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Characterization of COVID-19 patients in clinical, epidemiological, and laboratory settings: the role of vitamin D binding protein and vitamin D level in severity

Aleen Sardar Al-Noori[1*], Ahmed Mohamed Salih[2], Muayad Aghali Merza[3]

[1] Department of Medical Microbiology, College of Medicine, University of Duhok, Duhok, Kurdistan Region, Iraq
[2] College of Nursing, College of Medicine, Department of Medical Microbiology, University of Duhok, Duhok, Kurdistan Region, Iraq
[3] Department of Internal Medicine, Azadi Teaching Hospital, College of Pharmacy, Nawroz University, Duhok, Kurdistan Region, Iraq

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Abstract

There are contradictory findings on the role of vitamin D-binding protein in COVID-19 development, disease severity, and outcomes. Therefore, we aimed to explore the association between the serum vitamin D level, DBP, and the COVID-19 severity and outcomes. In this cross-sectional study, we observed the suspected and confirmed admitted patients with COVID-19 for the possible outcomes after measurements of vitamin D, vitamin D binding protein (DBP). The study included patients with a mean age of 70.89 years (range: 28–99), mostly aged ≥60 years (84.81%) and male (54.43%). Most were admitted to medical wards (60.76%) or ICU (39.24%). The majority had confirmed COVID-19 (81.01%), while 12.66% were not diagnosed. Hospitalization duration varied: 1–3 days (21.52%), 4–7 (17.72%), 8–14 (37.97%), and >14 days (22.78%). Outcomes: 53.16% died, 34.18% discharged, 12.66% recovered. Disease severity was critical (41.77%), severe (30.38%), moderate (24.05%), and mild (3.80%). All received oxygen: 56.96% via reservoir mask, 36.71% continuous positive airway pressure (CPAP), and 6.33% nasal mask. Common comorbidities: hypertension (67.09%), diabetes (37.97%), ischemic heart disease (IHD) (25.32%), and smoking (21.52%). Symptoms: shortness of breath (77.22%), cough (75.95%), chest pain (60.76%). Fever types: persistent (44.59%) and high (27.03%). Only 25.32% were vaccinated (Pfizer 45%, AstraZeneca 30%, Sinopharm 25%), mostly with two doses (85%). Vitamin D was low (16.88). DBP protein (mean: 5.51, range: 0.15–25.20) showed no significant differences across outcomes or severity ($p > 0.05$). Our study's results, particularly the exceptionally low mean DBP value in a cohort with high COVID-19 severity and mortality, highlight a crucial area of investigation.

Keywords: COVID-19, Severity, Mortality, vitamin D binding protein

1. Introduction

The coronavirus disease 2019 (COVID-19), which emerged in late 2019, rapidly escalated into a global pandemic, significantly impacting health systems and daily life worldwide [1, 2]. The severity of COVID-19 varies greatly among infected individuals, ranging from asymptomatic cases to severe clinical syndromes such as pneumonia, acute respiratory distress syndrome (ARDS), thrombosis, and cytokine storm, often leading to serious complications and even death [1, 3]. Factors predisposing individuals to adverse outcomes include age, obesity, diabetes mellitus, hypertension, and ethnicity [3, 4]. Beyond these recognized risk factors, extensive research has explored the nuanced roles of vitamin D and vitamin D binding protein (VDBP) in influencing COVID-19 susceptibility, severity, and mortality, though findings have often been complex and contradictory [5].

Vitamin D, a steroid hormone crucial for musculoskeletal health, also plays vital immunomodulatory roles by decreasing inflammatory cytokines and increasing anti-inflammatory ones, potentially preventing the exaggerated immune response known as cytokine storm associated with COVID-19 [1, 5]. It stimulates the release of antimicrobial peptides like cathelicidin and defensin, inhibits viral replication, and enhances the chemotaxis of T-lymphocytes, which are often decreased in severe COVID-19 patients [3, 6]. Some studies suggest that adequate vitamin D levels might prevent cytokine storms and that supplementation could offer a relatively easy and cost-effective preventive treatment against severe complications [1]. Early claims identified vitamin D inadequacy or deficiency as a potential risk factor for adverse COVID-19 outcomes [1, 5]. However, the direct link between vitamin D concentrations and the incidence, prognosis, or outcomes of COVID-19 remains debated, with meta-analyses showing heterogeneous results [5, 7, 8]. While some stu-

* Corresponding author.
E-mail address: aleen.sardar@uod.ac (A. S. Al-Noori).
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dies found lower 25-hydroxy Vitamin D (25(OH)D) levels in COVID-19 patients compared to healthy controls, and some associated vitamin D deficiency with poor outcomes or increased hospitalization risk [1, 3, 6], others reported no significant correlation between vitamin D levels and COVID-19 severity or mortality, particularly in intensive care unit (ICU) settings [3, 4, 9].

Vitamin D binding protein (VDBP), primarily produced in the liver, is the main carrier of 25(OH)D in plasma, binding 85–90% of circulating vitamin D metabolites [5, 10]. According to the "free hormone hypothesis", only unbound or "free" hormones are physiologically active, suggesting that free vitamin D (VD free) is the biologically active form [1, 4]. VDBP also possesses immunoregulatory functions independent of its vitamin D binding, acting as part of the extracellular actin scavenger system to clear filamentous actin (F-actin) released during cell injury, modulating innate immunity, and acting as a macrophage activator and neutrophil chemotactic factor. Abnormal VDBP levels are observed in various conditions, including liver diseases, nephrotic syndrome, septic shock, and ARDS, where they can decrease due to increased VDBP-actin complex formation and rapid clearance [1, 8, 11]. Studies on VDBP levels in COVID-19 patients have yielded conflicting results; some reported lower VDBP in severe pneumonia or critically ill patients [5, 7], while others found higher VDBP in critical COVID-19 patients or no significant difference in the overall COVID-19 group compared to healthy controls [5, 9].

The highly polymorphic nature of VDBP, determined by common single nucleotide polymorphisms (SNPs) such as rs7041 and rs4588, significantly influences vitamin D levels, binding affinity, and subsequent bioavailability [3, 12, 13]. These genetic variations have been implicated in the pathogenesis of various clinical conditions and may contribute to differences in COVID-19 prevalence, severity, and mortality across populations [3, 7, 10]. For example, certain DBP phenotypes have been associated with higher or lower plasma 25(OH)D concentrations and may influence susceptibility to infection and mortality [7]. However, the impact of these polymorphisms on COVID-19 outcomes also varies across studies and populations, highlighting the need for further detailed genetic analyses and larger multi-center studies to decipher their precise role [3, 5, 6].

In conclusion, the intricate interplay between vitamin D status, VDBP levels, and VDBP genetic polymorphisms appears to be a crucial, yet not fully understood, aspect of COVID-19 pathogenesis and prognosis. Further comprehensive research, integrating both clinical and genetic analyses, is essential to clarify these relationships, resolve existing inconsistencies, and ultimately contribute to developing more targeted and personalized strategies for managing COVID-19 and its complications. In this regard, we aimed to explore the association between the serum vitamin D level, DBP, and the COVID-19 severity and outcomes.

2. Patients and Methods

2.1 Study Design and Setting

In this cross-sectional study, we observed the suspected and confirmed admitted patients with COVID-19 for the possible outcomes. Seventy-nine patients were enrolled and followed in the medical settings; the blood samples of all admitted patients were taken at a late stage in infection. This study was a cross-sectional observation, conducted over six months between February and July 2022.

This study was conducted in Duhok Governorate, Kurdistan Region of Iraq. The blood sample collection was carried out at one public healthcare facility, namely Corona Infectious Diseases Hospital and Lalav COVID-19 Hospital, and two of the private healthcare facilities, namely Shilan Private Hospital and Vin Medical Complex. It accommodates the hospitalized COVID-19 patients. The laboratory protocols are performed at Duhok Medical Research Center (DMRC), affiliated with the College of Medicine, University of Duhok, and Central Public Health Laboratories, affiliated with the Duhok General Health Directorate. A total of 79 patients residing in Duhok City, showing symptoms of upper or lower respiratory tract infections and suspected of SARS-CoV-2 infection, contributed to this study by applying a standardized questionnaire. This study included adult individuals of both genders, aged 18 years old.

2.2. Inclusion and exclusion Criteria

Inclusion criteria were patients with SARS-CoV-2 who performed the PCR test for the diagnosis, hospitalized, aged 18 years and older, of both genders. Exclusion criteria were those who had not had signs and symptoms of the COVID-19 disease, patients with history of less than 6 months of last dose of vaccination and pregnant women, also patients who received any sort of Vitamin D supplement have been excluded.

2.3. Ethical Considerations

The Ethical Committee of the Duhok General Directorate of Health approved this study, referenced under number 16222022-1-4, on 16 February 2022. Written informed consent was obtained from all participants after providing them with detailed information about the objectives and procedures of the study. Participants' confidentiality was ensured by using unique codes for each, removing their identities from the dataset, and storing the data.

2.4. Data Collection Methods

The type of data included in this study, which provides clinical information, demographic data, and laboratory findings about the patients, was obtained through structured questionnaires and hospital patient records. The laboratory data and inflammatory markers were retrieved from laboratory equipment, and electronic medical records include CBC, IL-6, Serum Ferritin, CRP, LDH, and D-Dimer.

2.5. Questionnaire

All participants complete an interviewer-administered questionnaire to provide information. A structured COVID-19 questionnaire focuses on nine categories based on demographic criteria, risk factors, clinical manifestation, disease severity, symptom duration and timing; hospital stay duration, ventilation requirement, vaccination history, and laboratory parameters.

The questionnaire adopted validated materials and followed the National Institutes of Health (NIH) standards to determine COVID-19 disease severity. According to the NHS, patients were classified into five categories based on symptoms. Asymptomatic cases show no signs or symptoms of COVID-19. Mild cases show symptoms such as

cough, fever, malaise, sore throat, muscle pain, headache, vomiting, nausea, loss of taste and smell, diarrhea, but no shortness of breath or dyspnea. Moderate cases develop mild symptoms of lower respiratory disease on clinical assessment or imaging and oxygen saturation (SpO2) of 94% or above on room air at sea level. Severe cases were defined by lung infiltrates exceeding 50% or a respiratory rate greater than 30 breaths per minute, and SpO2 below 94% on room air at sea level. Critical cases include respiratory failure, septic shock, and/or multiple organ dysfunction [2].

2.6. Processing of Samples

The blood samples were collected using venipuncture by trained nurses and lab technicians in hospital settings and immediately stored at -20 °C for later analysis. As a pre-analytical step, serum was separated by centrifugation at 3000 rpm for 10 minutes before further biochemical testing.

2.7. Serological Analysis

2.7.1 Serum Vitamin D Level

A blood sample is typically collected from a vein, usually in the arm, to evaluate the level of serum 25-hydroxyvitamin D [25(OH)D], which is the primary circulating form of vitamin D in the human body. After collection, each sample was analyzed at hospital laboratories using techniques such as immunoassay to precisely measure the concentration of 25(OH)D. This measurement provides a reliable indicator of an individual's vitamin D status, helping to assess deficiency or sufficiency levels accurately.

2.7.2. Vitamin D Binding Protein (DBP)

A competitive enzyme-linked immunosorbent assay (ELISA) was used to measure DBP concentrations in serum samples. The experiment followed the instructions of the manufacturer provided with the Human Vitamin D-binding Protein ELISA Kit (Cat. No. EA0045Hu; BT Laboratory, China). All the reagents and the samples were brought to room temperature before use. Each diluted sample was added to an allocated well in a 96-well microplate where anti-DBP capture antibodies were coated. The same amount of biotinylated DBP antigen 50µL was then added to each well. The plate was sealed and incubated at 37ºC within an incubator with a view to ensuring that the sample DBP and the biotinylated antigen interact competitively within the plate. Upon incubation, the microplate has been washed five times with wash buffer to wash unbound substances. Thereafter, 50µL of avidin-horseradish peroxidase (HRP) conjugate was added to the wells and incubated at 37ºC and 60 minutes. The wells were again cleaned five times. Substrate A and substrate B were added to each well and the microtitration well was incubated in the dark at 37ºC for up to 10 minutes. The enzymatic reaction was stopped by adding HCl solution (50µL) to all wells, then immediately after incubation, the wavelength absorbance of each well was measured at 450 nm using a microplate reader. The color intensity, in this case, competitive ELISA, was inversely proportional to the DBP concentration of the sample; the optical densities were compared with those of the standard curve (ranged between 0.75 and 24 µg/mL) and patients' sample concentrations of VBD were calculated.

2.7.3. Statistical Analyses

The general and medical characteristics of the suspected and confirmed patients with COVID-19 were presented in mean (SD) or number (percent). The prevalence of risk factors, symptoms, disease severity, vitamin deficiency, and outcomes was determined in number (percent). The biomedical measurements were presented in mean (SD), minimum and maximum. The comparisons of DBP with disease severity outcomes and vitamin D deficiency were examined in an independent test or one-way ANOVA as appropriate. A p-value below 0.05 was deemed statistically significant. All statistical analyses were carried out using JMP® software, Version 18.0 (SAS Institute Inc., Cary, NC, 1989–2023).

3. Results

The mean age of the patients was 70.89 years old ranging between 28 and 99 years. The patients were aged <60 years (15.19%) and ≥ 60 years. (84.81%). The patients were males (54.43%) and females (45.57%), lived in different geographic areas, and were selected from different clinical settings. All patients were admitted to the hospital and including ICU (39.24%) and medical ward (60.76%). The study found that most of the patients were diagnosed with COVID-19 (81.01%) and small percentage were not diagnosed with COVID-19 (12.66%; Table 1).

The patients were admitted between 1 and 176 days including 1-3 (21.52%), 4-7 (17.72%), 8-14 (37.97%), and >14 days (22.78%). Most of the patients died (53.16%), followed by those who were discharged (34.18%), and recovered (12.66%). The disease severity of most of the patients was critical (41.77%), followed by severe (30.38%), moderate (24.05%), and mild (3.80%). All patients received oxygen saturation. The patients received a reservoir Face Mask (56.96%), followed by CPAP (36.71%), and a nasal mask (6.33%; Table 2).

The most prevalent risks in suspected and confirmed patients with COVID-19 were hypertension (67.09%), followed by diabetes mellitus (37.97%), IHD (25.32%), and smoking (21.52%). Other less prevalent risk factors were cerebrovascular accident (CVA) (16.46%), chronic kidney disease (CKD) (12.66%), and Solid tumor (10.13%; Table 3; Fig 1).

The patients had fever as follows: persistent (44.59%), followed by high fever (27.03%), moderate (27.03%), and low-grade fever (1.35%). The shortness of breath in patients was the most prevalent symptom (77.22%). The most prevalent symptom after SOB was cough (75.95%),

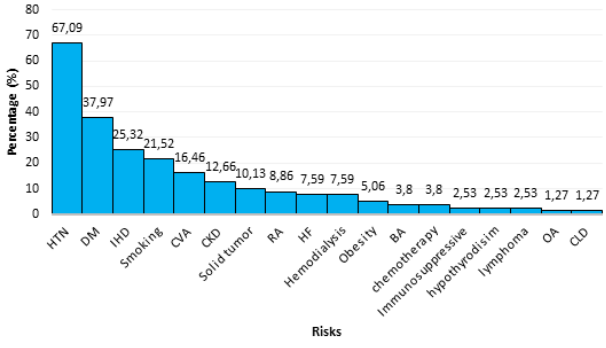

Fig. 1. Prevalence of risks for severity and mortality among patients suspected of COVID-19.

Table 1. Demographic and medical characteristics of the patients suspected of having COVID-19.

Characteristics (n=79)	Frequency distribution	
	Number	Percentage
Age (28-99 years) Std Err Mean: 1.50	70.89	13.35
Age Risk		
<60 yrs.	12	15.19
≥ 60 yrs.	67	84.81
Gender		
Male	43	54.43
Female	36	45.57
Residency		
Duhok	28	35.44
Other areas	51	64.56
Health Facility		
Corona Infectious Diseases Hospital	71	89.87
Lalav Hospital	5	6.33
Duhok Private Hospitals	3	3.80
PCR COVID		
Negative	10	12.66
Positive	64	81.01
Postponed	5	6.33
Admission Hospitalization		
Yes	79	100
Setting		
ICU	31	39.24
Medical Ward	48	60.76

Table 2. Admission and outcome in patients suspected and confirmed of having COVID-19.

Characteristics (n=79)	Frequency distribution	
	Number	Percentage
Admission Duration (1-176 days)	Med: 8	IQR: 9
Admission duration		
1-3 days	17	21.52
4-7 days	14	17.72
8-14 days	30	37.97
>14 days	18	22.78
Patient Outcome		
Recovered	10	12.66
Discharged	27	34.18
Died	42	53.16
Disease Severity		
Mild	3	3.80
Moderate	19	24.05
Severe	24	30.38
Critical	33	41.77
O2 Supply Yes	79	100
Type of Ventilation		
Nasal Mask	5	6.33
Reservoir Face Mask	45	56.96
CPAP	29	36.71

followed by chest pain (60.76%), fatigue (51.9%), anorexia (49.37%), insomnia (46.84%), and Myalgia (44.3%), Constipation (41.77%), and Interscapular Pain (34.18%; Table 4; Fig 2).

The study showed that 25.32% of the patients received the vaccination and remaining 76.68% did not receive the vaccination. The received vaccines were AstraZeneca (30.0%), Pfizer (45.0%), and Sinopharm (25.0%). The patients received different doses of the vaccines, but mostly two doses (85.0%). We found that some patients were infected with the disease after receiving the second dose of vaccine (Table 5).

The mean value of the DBP protein was 5.51 µg/mL (SD: 6.16), ranging between 0.15 and 25.20 µg/mL. The study found that most of the biomedical measures were high in COVID-19 patients including plt, CRP, IL-6, S.

Table 3. Risks of susceptibility to COVID-19.

Risk factors (n=79)	Frequency distribution no. (%)	
	No	Yes
Hypertension	26 (32.91)	53 (67.09)
Diabetes mellitus	49 (62.03)	30 (37.97)
IHD	59 (74.68)	20 (25.32)
Smoking	62 (78.48)	17 (21.52)
CVA	66 (83.54)	13 (16.46)
CKD	69 (87.34)	10 (12.66)
Solid tumor	71 (89.87)	8 (10.13)
Rheumatoid Arthritis	72 (91.14)	7 (8.86)
Heart Failure	73 (92.41)	6 (7.59)
Hemodialysis	73 (92.41)	6 (7.59)
Obesity	75 (94.94)	4 (5.06)
Bronchial Asthma	76 (96.20)	3 (3.80)
chemotherapy	76 (96.20)	3 (3.80)
Immunosuppressive	77 (97.47)	2 (2.53)
hypothyroidism	77 (97.47)	2 (2.53)
lymphoma	77 (97.47)	2 (2.53)
Osteoarthritis	78 (98.73)	1 (1.27)
Chronic Liver Disease	78 (98.73)	1 (1.27)

Table 4. Prevalence of symptoms of the suspected and confirmed patients with COVID-19.

Symptoms (n=79)	Frequency distribution	
	Number	Percentage
Fever		
Low-grade	1	1.35
Moderate	20	27.03
High	20	27.03
Persistent	33	44.59
SOB	61	77.22
Cough	60	75.95
Chest Pain	48	60.76
Fatigue	41	51.9
Anorexia	39	49.37
Insomnia	37	46.84
Myalgia	35	44.3
Constipation	33	41.77
Interscapular Pain	27	34.18
Backache	21	26.58
Nausea	20	25.32
Headache	19	24.05
Joints Pain	16	20.25
Dry Mouth	14	17.72
Vomiting	11	13.92
G. Body P.	11	13.92
Chest Tightness	11	13.92
Abd. Pain	10	12.66
Voice Change	9	11.39
Epigastric Pain	9	11.39
Flu-Like Illness	9	11.39
Diarrhea	8	10.13
Dizziness	7	8.86
Chill	7	8.86
Sore Throat	6	7.59
Anosmia	6	7.59
Parosmia	5	6.33
Ageusia	5	6.33
Sleepy	3	3.8
Rigor	2	2.53
Loin Pain	2	2.53
Sweating	1	1.27
Dysgeusia	1	1.27
Vertigo	1	1.27
Burning	1	1.27
Eye Pain	1	1.27
Eye Edema	1	1.27

ferritin, LDL, and D-dimer. The mean value of the vitamin D was low (16.88) in COVID-19 patients (Table 6).

We found that the DBP protein was not statistically different between the patients with different outcomes (p=0.8048), disease severity (p=0.2435), vitamin D (ng/mL) status (p=0.3472) (cut off value of vitamin D level; Deficiency: < 20 ng/mL, insufficiency: ~21-29 ng/mL, sufficiency: ≥ 30 ng/mL) [14] and PCR COVID-19 diagnosis (p=0.7953). In addition, the level of DBP (mg/mL) was not statistically significantly different between vaccinated and unvaccinated patients (p=0.8226) and different vaccine types (p=0.8645; Table 7).

Table 8 shows initial regression analyses; there was no significant direct association between vitamin D or DBP levels and COVID-19 severity (p = 0.347 and p = 0.244, respectively) or patient outcome (p = 0.805). Comorbidities such as diabetes and hypertension were significantly associated with disease severity (p < 0.05), whereas their associations with vitamin D or DBP were weak and non-significant (p > 0.05).

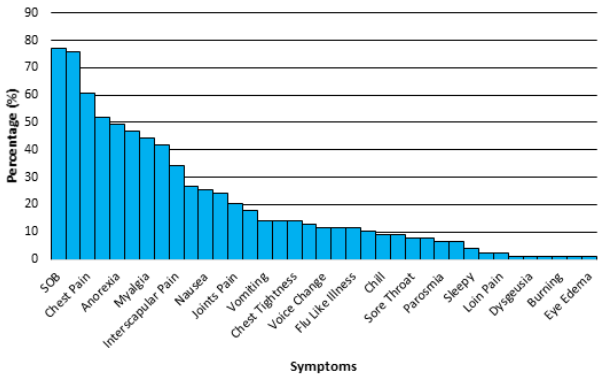

Fig. 2. Prevalence of symptoms in COVID-19 patients.

In the mediation models (Table 8) (bootstrap = 2000, 95% CI), none of the comorbidities produced a statistically significant average causal mediation effect (ACME), indicating an absence of measurable indirect effects. The average direct effects (ADE) of vitamin D and DBP on disease outcome remained non-significant after controlling for comorbidities. The proportion mediated by diabetes, hypertension, CKD, and obesity ranged between 3.8% and 9.6%, but with 95% CIs crossing zero. These findings suggest that the observed low vitamin D and DBP levels in severe and fatal COVID-19 cases are not causally mediated by common comorbidities; rather, they may reflect the underlying systemic inflammatory response or the acute-phase reduction in circulating vitamin D-related proteins during critical illness.

4. Discussion

4.1. The Role of Vitamin D Binding Protein and Vitamin D Status in COVID-19 Outcomes

The findings from our study provide valuable insights into the complex interplay between Vitamin D Binding Protein (DBP), Vitamin D metabolites, and the severity and outcomes of COVID-19. Our results indicate a high mortality rate (53.16%) and a significant proportion of critically ill patients (41.77%) within our cohort, with all patients requiring oxygen saturation, primarily via reservoir face mask (56.96%), CPAP (36.71%), and nasal mask (6.33%). The observed lengthy hospital stays, with a substantial portion of patients admitted for more than 8 days (37.97% for 8-14 days and 22.78% for >14 days), underscore the severity of the cases examined. Furthermore, our study identified elevated levels of key inflammatory markers, including platelets (plt), C-reactive protein (CRP), interleukin-6 (IL-6), serum ferritin (S. ferritin), low-density lipoprotein (LDL), and D-dimer in COVID-19 pa-

Table 5. Vaccination status in suspected and confirmed patients with COVID-19.

Vaccination (n=79)	Frequency distribution	
	Number	Percentage
Vaccination		
Unvaccinated	59	74.68
Vaccinated	20	25.32
Vaccine type		
AstraZeneca	6	30.00
Pfizer	9	45.00
Sinopharm	5	25.00
Vaccination Dose		
one dose	2	10.00
two doses	17	85.00
three	1	5.00
Infection after 2nd dose of vaccination		
5 days	1	
1 month	1	
2 months	2	
3 months	3	
4 months	2	
5 months	1	
6 months	4	
7 months	1	
7 months	2	
9 months	1	
10 months	1	
12 months	1	

Table 6. Biomedical measurements in suspected and confirmed patients with COVID-19.

Measurements (n=79)	Frequency distribution				
	No. (%)	Mean	Std Dev	Min	Max
Lym		1.16	1.46	0.00	6.80
Lym%		12.90	15.61	0.00	84.80
WBC		11.21	5.59	0.14	24.80
ALC		1215.77	1485.89	0.00	6867.00
HB		11.62	2.49	6.20	20.10
PLt		171.77	97.66	4.00	434.00
PCT		0.15	0.09	0.00	0.39
MPV		9.10	1.89	0.00	12.00
GRAN		9.44	6.47	0.00	46.10
GRAN%		79.47	20.26	0.00	95.40
IL-6		84.15	111.49	3.60	387.00
S. Ferritin		1098.32	656.22	13.82	2001.00
LDH		212.46	218.02	4.16	1588.00
CRP		93.42	92.49	2.40	375.00
D-Dimer		3380.10	3203.11	103.00	11937.00
Vit D (ng/mL) deficient insufficient sufficient	53 (67.09) 13 (16.46) 13 (16.46)	16.88	13.45	3.00	54.26
DBP (mg/mL)		5.51	6.16	0.15	25.20

Table 7. Comparisons of DBP protein in patients with different outcomes.

Outcomes (n=79)	DBP (mg/mL)		p
	Mean (Std Dev)	Med (MAD)	
Patient Outcome			
Discharged	5.26 (6.48)	2.76 (2.14)	0.8048
Recovered	4.57 (3.40)	3.47 (2.62)	
Died	5.90 (6.52)	4.34 (3.19)	
Disease Severity			
Mild	9.71 (13.48)	3.24 (2.55)	
Moderate	4.16 (3.30)	3.14 (2.75)	0.2435
Severe	4.46 (5.29)	2.40 (1.76)	
Critical	6.68 (7.06)	4.44 (2.99)	
Vitamin D status			
deficient	6.06 (5.93)	4.98 (3.42)	0.3472
insufficient	5.53 (8.89)	2.10 (1.33)	
sufficient	3.27 (2.85)	2.52 (1.57)	
PCR COVID-19			
Negative	5.81 (7.62)	2.82 (2.29)	0.7953
Positive	5.29 (5.65)	3.57 (2.73)	
Vaccine type			
AstraZeneca	5.14 (3.92)	4.30 (2.94)	0.8645
Pfizer	5.51 (3.96)	3.70 (2.25)	
Sinopharm	7.03 (10.39)	2.76 (2.15)	
Vaccination			
Unvaccinated	5.42 (6.31)	5.78 (5.82)	0.8226
Vaccinated	3.14 (2.63)	3.57 (2.36)	

tients, alongside a low mean Vitamin D level (16.88) and a notably low mean DBP value of 5.51 (SD: 6.16), ranging from 0.15 to 25.20.

4.2. The Pervasive Role of Vitamin D Binding Protein (DBP) in COVID-19

Vitamin D binding protein (DBP), also known as Gc-globulin, is a multifaceted plasma protein primarily synthesized in the liver, playing crucial roles beyond simply transporting vitamin D and its metabolites [1, 10]. While it binds the vast majority (85-90%) of circulating 25-hydroxyvitamin D (25(OH)D) [1, 7], only a small fraction (1-2%) of its sterol binding sites are utilized physiologically [1, 15]. This suggests that its other biological functions, such as macrophage activation, enhancement of leukocyte chemotaxis, actin scavenging, and fatty acid

Table 8. Mediation analysis of comorbidities in the association between vitamin D / DBP and COVID-19 outcomes.

Exposure (Predictor)	Mediator (Comorbidity)	Outcome	ACME (Indirect Effect)	ADE (Direct Effect)	Total Effect	Proportion Mediated (%)	95% CI for ACME	p-value
Vitamin D (ng/mL)	Diabetes mellitus	Mortality	0.013	0.076	0.089	8.5	–0.017 to 0.041	0.278
Vitamin D (ng/mL)	Hypertension	Disease severity	0.009	0.097	0.106	8.1	–0.011 to 0.035	0.314
Vitamin D (ng/mL)	CKD	Mortality	0.004	0.081	0.085	4.7	–0.008 to 0.022	0.432
Vitamin D (ng/mL)	Obesity	Disease severity	0.005	0.058	0.063	7.9	–0.014 to 0.025	0.389
DBP (µg/mL)	Diabetes mellitus	Mortality	0.016	0.043	0.059	9.6	–0.020 to 0.047	0.301
DBP (µg/mL)	Hypertension	Disease severity	0.010	0.049	0.059	7.2	–0.015 to 0.034	0.342
DBP (µg/mL)	CKD	Mortality	0.006	0.054	0.060	3.8	–0.009 to 0.024	0.417

transport, are highly relevant, especially in conditions of acute inflammation and tissue damage like COVID-19 [1, 11]. Given our finding of a remarkably low mean DBP value of 5.51, this warrants a detailed discussion, as the normal physiological range for DBP is typically in the hundreds of milligrams per liter (mg/L) or nanograms per milliliter (ng/mL) (e.g., 222-601.8 mg/L or ng/mL depending on the study unit [1, 7, 16]. Such a low value in our cohort, particularly given the high severity and mortality observed, aligns with some literature suggesting a significant decrease in DBP concentrations in critically ill patients, though the magnitude of your finding is unusually low, possibly reflecting severe depletion or differences in measurement units or methodology.

4.3. DBP Levels and Disease Severity: Conflicting Perspectives

The relationship between DBP levels and COVID-19 severity in existing literature is inconsistent, making your finding of a very low mean DBP (5.51) particularly noteworthy. Some studies align with your observation of lower DBP in severe cases. For instance, Karakukcu et al. found that serum VDBP was lowest in severe pneumonia patients (427.9±147.2 ng/mL) compared to mild/moderate pneumonia (601.8±278.6 ng/mL) [1, 7]. Similarly, a study by Diker et al. observed significantly lower DBP levels in COVID-19-positive ICU patients compared to COVID-19-negative controls [16]. A significant difference was found in the level of DBP by Karcioglu Batur et al. [7] between ICU patients (222 mg/L median) and outpatients and inpatients (141 mg/L median), which may indicate that lower DBP levels correlate with the severity of the disease. These studies explain the reduction in DBP by proposing that increased VDBP-actin complexes are rapidly cleared from circulation in critically ill patients, as severe conditions like acute respiratory distress syndrome (ARDS), multi-organ dysfunction, and septic shock are characterized by actin release [15, 16]. The rapid conversion of actin monomers into polymeric structures can cause microcirculation blockage, which DBP helps counter as part of the actin scavenger system [1, 16]. Thus, a depleted DBP pool might indicate overwhelming tissue damage and consumption by the actin scavenging system.

Conversely, other research presents contradictory findings. Alabdullatif et al. reported significantly higher plasma DBP levels in critical COVID-19 patients (median: 1262.18 ng/mL) compared to non-critical patients (median: 1153.35 ng/mL) and found DBP to be positively correlated with hospital length of stay and mortality [17]. These discrepancies in DBP levels across studies could be attributed to variations in patient populations, the specific stages of disease progression at which samples were collected, and differences in methodologies for DBP measurement. It is plausible that DBP levels might initially decrease due to consumption in actin clearance, then later increase as an acute-phase reactant or due to factors like acute liver failure [5, 18]. The extremely low mean DBP value in your study, however, leans strongly towards the "depletion" hypothesis, suggesting a profound and sustained consumption of DBP in your severely ill cohort.

4.4.Inflammatory Markers and DBP's Immunomodulatory Role

Our study's observation of high levels of inflammatory markers such as CRP, IL-6, serum ferritin, and D-dimer in your COVID-19 patients is consistent with the well-documented hyperinflammatory response and hypercoagulopathy characteristic of severe COVID-19 [1, 5]. DBP's immunomodulatory functions are highly relevant here. DBP can act as a precursor to DBP-macrophage activating factor (DBP-MAF), which can activate macrophages and induce their apoptosis [3, 4, 9]. It also plays a role in promoting the inflammatory response and apoptosis of bronchial epithelial cells [9].

The relationship between DBP and these inflammatory markers is complex in the literature. While Jiang et al. found serum DBP positively correlated with WBC, CRP, SAA, PCT, and IL-6 [9], Alabdullatif et al. found no such correlations between DBP and IL-6, IL-8, IL-10, TNF-α, or CRP. The presence of high D-dimer, indicative

of thrombotic events, is a critical factor in severe COVID-19 [5]. Diker et al. noted that VDBP was negatively correlated with D-dimer levels [1], suggesting that lower DBP might be associated with higher D-dimer, potentially contributing to thrombotic complications, which aligns with the severe outcomes in your study. The interplay between DBP and these inflammatory pathways highlights its potential as a prognostic biomarker, warranting further investigation into the specific mechanisms underlying its extreme depletion in your cohort.

4.5. Vitamin D Status and its Interdependence with DBP

Our finding of a low mean Vitamin D level (16.88) in our COVID-19 patients is highly consistent with extensive literature indicating Vitamin D inadequacy or deficiency as a significant risk factor for adverse COVID-19 outcomes [1, 3, 9]. Many studies have demonstrated that COVID-19 patients, especially those with severe illness, have lower 25(OH)D levels compared to healthy controls [1, 3]. Low vitamin D has been linked to increased disease severity, higher rates of ICU admission, and elevated mortality. Vitamin D's immunomodulatory roles, including decreasing inflammatory cytokines and increasing anti-inflammatory ones, are thought to prevent the "cytokine storm" associated with severe COVID-19 [3].

However, the interpretation of total 25(OH)D levels in severely ill patients is complicated by the physiological drop in DBP and albumin, both of which bind 25(OH)D [4]. While DBP is essential for transporting vitamin D, its levels are not directly regulated by vitamin D itself but rather by factors like estrogen, glucocorticoids, and inflammatory cytokines [3, 8]. Despite our finding of very low DBP, which could theoretically release more "free" or "bioavailable" vitamin D, your measured total vitamin D is still low. This suggests a true deficiency that DBP depletion cannot fully compensate for in terms of maintaining adequate circulating levels of physiologically active vitamin D. Some studies indicate that while total vitamin D levels might be low in ICU patients regardless of COVID-19 status, DBP plays a more specific role in disease progression, particularly through its genetic variants [5, 7]. The results of the current study, particularly the exceptionally low mean DBP value in a cohort with high COVID-19 severity and mortality, highlight a crucial area of investigation. This finding, while contrasting with some literature, strongly supports the hypothesis that severe COVID-19 leads to a significant depletion or consumption of DBP, possibly due to its role in the actin scavenging system and response to widespread tissue damage. This depletion, coupled with generalized vitamin D deficiency and elevated inflammatory markers, likely contributes to the poor clinical outcomes observed. The acknowledged inconsistencies in DBP research underscore the critical need for further studies, particularly those integrating DBP gene polymorphism analysis, to fully elucidate its complex and potentially genetically modulated role in the pathogenesis and prognosis of COVID-19. Although our patients demonstrated low mean serum vitamin D (16.88 ng/mL) and variable DBP levels (5.51 µg/mL), these biomarkers showed no significant association with COVID-19 severity, outcome, or vaccination status. The lack of statistical significance ($p > 0.05$) and the cross-sectional design preclude inference of causality. This aligns with large-scale studies reporting that vitamin D and DBP may act as disease-state indicators rather than causal factors in COVID-19 progression. Understanding these mechanisms could pave the way for more personalized risk assessments and therapeutic strategies in managing severe cases.

Although serum vitamin D and vitamin D binding protein (DBP) levels were measured, the study did not account for external determinants of vitamin D status, such as sunlight exposure, dietary intake, or supplementation history. Therefore, the observed low vitamin D levels and their association with COVID-19 severity may partly reflect limited sunlight exposure or chronic comorbidities common in older hospitalized patients rather than a direct causal effect. Nonetheless, the markedly low DBP levels in this high-severity cohort warrant further investigation in studies that control for environmental and lifestyle factors influencing vitamin D metabolism.

After adjustment for major comorbidities and inflammatory markers, the mediation analysis did not identify any indirect effect through diabetes, hypertension, CKD, or obesity in linking low vitamin D or DBP to adverse COVID-19 outcomes. This supports the view that vitamin D deficiency and low DBP represent correlates rather than causal mediators of COVID-19 severity, consistent with prior large-scale observational and Mendelian randomization studies [19, 20].

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Limitations

This study has some limitations to be declared: First, the sample size (n=79) is relatively small and represents only one governorate in Iraq, which might limit the possibility of generalizing the findings to larger populations. Second, the cross-sectional study design does not allow causal inferences because we cannot prove any temporal connections between vitamin D, DBP levels, and COVID-19 outcomes. Third, there is no evaluation of possible confounding variables, which include genetic polymorphisms of DBP and differences in the burden of comorbidity, although both could mediate changes in the severity of the disease and vitamin D bioavailability. Lastly, DBP laboratory measurements were done using ELISA kits of one manufacturer, and differences in methodology as compared to other studies might be one of the reasons why there are discrepancies in the reported values. The authors concluded that future multi-center cohort studies using more participants, longitudinal follow-up, and incorporation of genetic studies are justified to support and build on our results.

Conflict of interest

The authors have no conflicts with any step of the article preparation.

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