The Role of Free Vitamin D and Vitamin D Binding Protein in SARS-Cov-2 Infection in Children

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Abstract

Purpose: Many studies have discussed the effects of serum vitamin D deficiency in SARS-CoV-2 patients. This study aimed to investigate the relationship between SARS-CoV-2 infection severity and free vitamin D (FVD) and bioavailable vitamin D (BAVD) levels in children.

Methods: A prospective case-control study design was used. Participants were divided into three groups based on the World Health Organization (WHO) COVID-19 Clinical Progression Scale. Serum 25-hydroxyvitamin D (25OH vitamin D; ng/mL), albumin (g/L), and vitamin D binding protein (ng/mL) levels were evaluated to investigate the relationship between disease severity and FVD and BAVD levels.

Results: In total, 82 participants were included in the study. Of those, 24.4 % were uninfected (n = 20), 50% had a mild case of SARSS-CoV-2 (n = 41), and 25.6% had a moderate case (n = 21). There was a statistically significant difference in FVD and BAVD levels between the groups (p = 0.026). Median FVD (p = 0.007, Cohen's *d* = 0.84) and BAVD (p = 0.007, Cohen's *d* = 0.86) levels were significantly higher in the mild group compared to the moderate group. FVD and BAVD metabolites were moderately positively correlated with lymphocyte counts (FVD: r = 0.437, p < 0.001; BAVD: r = 0.439, p < 0.001).

Conclusion: This is the first study to demonstrate a relationship between SARS-CoV-2 symptom severity and FVD and BAVD levels. The relationship between FVD and BAVD levels and lymphocyte counts could play an important role in symptom severity and should be evaluated in further studies.

The study was registered with Clinical Trials (NCT05598957, 10/06/2022).

Introduction

Vitamin D is a steroid hormone that plays a critical role in bone health and immune system modulation [1, 2]. Vitamin D deficiency (VDD) is considered an important global public health problem that affects people of all ages [3]. Many studies have investigated the relationship between acute respiratory syndrome coronavirus 2 (SARS-CoV-2) clinical severity [4–6] and VDD, as well as the possible protective effects of vitamin D supplementation [7]. Such studies have argued that the frequency of VDD is significantly higher in SARS-CoV-2 patients with severe symptoms [8], suggesting a potential relationship between VDD and SARS-CoV-2 prognosis [4, 9, 10]. Vitamin D acts as a virus entry inhibitor by interacting with the angiotensin-converting enzyme-2 receptor, which acts as a virus entry point due to its protein spike (S) end [11]. Speeckaert et al. [12] suggested that, coupled with the possible impact of vitamin D on SARS-CoV-2 infection pathogenesis, vitamin D binding protein (VDBP)-regulated concentrations of bioavailable vitamin D (BAVD) and free vitamin D (FVD) could modulate the human immune system response to viral infections. Therefore, BAVD and FVD may play a role in the relationship between SARS-CoV-2 and vitamin D.

Although 25-hydroxyvitamin D (25OH vitamin D) levels are the most effective indicator of vitamin D levels, FVD and BAVD levels should be considered when discussing immunomodulation [2, 13]. The free hormone hypothesis proposes that protein-bound hormones demonstrate weaker activity than those released from binding proteins. However, albumin binding is considered relatively weak compared to the strength of specific binding proteins. Therefore, albumin-bound hormones are referred to as being "bioavailable" [14]. In healthy subjects, approximately 85% of the vitamin D metabolites are bound with a high affinity to VDBP, while approximately 15% are bound to albumin with low affinity [13]. Therefore, bioavailable 25OH vitamin D levels may be a more effective indicator of the relationship between particular diseases and vitamin D levels than serum vitamin D concentrations.

Although many studies have investigated the relationship between vitamin D levels and SARS-CoV-2 disease severity [4–6, 15], to the authors' knowledge, none have investigated the relationship between disease severity and BAVD and FAVD levels in children [12]. Therefore. This study aimed to investigate the relationship between SARS-CoV-2 infection severity and FVD and BAVD levels in children.

Methods

A prospective case-control study design was used. Cases were selected from children who were admitted to the Haseki Training and Research Hospital Pediatric Infection Inpatient and Outpatient Clinic between 01 July 2021 and 31 June 2022. To be included in the study, participants had to have presented to the outpatient clinic with suspected SARS-CoV-2 infection, be aged between 0–18 years old, provide consent to participate, and not have a chronic disease (e.g., cystic fibrosis). Participants who returned a positive SARS-CoV-2 polymerase chain reaction (PCR) or antibody test were placed in either of the study groups (mild and moderate), while those who returned negative tests were placed in the control group. Only one patient demonstrated severe symptoms during the period of data collection; these patients were not included in the study (Fig. 1).

In total, 82 children were included in the study. Participants were divided into three groups according to World Health Organization (WHO) COVID-19 Clinical Progression Scale [16]. Patients who were asymptomatic but returned positive PCR tests (i.e., viral RNA was detected) were placed in the mild group (Group 2). Patients who returned positive PCR tests and were hospitalized, but did not require oxygen therapy or oxygen masks, or nasal prongs cases, were placed in the moderate group (Group 3). Patients who underwent a PCR test due to contact with another COVID-19 case but had no symptoms or complaints and returned negative tests (i.e., no viral RNA was detected) were placed in the control group (Group 3). No severe cases were included in the study (Fig. 1).

All participants in the mild and moderate groups underwent routine laboratory screening, and the laboratory data were available in their hospital records. The white blood (including neutrophils and lymphocytes; uL), thrombocyte (uL), C-reactive protein (CRP; mg/L), high-sensitivity CRP (Hs-CRP; ng/mL), procalcitonin (ug/L), sedimentation (mm/hour), fibrinogen (g/L), and D-dimer (ug/L) levels were noted from the records of all participants in the mild and moderate groups. Due to ethical issues, all patient data were anonymized, and all patient records were attributed to ID numbers instead of names.

Patient characteristics, such as age and gender, were taken from the patient logs on the hospital information management system. To investigate the relationship between SARS-CoV-2 disease severity and FVD and BAVD levels, 25OH vitamin D (ng/mL), albumin (g/L), and VDBP (ng/mL) using samples that underwent centrifugation in a dry tube. The 25OH vitamin D levels were measured via an immune inhibition assay (DXI800 instrument, Beckman Coulter, Brea,

CA, USA), while VDBP levels were evaluated using the enzyme-linked immunosorbent assay (ELISA) technique and a VDBP kit (Immundiagnostik AG, Cat: K2314). BAVD and FVD levels were calculated using the Bikle [17] and Vermeulen [18] equations that have been validated by previous studies.

All participants (both parents and children) signed an informed consent form to participate in the study. Ethics committee approval was obtained from the local ethics committee with a protocol decision numbered 2022/10/17.

Statistical Analysis

Data were analyzed using the SPSS Statistics 28.0 program for Windows. Study variables were investigated using visual and analytical methods (Kolmogorov-Smirnov and Shapiro-Wilk's tests) to determine whether they were normally distributed. Descriptive variables were presented using differently based on whether they were categorical or numerical. Categorical variables were presented as numbers and percentages, while numerical variables were presented as medians and interquartile ranges (IQRs). Differences between categorical variables and outcome variables were assessed using Chi-square tests. As the numerical variables were not normally distributed, a one-way ANOVA test was used to compare two independent groups. The Kruskal-Wallis test was used to compare more than two independent variables. Differences between two group means were calculated using Cohen's *d*. No parameters were normally distributed; therefore, correlation coefficients were calculated using Spearman's rank-order correlation coefficient. Statistical significance was accepted as $p < 0.05$.

Results

In total, 82 participants were included in the study. The mean participant age was 150 ± 56.7 months, and 53.3% (n = 47) of participants were female. Regarding the number of participants allocated to each group, 24.4% (n = 20) of participants were placed in the control group, 50% (n = 41) were placed in the mild group, and 25.6% (n = 21) were placed in the moderate group (Fig. 1).

There was no significant relationship between VDBP and 25OH vitamin D levels in all groups. However, albumin levels were significantly higher in the control group compared to the mild and moderate groups ($p < 0.001$). Pairwise comparisons revealed that this difference was caused by the high albumin levels in the control group compared to the mild and moderate groups (control vs. mild: 48 vs. 44 g/L, $p = 0.01$; control vs. moderate: 48 vs. 44 g/L, $p < 0.001$; Table 1, Fig. 2). A significant difference was found in the BAVD and FVD levels calculated by Vermeulen (FVDv) and Bikle (FVDb) methods between the mild and moderate groups ($p = 0.026$). Median FVD and BAVD levels were significantly lower in the moderate group compared to the mild group (FVDv 4.04 vs. 2.59 pg/ml, $p = 0.007$, Cohen's $d = 0.79$; FVDb 4.00 vs. 2.58 pg/ml, $p = 0.007$, Cohen's $d = 0.84$; BAVD 1.61 vs. 1.01 pg/ml, $p = 0.007$, Cohen's $d = 0.86$; Table 1 and Table 2).

Table 1
Sociodemographic and Vitamin D related laboratory findings of groups

		Group 1 Uninfected group (n = 20)	**Group 2 Mild group (n = 41)**	**Group 3 Moderate group (n = 21)**	**p**
Age	Mean ± SD	138.85 ± 54.21[a]	136.17 ± 63.55[a]	186.24 ± 16.54[b]	**0.020***
Gender Female	n, %	11 (55)	22 (53.7)	14 (66.7)	0.601**
Male	n, %	9 (45)	19 (46.3)	7 (33.3)	
25OH Vitamin D (ng/ml)	Median(25-75p)	11.60 (7.45–18.60)	13.40 (10.70–16.60)	11.00 (7.10-12.65)	0.076***
VDBP (ng/ml)	Median (25-75p)	265300 (221800–306375)	228200 (167900–298600)	268500 (184450–361850)	0.103***
Albumin (g/l)	Median (25-75p)	48 (47–49) [a]	44 (43–46) [b]	44 (41–45) [b]	**< 0.001****
Free Vitamin D Bikle [17] (pg/ml)	Median (25-75p)	2.90 (1.64–5.32) [a]	4.00 (2.52–5.58) [a, b]	2.58 (1.91–3.42) [a, c]	**0.026****
Free Vitamin D Vermeulen [18] (pg/ml)	Median (25-75p)	2.92 (1.65–5.38) [a]	4.04 (2.54–5.65) [a, b]	2.59 (1.92–3.45) [a, c]	**0.026****
Bioavailable Vitamin D (pg/ml)	Median (25-75p)	1.20 (0.73–2.05) [a]	1.61 (1.01–2.33) [a, b]	1.01 (0.74–1.40) [a, c]	**0.026****

* One-way Anova test, ** Ki-square test, ***Kruskal-Wallis test, Abbreviations SD: Standard Deviation, 25OH Vitamin D: 25-Hydroxyvitamin D, VDBP: Vitamin D Binding Protein. Each letter represents statistically significant difference.

Table 2
The difference between the groups according to the Vitamin D metabolites

		Group 1 vs. 2	Group 1 vs. 3	Group 2 vs. 3
		Uninfected vs. Mild group	Uninfected vs. Moderate group	Mild vs. Moderate group
Free Vitamin D Vermeulen [18] (pg/ml)	p	0,121	0,482	**0,007**
	Cohen'sd	**-0,12**	**0,40**	**0,79**
Free Vitamin D Bikle [17] (pg/ml)	p	0,121	0,500	**0,007**
	Cohen'sd	**-0,13**	**0,40**	**0,84**
Bioavailable Vitamin D (pg/ml)	p	0,168	0,332	**0,007**
	Cohen'sd	**-0,07**	**0,45**	**0,86**

Cohen's *d*, Practical/Clinical + and effect; 0.25 = educationally significant (e.g., something was learnt), 0.50 = practically / clinically significant (e.g., something really changed)

As expected, the median white blood count of the mild group was significantly higher than that of the moderate group (6350 vs. 4600 uL, respectively, p = 0.017). This difference could be attributed to the difference in the median lymphocyte counts of the mild and moderate groups (2520 vs. 1700 uL, respectively, p < 0.001). Although no significant differences in Hs-CRP and procalcitonin levels were observed, CRP and sedimentation levels were significantly higher in the moderate group compared to the mild group (CRP: 0.70 vs. 5.10 mg/L, respectively, p = 0.004; sedimentation: 8.00 vs. 15.50 mm/hour, respectively, p = 0.005). Furthermore, fibrinogen and D-dimer levels were significantly higher in the moderate group compared to the mild group (fibrinogen: 276.00 vs. 367.00 g/L, respectively, p = 0.004; D-dimer: 0.32 vs. 0.51 ug/L, respectively, p = 0.020; Table 3).

Table 3
Laboratory findings of study groups

Median (25-75p)	**Group 2** **Mild group** **(n = 41)**	**Group 3** **Moderate group** **(n = 21)**	**P***
White Blood Count (uL)	6350 (5075–8970)	4600 (3765–7550)	**0.017**
Neutrophile	3250 (2315–4195)	2370 (1845–3770)	0.243
Lymphocyte	2520 (1995–3550)	1700 (1330–2435)	**< 0.001**
Thrombocyte (uL)	253000 (215500–319500)	222000 (192500–293500)	0.228
CRP (mg/l)	0.70 (0.40–2.60)	5.10 (0.95–8.80)	**0.004**
Hs-CRP (ng/ml)	11.10 (8.70–13.70)	9.80 (8.05–10.65)	0.090
Procalcitonin (ug/L)	0.03 (0.02–0.04)	0.02 (0.02–0.04)	0.960
Sedimentation (mm/hour)	8.00 (4.50–13.00)	15.50 (8.25–26.75)	**0.005**
Fibrinogen (g/L)	276.00 (235.00-347.00)	367.00 (281.00-409.00)	**0.004**
D-Dimer (ug/L)	0.32 (0.25–0.47)	0.51 (0.28–1.17)	**0.020**

* One-way Anova test, Abbreviations SD: Standard Deviation, CRP: C-reactive protein, Hs-CRP: High sensitive c-reactive protein.

The bivariate correlations of laboratory findings and vitamin D variables are summarized in Table 4. All FVD and BAVD metabolites were moderately positively correlated with lymphocyte counts (FVDv: r = 0.437, p < 0.001; FVDb: r = 0.437, p < 0.001; BAVD: r = 0.439, p < 0.001).

Table 4
Bivariate correlation of variables

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Hs-crp	1												
2. WBC	0.284*	1											
3. Neutrophile	0.275*	0.883**	1										
4. Lymphocyte	0.216	0.778**	0.410**	1									
5. Thrombocyte	0.188	0.776**	0.654**	0.652**	1								
6. Sedimentation	-0.209	-0.292*	-0.174	-0.331*	-0.178	1							
7. CRP	-0.017	0.220	0.372**	-0.013	0.287*	0.138	1						
8. Prokalsitonin	-0.090	0.120	0.211	-0.009	-0.100	-0.004	0.661**	1					
9. Fibrinogen	-0.024	0.040	0.284*	-0.235	0.116	0.388**	0.658**	0.348**	1				
10. D-Dimer	-0.100	-0.187	-0.075	-0.248	-0.135	0.169	0.246	0.259	0.331*	1			
11. FVD-Vermulen[8]	0.218	0.228	0.048	0.437**	0.128	-0.094	0.001	0.043	-0.085	-0.176	1		
12. FVD-Bikle[7]	0.218	0.228	0.049	0.437**	0.128	-0.093	0.001	0.044	-0.086	-0.176	1.000**	1	
13. BVD	0.245	0.231	0.054	0.439**	0.120	-0.133	-0.032	0.027	-0.122	-0.185	0.993**	0.993**	1
14. 25OH vitamin D	0.233	0.079	-0.052	0.266	-0.019	-0.146	-0.010	-0.005	-0.085	-0.242	0.753**	0.752**	0.756**
15. Albumin	0.240	0.065	0.036	0.085	-0.052	-0.256	-0.252	-0.111	-0.277*	-0.095	0.080	0.080	0.188
16. VDBP	-0.110	-0.155	-0.175	-0.120	-0.207	0.078	-0.051	0.007	-0.018	-0.070	-0.490**	-0.492**	-0.487**

Spearman's Rho Correlation analysis test * Correlation is significant at the 0.05 level (2-tailed), ** Correlation is significant at the 0.01 level (2-tailed). Abbrevia sensitive c-reactive protein, WBC: white blood count, CRP: C-reactive protein, FVD: free vitamin D, BVD: Bioavailable Vitamin D, 25OH Vit D: 25-Hydroxyvitamin Binding Protein.

p values in each figure represent the Kruskal-Wallis test significance level between the three groups.

* p:<0.05, ** p: <0.01, *** p: <0.001; Abbreviations: 25OH Vitamin D: 25-Hydroxyvitamin D, VDBP: Vitamin D Binding Protein.

Discussion

This is the first study to demonstrate a relationship between SARS-CoV-2 symptom severity and FVD and BAVD levels. Median FVD and BAVD levels were significantly lower in more severe SARS-CoV-2 patients and were moderately positively correlated with lymphocyte counts, thereby suggesting that VDD plays a role in the clinical severity of SARS-CoV-2.

Besides the already well-known protective immunomodulatory effects of vitamin D [1], VDBP may play several roles in the course of COVID-19 and other viral infections, such as macrophage activation and chemotaxis [1, 19]. Reduced serum VDBP concentrations have been reported in patients with sepsis and acute respiratory distress syndrome [20]. As a multifunctional protein, VDBP is the major carrier of vitamin D metabolites and acts as an actin scavenger; actin is a neutrophil chemotactic factor and macrophage activator [21]. Serum VDBP concentrations and the D vitamin binding protein (DBP) genotype impact bioavailable 25OH vitamin D concentrations. The DBP gene family is characterized by considerable polymorphism, with three major alleles determined by the single nucleotide polymorphisms (SNPs) rs7041 and rs4588 (DBP1F [rs7041-T/rs4588-C], DBP1S [rs7041-G/rs4588-C], and DBP2 [rs7041-T/rs4588-A]). There are over 120 DBP variants. DBP phenotypes are associated with discriminatory differences in 25OH D, 1,25-dihydroxy vitamin D, and VDBP plasma concentrations. These concentrations are highest in individuals with the DBP1-1 phenotype, moderate in the DBP2-1 phenotype, and lowest in the DBP2-2 phenotype. The GT genotype at rs7041 exhibits a positive correlation with COVID-19 prevalence and mortality, whereas a negative correlation is seen with the TT genotype. DBP1 carriers might be less susceptible to infection and mortality due to SARS-CoV-2. The association between DBP1 allele frequency and a lower prevalence of and mortality due to SARS-CoV-2 could be partially explained by the potential protective effects of vitamin D [22]. Although there was no difference between the VDBP levels of the groups, FVD and BAVD levels were lower in the moderate group compared to the mild group. As VDBP concentrations and DBP genotypes are affected by FVD and BAVD concentrations, these results may be due to DBP gene polymorphisms.

Previous studies have reported that the positive effects of vitamin D on the innate and adaptive immune system and immune response modulation may prevent lung and cardiovascular system damage and decrease thrombotic events [23]. Furthermore, vitamin D may prevent virus entry and replication by protecting the integrity of physical barriers and may reduce organ damage and thrombotic events by increasing levels of angiotensin-converting enzyme 2 (ACE2), nitric oxide, and antioxidants or by reducing inflammatory cytokine and free radical levels [23]. A recent meta-analysis found that low levels of vitamin D increased the risk of severe SARS-CoV-2 disease in pediatric patients by 5.5. times and pediatric patients with VDD were at a greater risk of SARS-CoV-2

infection than patients with normal vitamin D levels [24, 25]. However, another recent systematic review and meta-analysis investigating the relationship between vitamin D and SARS-CoV-2 severity determined that the currently available results are still too controversial and insufficient for vitamin D to be used in intensive care units (ICUs) [26]. All studies included in these meta-analyses assessed either serum or plasma vitamin D concentrations, which may explain the conflicting results. Therefore, determining the FVD and BAVD concentrations may provide greater clarification on whether vitamin D should be administered in ICUs.

The current study detected a weak positive correlation between lymphocyte count and FVD and BAVD levels. Similar to these findings, a study investigating VDD, SARS-CoV-2 clinical severity, and inflammatory markers in children found children with higher clinical severity had significantly lower vitamin D levels and significantly higher levels of inflammatory markers. This study concluded that low 25OH vitamin D levels were associated with higher levels of inflammatory markers and that vitamin D may affect the clinical course of SARS-CoV-2 in children and adolescents, possibly by regulating the systemic inflammatory response [4].

Considering that serum DBP concentrations may affect FVD and BAVD levels during infection periods, it may be insufficient to evaluate the immunomodulatory functions of vitamin D using only 25OH vitamin D levels (4, 19). Both serum DBP and albumin concentrations are known to induce negative acute phase responses during the acute phase of illness [13]. In this study, although no significant difference was observed in the DBP levels of groups, albumin levels were significantly higher in the control group. This finding is in line with the literature that albumin acts like a negative acute phase reactant.

In a study comparing the severity of SARS-CoV-2 and influenza A infections in adults using total 25OH vitamin D and FVD levels, serum 25OH vitamin D levels were found to be significantly lower among patients who received invasive mechanical ventilation [27]. A similar relationship was observed in those with more severe infections. Furthermore, a decrease in FVDs has been shown to significantly increase the possibility of patients requiring invasive mechanical ventilation requirement and mortality rates [27]. In the current study, lower FVD concentrations were observed in the moderate group compared to the mild group. As FVD concentration may affect disease severity, it may be useful to evaluate FVD levels in moderate and severe patients in the ICU.

The current study found that lower serum vitamin D levels, particularly VDD, were associated with clinical severity and significantly associated with higher levels of inflammatory markers, like CRP and fibrinogen, and lower lymphocyte counts [4]. Similarly, a previous study observed increased CRP in hospitalized pediatric SARS-CoV-2 patients with low vitamin D concentrations, although this relationship was non-significant [15]. In line with the existing literature, FVD and BAVD metabolites were moderately positively correlated with lymphocyte counts in the current study. Although there was a significant relationship between lymphocyte counts and FVD and BAVD levels, no relationship was detected between inflammatory markers and FVD and BAVD levels. Further studies are needed to clarify these interactions.

There are several limitations of the current study. First, no inflammatory markers were detected in the control cases. Second, the sample size was relatively small. Due to the vulnerable nature of children, no additional interventions were used, as they could have placed them at risk. Furthermore, no severe patients were included in the study group. This was due to the case-control study design and the fact that only one severe case was reported at the outpatient and inpatient clinics during the period of data collection, which was an insufficient number of cases to form a study group.

Although previous studies have investigated the relationship between serum vitamin D levels and SARS-CoV-2 severity in children, the current study is the first to demonstrate a relationship between symptom severity and FVD and BAVD levels. The relationship between increased FVD and BAVD levels and lymphocyte counts may play an important role in determining SARS-Cov-2 severity and must be evaluated with further studies. Based on the findings, vitamin D supplementation may help lessen SARS-CoV-2 severity among the pediatric population.

Declarations

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Availability of data and material: When needed, all data can be shared with the consent of the participants.

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Ethics approval: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the TC Health Sciences University Hamidiye Clinical Ethics Committee with protocol decision numbered 2022/10/17 and registered with Clinical Trials (NCT05598957, 10/06/2022).

Consent to participate: Written informed consent was obtained from the parents.

Consent to publish: All participants confirmed and signed the informed consent form to the publication of the study.

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Figures

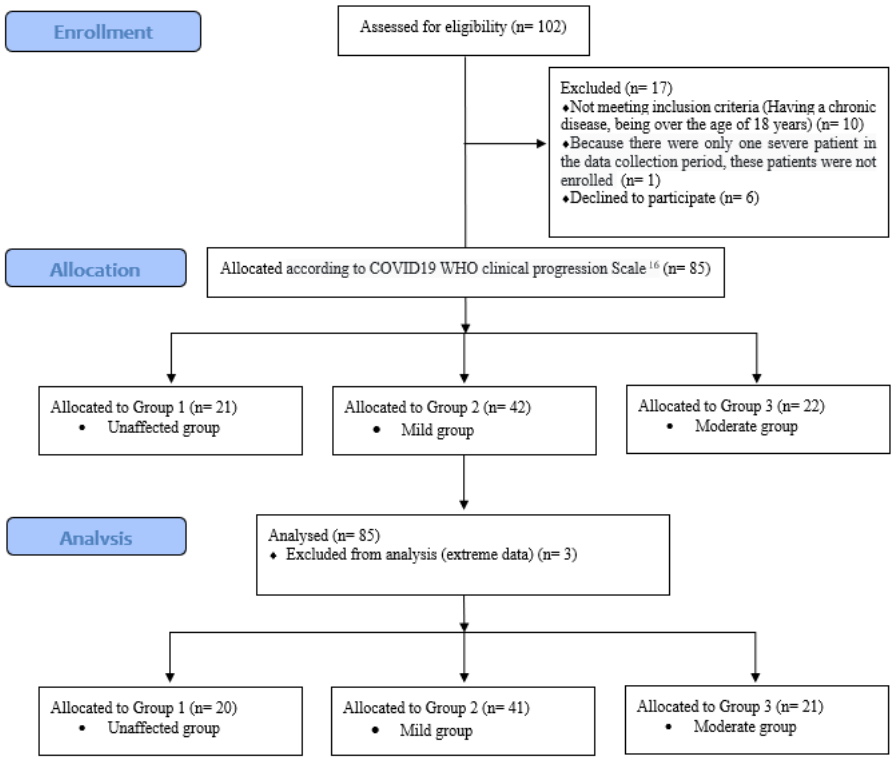

Figure 1

Flow chart of the study according to COVID19 WHO clinical progression Scale [16]

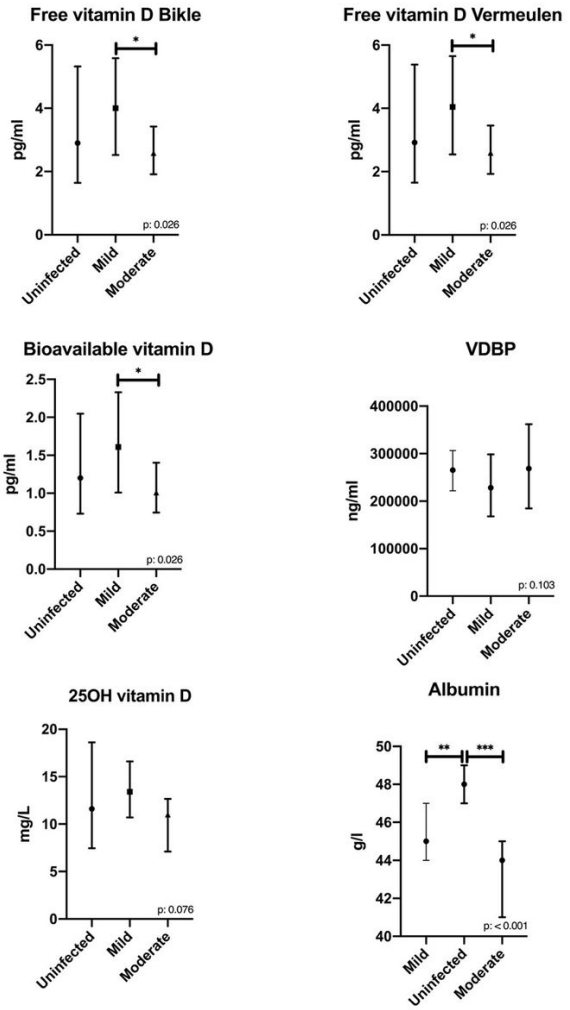

Figure 2

Vitamin D, Vitamin D metabolites and albumin levels according to the severity of SARS-CoV-2 infection