D: Total 25(OH) Vitamin D; VDBP: Vitamin D binding protein; CRP: C-reactive protein; WBC: White blood cell; VD_{free}: Free Vitamin D; VD_{bio}: bioavailable Vitamin D.

interest [23]. A meta-analysis study showed that the mean Vitamin D level in COVID-19 patients was 21.9 nmol/L (15.36–28.45), and patients with poor prognosis had significantly lower Vitamin D levels than those with good prognosis [24]. However, the studies included in this meta-analysis are heterogeneous in Vitamin D measurement methods which can affect the results [25].

D'Avolio et al. [26] reported that individuals with significantly lower 25(OH)D levels had a higher risk of SARS-CoV-2 infection and COVID-19 was more strongly associated with 25(OH)D levels than other respiratory tract infections. Recent studies have shown that COVID-19 patients with Vitamin D deficiency had poor outcomes, while those with high Vitamin D levels had better outcomes [27, 28]. Jain et al. and Merzon et al. [29, 30] noted that COVID-19 patients with Vitamin D deficiency and poor outcomes may benefit from Vitamin D supplementation. Consistent with these findings, in this study, we demonstrated that COVID-19 patients have lower 25(OH)D levels than healthy controls.

It has been reported that T regulatory lymphocytes (Tregs), which provide defense against viral agents and uncontrolled inflammation during viral infections, are significantly decreased in COVID-19 patients, especially in severe disease and can be increased with Vitamin D supplementation [12, 31, 32]. Vitamin D can also induce ACE2/Ang-(1–7)/MasR axis (anti-inflammatory axis) and inhibit the ACE/Ang II/AT1R axis (inflammatory axis) by targeting renin-angiotensin system imbalance and ACE2 downregulation in COVID-19. Reduction in AT1 receptor expression has a potential protective role against acute lung injury by reducing inflammation, fibrosis, and apoptosis [33].

Vitamin D deficiency increases the risk of viral upper respiratory tract infections and pneumonia [34, 35]. A meta-analysis of 11321 participants in 25 randomized controlled trials showed that the Vitamin D supplemented patients with very low serum 25(OH)D levels (<25 nmol/L) had a lower risk of exposure to acute respiratory tract infections [36].

In the literature, according to the best of our knowledge, there is only one study evaluating VD_{free}, VD_{bio} and VDBP in COVID-19 patients. In this recent study, Subramanian et al. [37] divided COVID-19 patients into two groups, alive and deceased, according to 28-day mortality status. They found mortality rates higher in those with 25(OH)D <25 nmol/L, but none of serum VDBP, VD_{free}, and VD_{bio} were associated with mortality. Unlike in this study, we evaluated the association of VDfree, VDbio and VDBP concentrations with the disease severity, not mortality. We found serum Vitamin D metabolites, VD_{free} and VD_{bio} concentrations were lower in COVID-19 patients. Although VDBP levels did not differ in the whole patient group with the control group, there was a significant difference in the COVID-19 subgroups constructed according to disease severity. The highest levels were in patients with mild/moderate pneumonia followed in the inpatient clinic, and the lowest levels were in patients with severe pneumonia followed in the ICU. In severe COVID-19, sustained activation of actin and lectin pathway results in highly destructive complement-mediated thrombotic microvascular injury [16]. Indeed, ARDS, multi-organ dysfunction and septic shock occurring in severe disease, are clinical syndromes characterized by actin release [18]. The rapid conversion of actin monomers to polymeric structures can cause microcirculation blockage, which can be countered by the actin scavenging system consisting of VDBP and gelsolin [1]. Low VDBP

concentrations in critically ill COVID-19 patients followed in ICU according to asymptomatic or mild/moderately ill COVID-19 patients can be explained by the increased VDBP-actin complexes. Our serum VDBP measurement method is intended for the quantitative determination of only free and not actin bound VDBP. VDBP binds to actin with a very high affinity and VDBP-actin complexes are cleared from the circulation more rapidly than VDBP alone. The value and prognostic significance of low VDBP concentration as a marker of disease severity has been demonstrated previously in sepsis [38]. However, lower serum VDBP concentration in severe COVID-19 patients is the 1st time reported in an observational study with this paper. In severe pneumonia patients, higher 25(OH)D and VDfree concentrations-even still in inadequacy levels and not statistically significant - may also be related to lower VDBP concentrations, which binds and carries 25(OH)D into target tissues or maybe an adaptation mechanism of Vitamin D to immune response [39]. Although the role of free or bioavailable Vitamin D in critical illness is not yet completely clear, it is noteworthy that the decrease in circulating VDBP and total Vitamin D is accompanied by stable concentrations of free Vitamin D [14].

In addition, VDBP has two common single nucleotide polymorphisms (rs7041 and rs4588) and three isotypes (DBP1F, DBP1S, and DBP2), meaning it is a highly polymorphic plasma protein. Recently, Speeckaert et al. [19] found that DBP1-carriers had the highest plasma concentrations of Vitamin D metabolites have a better prognosis.

There were some limitations of this study: (1) the inability to examine the VDBP polymorphisms, (2) relatively small sample size in in the severity subgroups.

Conclusion

In this study, we demonstrated that COVID-19 patients have lower plasma 25(OH)D levels and lower 25(OH)D metabolites VD_{free}, VD_{bio} which are physiologically active. In addition, serum VDBP concentrations significantly decrease in critically ill patients which need further studies to be associated in the etiopathogenesis of the disease severity.

Conflict of Interest: The authors declare that there is no conflict of interest.

Ethics Committee Approval: The study was approved by The Kayseri City Hospital Clinical Research Ethics Committee (No: 329, Date: 18/03/2021).

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