Vitamin D, vitamin D—binding protein, free vitamin D and COVID-19 mortality in hospitalized patients

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ABSTRACT

Background: Vitamin D deficiency has been associated with worse coronavirus disease 2019 (COVID-19) outcomes, but circulating 25-hydroxyvitamin D [25(OH)D] is largely bound to vitamin D–binding protein (DBP) or albumin, both of which tend to fall in illness, making the 25(OH)D status hard to interpret. Because of this, measurements of unbound ("free") and albumin-bound ("bioavailable") 25(OH)D have been proposed.

Objectives: We aimed to examine the relationship between vitamin D status and mortality from COVID-19.

Methods: In this observational study conducted in Liverpool, UK, hospitalized COVID-19 patients with surplus sera available for 25(OH)D analysis were studied. Clinical data, including age, ethnicity, and comorbidities, were extracted from case notes. Serum 25(OH)D, DBP, and albumin concentrations were measured. Free and bioavailable 25(OH)D were calculated. Relationships between total, free, and bioavailable 25(OH)D and 28-day mortality were analyzed by logistic regression.

Results: There were 472 patients with COVID-19 included, of whom 112 (23.7%) died within 28 days. Nonsurvivors were older (mean age, 73 years; range, 34–98 years) than survivors (mean age, 65 years; range, 19–95 years; $P = 0.003$) and were more likely to be male (67%; $P = 0.02$). The frequency of vitamin D deficiency [25(OH)D < 50 nmol/L] was similar between nonsurvivors (71/112; 63.4%) and survivors (204/360; 56.7%; $P = 0.15$) but, after adjustments for age, sex, and comorbidities, increased odds for mortality were present in those with severe deficiency [25(OH)D < 25 nmol/L: OR, 2.37; 95% CI, 1.17–4.78] or a high 25(OH)D [≥100 nmol/L; OR, 4.65; 95% CI, 1.51–14.34] compared with a 25(OH)D value of 50–74 nmol/L (reference). Serum DBP levels were not associated with mortality after adjustments for 25(OH)D, age, sex, and comorbidities. Neither free nor bioavailable 25(OH)D values were associated with mortality.

Conclusions: Vitamin D deficiency, as commonly defined by serum 25(OH)D levels (<50 nmol/L), is not associated with increased mortality from COVID-19, but extremely low (<25 nmol/L) and high (>100 nmol/L) levels may be associated with mortality risks. Neither free nor bioavailable 25(OH)D values are associated with mortality risk. The study protocol was approved by the London-Surrey Research Ethics Committee (20/HRA/2282).   *Am J Clin Nutr* 2022;00:1–11.

Keywords: vitamin D, COVID-19 mortality, vitamin D–binding protein, free vitamin D, bioavailable vitamin D

Introduction

Coronavirus disease 2019 (COVID-19) has accounted for nearly 200 million infections and over 4.25 million deaths worldwide as of 22 November 2021 (1). Factors associated with

adverse outcomes include older age; male sex; comorbidities such as obesity, diabetes, and hypertension; and Black or Asian race (2). These factors are all independently associated with vitamin D deficiency (2) or, in males, with a reduced impact of vitamin D on the immune response (3). A role for vitamin D is further supported by correlations between latitude or UVB exposure and both COVID-19 infection growth rates (4) and age-adjusted mortality (5, 6), although such associations might be confounded by unmeasured factors.

Vitamin D, a cholesterol-derived steroid hormone, plays critical roles in musculoskeletal health and in modulating innate and adaptive immunity (6). In keeping with this, vitamin D status has been shown to influence the risk of acquiring respiratory viral infections (7). Vitamin D deficiency is also associated with the severity of respiratory disease: for example, the need for intensive care in infants hospitalized with bronchiolitis (8). Vitamin D deficiency upregulates proinflammatory cytokines such as TNF, type 1 IFN, and IL-6 (9, 10). Moreover, genetically modified mice lacking a vitamin D receptor show greatly increased susceptibility to lipopolysaccharide-induced experimental lung damage (11).

Various studies have examined associations between vitamin D status and the risk of acquiring COVID-19 or its severity (12–17), but they are heterogenous and inconsistent. Studies have often been limited by sample size or by historical measurements of vitamin D, sometimes from many years before. Furthermore, studies of vitamin D concentrations in individuals who are already ill have to be interpreted with caution because of the possible fall in vitamin D–binding protein (DBP), and hence total 25-hydroxycholecalciferol [25(OH)D], as part of a negative acute-phase response (18, 19).

Vitamin D status is determined by the serum concentration of 25(OH)D, which is largely protein bound, with approximately 85% to DBP and 15% to albumin. Binding to albumin is less avid, and albumin-bound vitamin D is therefore commonly designated as "bioavailable." Both serum DBP (20) and albumin (21) concentrations may fall in illness as a negative acute-phase response. Only 0.03% of circulating 25(OH)D is unbound or "free" (18). Measurement of free 25(OH)D is challenging due to its very low serum concentration, and has historically relied on cumbersome radioactive tracer–based methods (18). Thus, computational methods that rely on concentrations of total ligands and DBP and their in vitro measured affinity constants are often used to calculate free and bioavailable 25(OH)D (19). Both free 25(OH)D and active 1,25(OH)$_2$D freely diffuse across the lipid bilayer of cell walls. Many cells and tissues, not just the kidneys, possess the 1α-hydroxylase-activating enzyme (CYP27B1). Perhaps consequently, adverse biological consequences of vitamin D deficiency tend to correlate with low serum concentrations of 25(OH)D but not with serum concentrations of activated 1,25(OH)$_2$D (22).

Given these complexities, plus uncertainties around the 25(OH)D concentration used to define deficiency (23), it is perhaps not surprising that studies of vitamin D during COVID-19 have produced contradictory results. We have therefore investigated the associations between total, free, and bioavailable 25(OH)D and COVID-19 mortality.

Methods

We conducted a single-center study of COVID-19 patients who were hospitalized between 18 March and 2 November 2020 across the 2 acute hospital sites of the Liverpool University Hospitals NHS Foundation Trust. We included all patients who had surplus sera available for analyses of 25(OH)D concentrations. The population studied were unvaccinated. We collected baseline clinical information, including demographic data such as age, sex, BMI, and comorbidities (diabetes; hypertension; chronic respiratory illness, including asthma, chronic obstructive airways disease, and interstitial lung disease; chronic cardiovascular or cerebrovascular disease; neurological disease, including dementia or cerebrovascular accident; chronic kidney disease; chronic liver disease; and active malignancy). We used self-reported data on ethnicity, which is held in standard demographics of medical records. Data on clinical progress during hospitalization, including the need for supplemental oxygen, respiratory support in the form of continuous positive airways pressure or mechanical ventilation, and death within 28 days of a positive severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) test were recorded. We also collected information on medical treatment of COVID-19 (remdesivir and/or dexamethasone) or prescribed vitamin D supplementation prior to measurement of serum 25(OH)D levels.

Sample size

The sample size was determined by the number of suitable patients available for the study, and no formal power calculation was made. In total, data on 472 patients were available: of these, 360 were still alive 28 days after testing positive for SARS-CoV-2, and 112 had died. A retrospective power estimate based on the 112 patients who died and the 360 who survived suggested that we could have determined a difference of 6 nmol/L in the serum 25(OH)D levels between the survivor and nonsurvivor groups, using a standard significance level of 5% and a power of 80%. DBP was measured in 419 patients, as the remainder did not have sufficient residual serum to measure DBP. The primary outcome measure was mortality within 28 days of a positive SARS-CoV-2 test.

Measurement of serum 25(OH)D and DBP

Serum samples were aliquoted and stored at −80°C until they could be analyzed for DBP and total 25(OH)D. Concentrations of DBP were measured using a commercial polyclonal antibody ELISA kit (Immundiagnostik AG) according to the manufacturer's protocol. The 25(OH)D$_2$ and 25(OH)D$_3$ concentrations were measured using established in-house LC-MS/MS methodology. The vitamin D2 and D3 values were summated to determine total 25(OH)D. Serum albumin concentrations were measured using a colorimetric bromocresol green–based method (Roche Diagnostics) using Roche Cobas c701 instrumentation. The levels of bioavailable 25(OH)D were calculated from total measured 25(OH)D, DBP, and serum albumin

concentrations using the following equation, as described previously (19):

$$\text{free vitamin D metabolite} = \text{total vitamin D metabolite}/1$$
$$+ (\text{Ka}_{\text{alb}} \times \text{albumin}) + (\text{Ka}_{\text{DBP}} \times \text{DBP}) \quad (1)$$

Here, Ka is the affinity constant. We used values of 7×10^8 M^{-1} for Ka$_{\text{DBP}}$ and 6×10^5 M^{-1} for Ka$_{\text{alb}}$.

Bioavailable 25(OH)D was calculated using the following affinity constants: DBP for 25(OH)D = 7×10^8 M^{-1}, DBP for 1,25(OH)$_2$D = 4×10^7 M^{-1}, albumin for 25(OH)D = 6×10^5 M^{-1}, and albumin for 1,25(OH)$_2$D = 5.4×10^4 M^{-1} and the variables were concentrations of 25(OH)D, 1,25(OH)$_2$D (assigned 0.1 nM in all cases), DBP, and albumin. A MATLAB program implements a system of coupled, nonlinear, algebraic equations for each vitamin D metabolite (19) for extracellular steady state free-ligand modeling, yielding calculated values for the bioavailable and free 25(OH)D metabolites of interest (19).

Statistical analyses

Initially, the baseline demographics and clinical characteristics were reported using standard summary statistics. As all the continuous variables, except patient age, were not normally distributed, the median values along with the IQRs were reported for these variables; counts and percentages were reported for categorical variables. Logistic regression models were then used to determine associations between the demographic and clinical variables and the primary outcome of 28-day mortality. Each possible explanatory variable was tested in a univariate analysis to determine its strength of association with the outcome of interest. Serum concentrations of total 25(OH)D were initially assessed as continuous variables and then by categories defined according to the various published definitions of vitamin D deficiency and sufficiency: that is, <25, 25–49, 50–74, and 75–99 nmol/L plus a category with high values ≥ 100 nmol/L and a group for high values ≥ 100 nmol/L, previously shown to be associated with increased fibroblast growth factor (FGF) 23, which suppresses vitamin D activation via the 1-alpha-hydroxylase (24–26), with significance determined in the univariate analysis using a chi-square goodness-of-fit. Free 25(OH)D, bioavailable 25(OH)D, and DBP were categorized into quintiles and similarly examined for associations with mortality. All variables identified as being possibly associated with the outcome ($P < 0.1$) were then taken forward into a multivariable model. The 2 clinical variables that were included in the final model (chronic kidney disease and neurological diseases) have been previously associated with worse clinical outcomes (27). Two different multivariable models were fitted, 1 with vitamin D as a continuous variable and the other with vitamin D as a categorized variable. A further analysis was performed by logistic regression, but with vitamin D as a continuous rather than categorized variable, again with adjustments for age and sex, and plotted with cubic spline smoothing using the package mgcv (28) in R (29). Correlations between 25(OH)D and biochemical parameters were assessed using Spearman rank correlation. Analyses were carried out using SPSS (Version 27.0, IBM Corp), or R version 4.1.1 (29). A 2-sided P value < 0.05 was considered statistically significant.

Ethical approval

The study protocol was approved by the London-Surrey Research Ethics Committee (20/HRA/2282).

Results

Cohort demographics

We included 472 patients with COVID-19, of whom 268 (56.8%) were men. This represented 47.5% of the 992 patients who had been admitted with COVID-19 during the study period of 18 March to 2 November 2020. We only included patients who had surplus sera available for 25(OH)D analysis (for patient flowchart, see **Supplemental Figure 1**). The 28-day mortality rate was 23.7%. The mean age at death was 73 years (SD, 19 years), and the 28-day mortality rates were 28.0% and 18.5% among men and women, respectively. The univariate analysis showed a significant association between an increased risk of mortality by 28 days and older age, male sex, and preexisting neurological disease (**Table 1**). There were no significant associations found with BMI, ethnicity, and other comorbidities (Table 1). There was a trend towards increased mortality in patients with chronic kidney disease, although this was not significant ($P = 0.06$; Table 1). Increased mortality was recorded in patients with higher baseline concentrations of D-dimer, troponin, C-reactive protein (CRP), and creatinine and in those with lower baseline concentrations of albumin (**Table 2**). Of the entire cohort, 76 patients (16.1%) received remdesivir and 128 (27.1%) received dexamethasone (**Table 3**). Oxygen therapy was received by 69.1% during their hospital stay, and 76 (16.1%) were treated in the intensive care unit, among whom 56 (11.7% of total) received mechanical ventilation (Table 3).

Vitamin D status

The overall prevalence of vitamin D deficiency [25(OH)D < 50 nmol/L] in the cohort was 58.1%, while 25.0% were severely deficient [25(OH)D < 25 nmol/L]. There was no significant difference between 25(OH)D concentrations amongst nonsurvivors (median, 39.5 nmol/L; IQR, 48.5 nmol/L) compared with survivors (median, 43.0 nmol/L; IQR, 41.0 nmol/L; $P = 0.11$; **Table 4**). Unadjusted, there was also no significant difference between frequencies of 25(OH)D vitamin D deficiency [25(OH)D < 50 nmol/L] amongst those who died (71/112; 63.4%) compared with survivors (204/360; 56.7%; $P = 0.15$). Total 25(OH)D, analyzed by 5 concentration categories according to the different definitions of sufficiency [<25, 25–49, 50–74, and 75–99 nmol/L, plus a group for high values ≥ 100 nmol/L, previously shown to be associated with increased FGF23 (24–26)], did suggest an association with 28-day mortality, with the lowest mortality for those with a 25(OH)D concentration of 50–74 nmol/L ($P = 0.04$; **Table 4**; **Figure 1**). This pattern remained after excluding patients who received prescribed vitamin D supplementation (**Supplemental Figure 2**). A similar analysis with 25(OH)D as a continuous variable was not significant. There was no significant association between free or bioavailable 25(OH)D and mortality (Table 4).

A multivariable analysis was therefore performed to investigate the associations between mortality and 25(OH)D concentration ranges, with adjustments for age, sex and comorbidities.

TABLE 1 Clinical characteristics of included subjects[1]

Variable	Alive	Deceased	P value
N	360	112	
Age			
Mean (SD)	65 (26)	73 (19)	0.003
Range	19–95	34–98	
Sex			
Female	167 (46.4%)	37 (33.0%)	0.02
Male	193 (53.6%)	75 (67.0%)	
BMI, kg/m^2			
Mean (SD)	27 (9)	26 (8)	>0.999
Underweight	25 (6.9%)	12 (10.7%)	0.627
Normal	97 (26.9%)	30 (26.7%)	
Overweight	74 (20.6%)	22 (19.6%)	
Obese	110 (30.6%)	33 (29.5%)	
Not recorded	54 (15%)	15 (13.4%)	
Ethnicity			
Black and minority	41 (11.3%)	12 (10.7%)	>0.999
White	302 (83.9%)	94 (83.9%)	
Not recorded	17 (4.7%)	6 (5.3%)	
DM			
No	270 (75.0%)	77 (69.8%)	0.22
Yes	90 (25.0%)	35 (30.2%)	
Hypertension			
No	203 (56.4%)	55.(49.1%)	0.19
Yes	156 (43.6%)	57 (50.9%)	
Not recorded	1 (0.2%)	—	
CKD			
No	301 (83.8%)	77 (68.8%)	0.06
Yes	58 (16.2%)	25 (31.2%)	
Not recorded	1 (0.2%)	—	
IHD			
No	294 (81.9%)	88 (78.6%)	0.49
Yes	65 (18.1%)	24 (21.4%)	
Not recorded	1 (0.2%)	—	
Smoking status			
Non-/ex-smoker	311 (86.4%)	99 (88.4%)	0.113
Current smoker	39 (10.8%)	5 (4.5%)	
Not recorded	10 (2.8%)	8 (7.1%)	
Respiratory			
No	256 (71.1%)	83 (74.1%)	0.576
Yes	104 (28.9%)	29 (25.9%)	
Neurological			
No	291 (81.1%)	76 (67.9%)	0.003
Yes	68 (18.9%)	36 (32.1%)	
Not recorded	1 (0.2%)	—	
Liver disease			
No	339 (94.4%)	103 (92.0%)	>0.999
Yes	20 (5.6%)	9 (8.0%)	
Not recorded	1 (0.2%)	—	
Active malignancy			
No	330 (91.9%)	102 (91.1%)	0.84
Yes	29 (8.1%)	10 (8.9%)	
Not recorded	1 (0.2%)	—	

[1]A univariate analysis was performed to compare variables across alive and deceased categories using a chi-squared test for categorical variables and a Mann-Whitney U test for continuous variables. Categorical data are represented as counts and percentages. CKD, chronic kidney disease; DM, diabetes mellitus; IHD, ischemic heart disease.

This showed increased mortality in those with 25(OH)D concentrations < 25 nmol/L (OR, 2.37; 95% CI, 1.17–4.78; $P = 0.016$) and in those with 25(OH)D concentrations >100 nmol/L (OR, 4.65; 95% CI, 1.51–14.34; $P = 0.007$) compared with 25(OH)D concentrations of 50-74 nmol/L, with the latter selected as reference since it reflects the typical summer range for 25(OH)D in healthy adults (30). It is important to note, though, that the number of patients with 25(OH)D concentrations >100 nmol/l was small ($n = 18$). Levels of 25(OH)D between 25–49 and 75–99 nmol/L were not associated with significant differences in mortality compared with levels of 50–74 nmol/L (**Table 5**). A logistic regression analysis of mortality, with adjustments for age and sex, but with vitamin D set as a continuous variable, did not show an association between mortality and 25(OH)D levels (**Figure 2**). Free 25(OH)D and bioavailable 25(OH)D concentrations, grouped by quintiles, remained unassociated with mortality in a model adjusted for age, sex, and comorbidities (**Supplemental Tables 1 and 2**).

DBP and mortality

On univariate analysis, median DBP concentrations were lower in patients who died ($P = 0.005$; Table 4; **Figure 3**). However, a multivariable analysis with adjustments for 25(OH)D, age, sex, and significant comorbidities did not show an association between DBP and mortality (**Supplemental Table 3**).

Associations of total, free, and bioavailable 25(OH)D with serum DBP, CRP, and albumin

We investigated associations between total, free, and bioavailable 25(OH)D; DBP; and albumin, with baseline CRP as a marker of inflammation. The relationship between the DBP concentration and free 25(OH)D concentration was significant (Spearman's rho = −0.572) but only sizeable at very low concentrations of DBP (<500 mg/L; Figure 3). There was no significant relationship between free 25(OH)D and serum albumin concentrations (Spearman's rho = 0.048; **Supplemental Figure 3**).

Serum 25(OH)D concentrations correlated with baseline serum CRP values (Spearman's rho = −0.102), in keeping with a negative acute-phase response but with a modest size effect; the mean 25(OH)D concentration fell from approximately 50 nmol/L at CRP levels < 5 mg/L to approximately 40 nmol/L at a CRP of 200 mg/L (**Figure 4**A). Serum 25(OH)D concentrations correlated with DBP concentrations (Spearman's rho = 0.127), but again with a modest size effect (Figure 4B). DBP concentrations showed a modest correlation with CRP (Spearman's rho = −0.084; Figure 4C). Serum 25(OH)D concentrations correlated with serum albumin (Spearman's rho = 0.166), with a stronger size effect than that observed for the DBP association, and the mean 25(OH)D concentration falling from approximately 50 nmol/L at an albumin concentration of 40 g/L to approximately 40 nmol/L at an albumin concentration of 30 g/L (Figure 4D). There was also an association between serum albumin concentration and baseline CRP value (Spearman's rho = −0.212; **Supplemental Figure 4**). However, the difference in serum albumin concentrations between survivors (median, 38 g/L; IQR, −7 g/L) and nonsurvivors (median, 35 g/L; IQR, −8 g/L), although significant, was only modest (Table 2).

TABLE 2 Biochemical and radiological parameters among survivors and nonsurvivors[1]

Variable	Alive, $n = 360$ (76.3%)	Deceased, $n = 112$ (23.7%)	P value
D-dimer, ng/ml, median (IQR)	781 (836)	1668 (2774)	<0.001
Troponin, ng/L, median (IQR)	21 (21.25)	23 (37.5)	0.003
CRP, mg/L, median (IQR)	49.5 (88.75)	111 (125)	<0.001
Albumin, g/L, median (IQR)	38 (7)	35 (8)	<0.001
ALT, iu/L, median (IQR)	24 (23)	22 (21)	0.47
Creatinine, umol/L, median (IQR)	73 (35)	101.5 (76)	<0.001
eGFR, ml/min/1.73m^2			
≤90	220 (62.0%)	85 (77.3%)	
>90	135 (38.0%)	25 (22.7%)	0.003
CXR changes			
No	63 (42.6%)	7 (18.0%)	
Yes	85 (57.4%)	32 (82.0%)	0.005
CT changes			
No	4 (10.8%)	—	
Yes	33 (89.2%)	4 (100.0%)	>0.999

[1] Values are based on values at hospital admission. COVID-19 changes on chest X-ray or CT scan were considered present on any imaging during hospital admission. A univariate analysis was performed to compare variables across alive and deceased categories using a chi-squared test for categorical variables and a Mann-Whitney U test for continuous variables. Categorical data are represented as counts and percentages and continuous variables as medians and IQRs. ALT, alanine aminotransferase; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; CT, computed tomography; CXR, chest X-ray; eGFR, estimated glomerular filtration rate.

Vitamin D supplementation and mortality

Prescribed vitamin D supplements were being taken by 131 (27.8%) patients. Data were not available for use of

TABLE 3 Treatments received by COVID-19 patients[1]

Variable	Alive, $n = 360$ (76.3%)	Deceased, $n = 112$ (23.7%)	P value
Remdesivir			
No	305 (85.0%)	91 (81.3%)	
Yes	55 (15%)	21 (18.7%)	0.37
Dexamethasone			
No	268 (74.7%)	76 (67.9%)	
Yes	92 (25.3%)	36 (32.1%)	0.18
Vitamin D supplements			
No	256 (71.1%)	80 (71.4%)	>0.999
Yes	100 (27.8%)	31 (27.7%)	
Not recorded	4 (1.1%)	1 (0.8%)	
Oxygen			
No	144 (40.0%)	2 (1.8%)	
Yes	216 (60.0%)	110 (98.2%)	<0.001
ICU			
No	318 (88.3%)	78 (68.8%)	
Yes	42 (11.7%)	34 (31.2%)	<0.001
CPAP			
No	321 (89.2%)	88 (78.6%)	
Yes	39 (10.8%)	24 (21.4%)	0.48
Mechanical ventilation			
No	334 (93.0%)	82 (73.2%)	
Yes	26 (7.0%)	30 (26.8%)	<0.001

[1] A univariate analysis was performed to compare variables across alive and deceased categories using a chi-squared test for categorical variables and a Mann-Whitney U test for continuous variables. Categorical data are represented as counts and percentages and continuous variables as medians and IQRs. COVID-19, coronavirus disease 2019; CPAP, continuous positive airways pressure; ICU, intensive care unit.

supplements purchased over the counter. The mean age of death was significantly higher (80.2 years; SD, 11.9 years) amongst those taking prescribed supplements compared with those not taking prescribed supplements (71.1 years; SD, 12.0 years; $P < 0.001$), but the average age of all people taking prescribed supplements (71.5 years; SD, 16.0 years) was greater than that of those not taking prescribed supplements (62.0 years; SD, 18.1 years; $P < 0.0001$), and the analysis of 28-day mortality did not show a significant association with prescribed supplements after adjustments for age (OR, 0.67; 95% CI, 0.40–1.12; $P = 0.12$). There was an association between prescribed supplement usage and 25(OH)D concentrations (supplement usage of 0% in those with a 25(OH)D concentration ≤25 nmol/L; 23% in those with a concentration of 25–49 nmol/L; 32% in those with a concentration of 50–74 nmol/L; 59% in those with a concentration of 75–99 nmol/L; and 53% in those with a concentration ≥100 nmol/L).

Discussion

In this retrospective, single-center cohort study, we confirm that older age and male sex are associated with increased 28-day mortality from COVID-19, consistent with the results of other studies (30–32). We found no significant relationship between overall 25(OH)D deficiency (<50 nmol/L) and mortality from COVID-19. However, when 25(OH)D values on admission were categorized according to the varying definitions of deficiency (<25, <50, or <75 nmol/l) plus an additional category for high values > 100 nmol, a multivariable analysis, adjusted for age, sex, and comorbidities, showed increased mortality amongst those with very low (<25 nmol/l) or high (>100 nmol/l) serum 25(OH)D concentrations compared to those with 25(OH)D concentrations of 50–74 nmol/L, which was selected as the reference since it approximates the typical range for 25(OH)D seen from June to December in healthy, British adults (30). The

TABLE 4 Levels of total 25(OH)D, DBP, and free and bioavailable 25(OH)D among COVID-19 patients[1]

Variable	Alive	Deceased	P value
25(OH)D, nmol/L, median (IQR)	43.0 (41.0)	39.5 (48.5)	0.11
25(OH)D deficiency < 50 nmol/L, n (%)	204 (56.7%)	71 (63.4%)	0.15
25(OH)D by categories	—	—	0.04
<25 nmol/L, n (%)	85 (23.6%)	33 (29.5%)	
25–49 nmol/L, n (%)	119 (33.1%)	38 (33.9%)	
50–74 nmol/L, n (%)	99 (27.4%)	16 (14.3%)	
75–99 nmol/L, n (%)	47 (13.0%)	17 (15.2%)	
>100 nmol/L, n (%)	10 (2.8%)	8 (7.4%)	
Free 25(OH)D, pmol/L, median (IQR)	3.31 (3.93)	3.73 (4.92)	0.28
Free 25(OH)D by quintile categories	—	—	0.32
0–1.55 pmol/L, n (%)	66 (20.4%)	19 (21.1%)	
1.56–2.68 pmol/L, n (%)	68 (21.0%)	12 (13.3%)	
2.69–4.10 pmol/L, n (%)	67 (20.7%)	18 (20.0%)	
4.11–6.11 pmol/L, n (%)	64 (19.8%)	17 (18.9%)	
>6.11 pmol/L, n (%)	59 (18.2%)	24 (26.7%)	
Bioavailable 25(OH)D, nmol/L, median (IQR)	1.07 (1.26)	1.01 (1.34)	0.81
Bioavailable 25(OH)D by quintile categories	—	—	0.39
0–0.459 nmol/L, n (%)	62 (19.1%)	20 (22.2%)	
0.46–0.81 nmol/L, n (%)	70 (21.6%)	12 (13.3%)	
0.82–1.3 nmol/L, n (%)	64 (19.8%)	23 (25.6%)	
1.31–2.037 nmol/L, n (%)	64 (19.8%)	16 (18.9%)	
>2.038 pmol/L, n (%)	64 (19.8%)	19 (21.1%)	
DBP, mg/L, median (IQR)	858 (707.8)	707 (441.0)	0.005
DBP by quintile categories	—	—	0.003
0–579.9 mg/L	66 (20.3%)	21 (22.3%)	
580–733.6 mg/L	69 (21.2%)	16 (17.0%)	
733.61–960.4 mg/L	51 (15.7%)	30 (31.2%)	
960.41–1615 mg/L	74 (22.8%)	10 (11.9%)	
>1615 mg/L	65 (20.0%)	17 (18.1%)	

[1]Note that sufficient sera for DBP, and hence free and bioavailable 25(OH)D, were only available for 325/360 (90.3%) of those who were alive and 94/112 (84%) of those who were deceased. A univariate analysis was performed using a chi-squared goodness-of-fit test. Categorical data are represented as counts and percentages and continuous variables as medians and IQRs. Levels of total 25(OH)D were categorized based on published definitions of adequacy and free 25(OH)D; bioavailable 25(OH)D and DBP were categorized as quintiles. COVID-19, coronavirus disease 2019; DBP, vitamin D–binding protein; 25(OH)D, 25-hydroxyvitamin D.

increased mortality in patients with serum 25(OH)D concentrations < 25 nmol/L is consistent with that reported in some other studies (15, 31), but patients with high serum 25(OH)D concentrations ≥ 100 nmol/L also had increased mortality in our cohort. This needs to be interpreted with caution, though, since the number of patients was small (n = 18) in the highest category (>100 nmol/l). Moreover, a multivariable analysis with 25(OH)D as a continuous variable did not reach significance.

Many studies have reported on 25(OH)D status, in terms of deficient compared with sufficient concentrations, in association with COVID-19 outcomes in hospitalized patients, with contradictory findings (12, 13). There are also some data on COVID-19 outcomes in relation to 25(OH)D concentration ranges in hospitalized patients: a study from New York showed a significantly increased likelihood of needing oxygen support in patients with 25(OH)D concentrations < 50 nmol/L (16); a Brazilian study showed a nonsignificant trend towards increased mortality at both very low and very high 25(OH)D levels (17); an Italian study reported a progressive fall in mortality with high 25(OH)D levels but was based only on 9 deaths (14); and finally, another study from Italy reported a progressive increase in mortality with increasing 25(OH)D concentrations

after adjustments for various factors, including baseline CRP and severe pneumonia (32).

U-shaped associations between serum 25(OH)D concentrations and various clinical outcomes, including all-cause mortality, have been reported previously but are highly controversial. The US Academy of Medicine noted in 2010 that several studies had reported such an association, and concluded that the "data are clearly suggestive of a U-shaped or reverse-J-shaped risk curve between serum 25OHD level and all-cause mortality; increases in risk are suggested at thresholds in the range of 75 to 120 nmol/L for the White population, with lower levels for the Black population" (33). A subsequent general-practice study from Denmark of 247,574 people, with an average age of 51 years who were followed over 3 years, confirmed a U-shaped association, with the lowest all-cause mortality rates again seen for those with 25(OH)D concentrations of 50–60 nmol/L (25). However, a study of 24,094 hospitalized patients from Boston showed a U-shaped association, with significantly increased mortality only seen at serum 25(OH)D concentrations ≥ 60 ng/ml, equivalent to 150 nmol/L (24). A U-shaped association between serum 25(OH)D and a 2.7-fold increased risk of bone fracture has also been shown in older
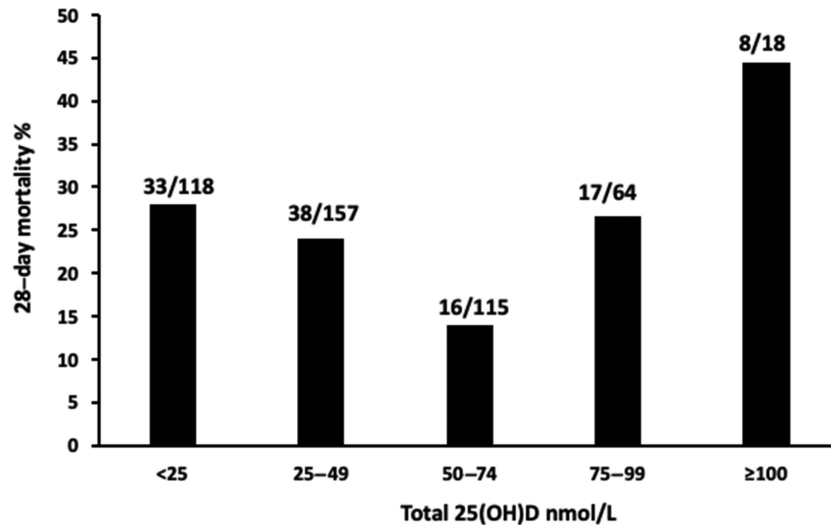
FIGURE 1 Bar graph of 28-day mortality (%) in patients, grouped according to serum 25(OH)D concentration on admission (unadjusted). It can be seen that the mortality rate was lowest among patients with a serum 25(OH)D concentration between 50–74 nmol/L. Serum 25(OH)D was measured in 472 individuals with COVID-19, among whom 360 were survivors and 112 were nonsurvivors. COVID-19, coronavirus disease 2019; 25(OH)D, 25-hydroxyvitamin D.

men at 25(OH)D concentrations > 72 nmol/L (34), and a dose-ranging study of vitamin D supplementation for prevention of falls has reported worse outcomes with higher dosing regimens (35). Also, a meta-analysis of standardized serum 25(OH)D measurements in 26,916 individuals from 8 prospective European studies showed the lowest all-cause mortality rate for 25(OH)D concentrations of 75–100 nmol/l, but with no significant increase in mortality at levels >100 nmol/l (36). There is a biologically plausible explanation for the U-shaped curve: the phosphaturic hormone FGF23 is typically induced when the serum 25(OH)D concentration rises above 100 nmol/L (37), which leads to a marked suppression of the vitamin D–activating 1-alpha-hydroxylase.

In our study, there was no correlation between serum free vitamin D and mortality. There are several possible explanations for this: *1*) protein-bound vitamin D can be taken up by immune cells expressing megalin (38) and activated by these cells through endogenous 1α-hydroxylase activity; and *2*) serum concentrations of free 25(OH)D are more than 10,000-fold lower than those of total 25(OH)D, while bioavailable—that

is, albumin-bound 25(OH)D—for which there was also no association with mortality in our patients, is present at even lower serum concentrations. A limitation of our study is that we did not directly measure free 25(OH)D levels but, as indicated previously, this is an extremely difficult assay. Moreover, previous studies have suggested that measurement of free and bioavailable 25(OH)D may not offer additional advantages over standard measurement of total 25(OH)D for diagnosing vitamin D deficiency (39).

Our study has several important strengths. Using a large sample size comprising 472 subjects has made this the largest study to date to investigate the association between serum vitamin D status and COVID-19 outcomes. In addition, we have also estimated DBP and free and bioavailable 25(OH)D concentrations. Importantly, vitamin D levels were measured contemporaneously, unlike in some other studies, where associations were derived from previously measured vitamin D levels (16, 40). There are, however, some limitations. Firstly, our study is retrospective and we only included patients with available surplus sera, which may have introduced bias. Moreover, the

TABLE 5 Multivariable analysis of age, sex, comorbidities, and total 25(OH)D with 28-day mortality[1]

Variable	Regression Coefficient	OR	95% CI	*P* value
Age	0.041	1.04	1.03–1.06	<0.001
Male sex	0.685	1.98	1.22–3.22	0.006
CKD	− 0.345	0.71	0.41–1.22	0.213
Neurological disease	− 0.280	1.35	0.45–1.27	0.288
25(OH)D, 50–74 nmol/L	—	1.00 (ref)	—	0.049
25(OH)D, <25 nmol/L	0.861	2.37	1.17–4.78	0.016
25(OH)D, 25–49 nmol/L	0.626	1.87	0.95–3.67	0.069
25(OH)D, 75–99 nmol/L	0.786	2.19	0.97–4.98	0.060
25(OH)D, ≥100 nmol/L	1.538	4.65	1.51–14.34	0.007

[1]A logistic regression analysis was performed to determine associations between the variables and 28-day mortality. CKD, chronic kidney disease; 25(OH)D, 25-hydroxyvitamin D.
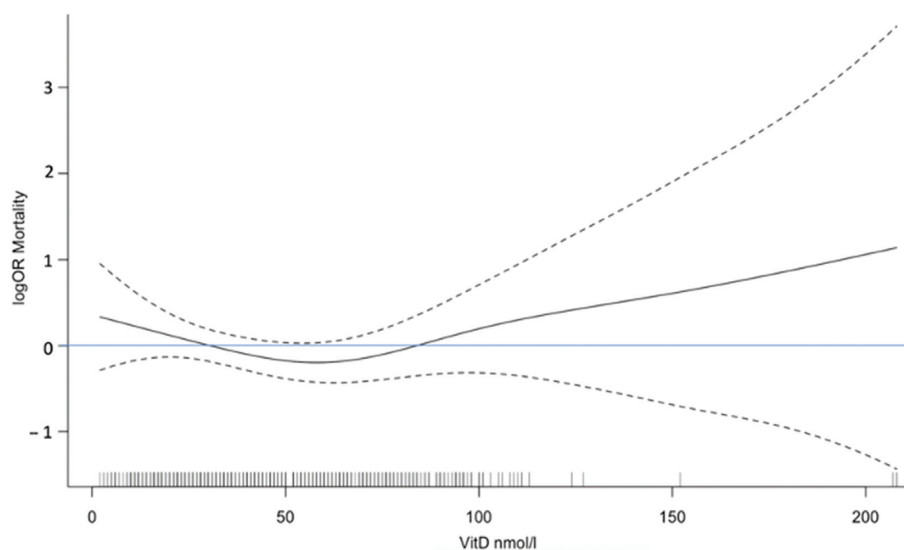
FIGURE 2 Log OR for 28-day mortality according to serum 25(OH)D concentration on admission, compared with mean vitamin D value (47.4 nmol/L) as the reference, adjusted for age and sex, analyzed by logistic regression with cubic spline smoothing, and showing 95% CIs. This analysis, performed with 25(OH)D as a continuous variable, did not show a significant association with mortality from COVID-19. Serum 25(OH)D was measured in 472 individuals with COVID-19, among whom 360 were survivors and 112 were nonsurvivors. COVID-19, coronavirus disease 2019; 25(OH)D, 25-hydroxyvitamin D.

sample size, though considerable, is probably underpowered to detect small differences in overall serum 25(OH)D levels between the groups; thus, according to a retrospective analysis, to detect with statistical significance a difference of 3.5 nmol/l 25(OH)D, as found between survivors and nonsurvivors in this study, would have required over 500 patients per group. Our study was also probably underpowered to evaluate any

interaction between COVID-19 severity, vitamin D levels, and other known risk factors, such as obesity, ethnicity, and the presence of comorbidities such as hypertension, diabetes, and chronic kidney disease. We noted a lower proportion of current smokers in patients who died from COVID-19, although this did not reach statistical significance. This is in keeping with a large UK study that showed a lower risk of COVID-19
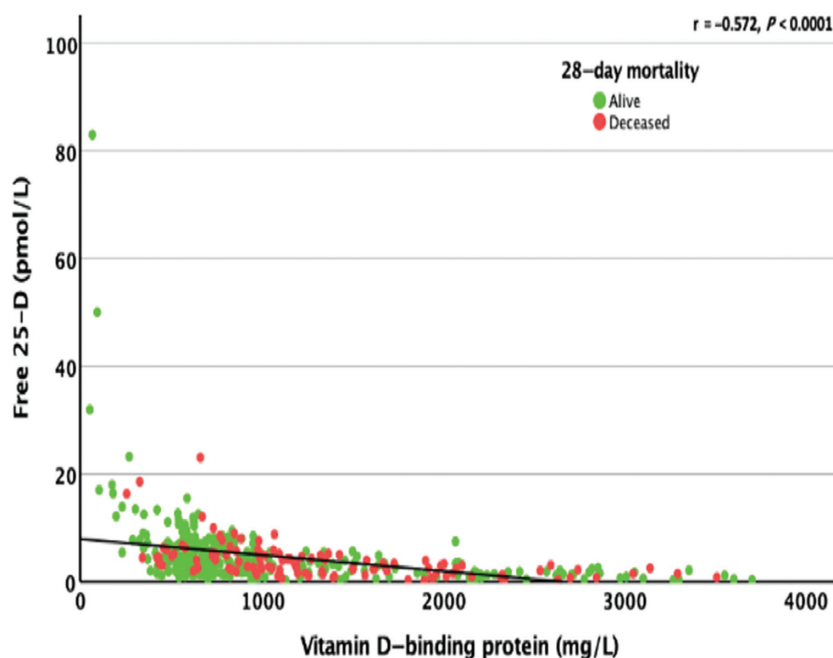

FIGURE 3 Association between serum-free 25(OH)D and DBP concentrations. The correlation between free 25(OH)D and DBP was assessed using the Spearman rank correlation. DBP and free 25(OH)D values were only available for 325/360 (90.3%) of those who were alive and 94/112 (84%) of those who were deceased. DBP, vitamin D–binding protein; 25(OH)D, 25-hydroxyvitamin D.
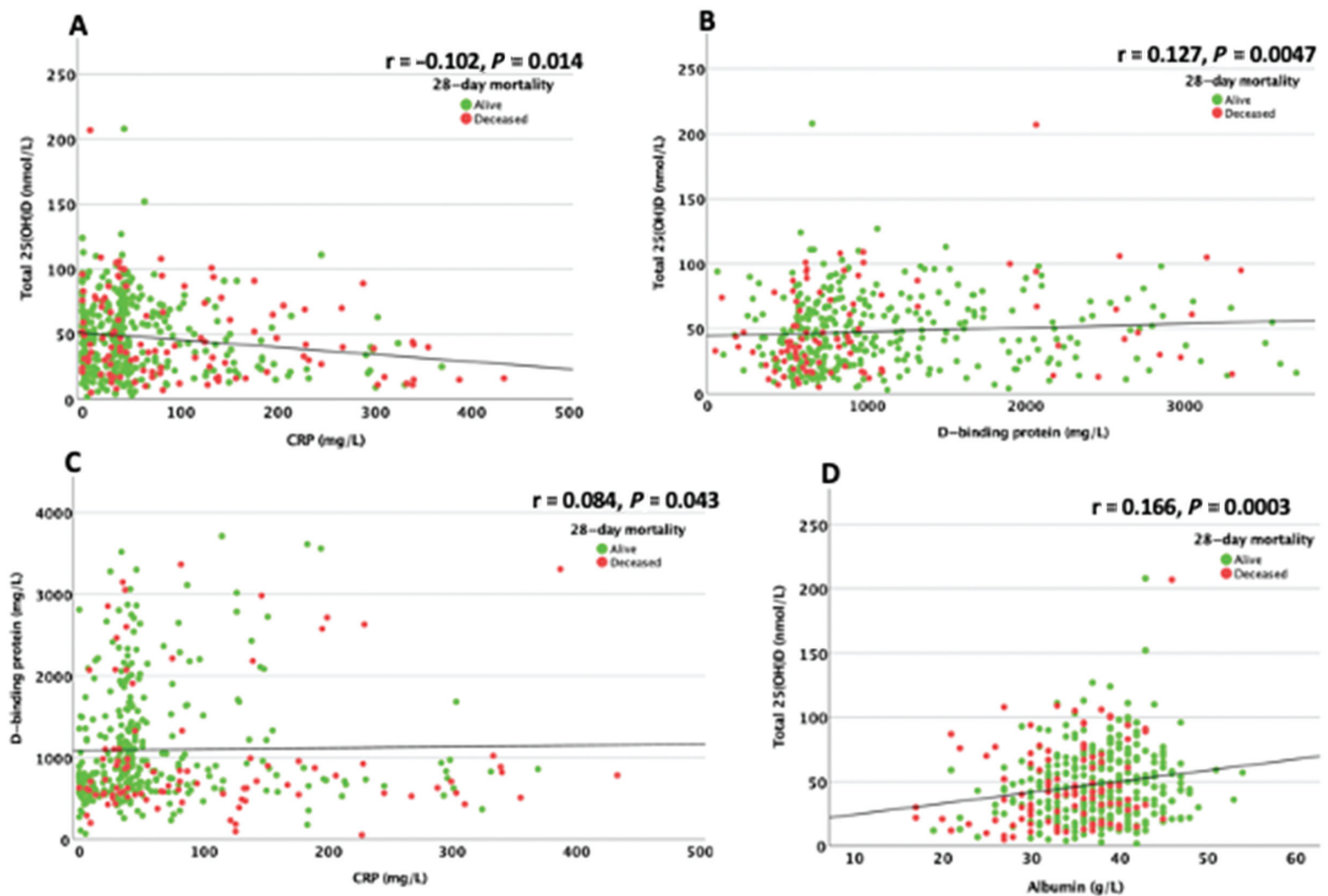
FIGURE 4 Association between serum 25(OH)D and CRP serum albumin and associations between DBP and serum 25(OH)D and CRP. (A) Association between serum 25(OH)D concentrations and baseline CRP. There is only a modest correlation between CRP and total 25(OH)D. The correlation between total 25(OH)D and CRP was assessed using Spearman rank correlation. Both serum 25(OH)D and CRP were measured in 472 individuals with COVID-19, among whom 360 were survivors and 112 were nonsurvivors. (B) Associations between serum 25(OH)D concentrations and serum DBP. The correlation between free 25(OH)D and DBP was assessed using Spearman rank correlation. DBP and free 25(OH)D were only available for 325/360 (90.3%) of those who were alive and 94/112 (84%) of those who were deceased. (C) Association between serum DBP and CRP. The correlation between DBP and CRP was assessed using Spearman rank correlation. DBP measurements were only available for 325/360 (90.3%) of those who were alive and 94/112 (84%) of those who were deceased. (D) Association between serum 25(OH)D and serum albumin. The correlation between 25(OH)D and albumin was assessed using Spearman rank correlation. Both serum 25(OH)D and albumin were measured in 472 individuals with COVID-19, among whom 360 were survivors and 112 were nonsurvivors. COVID-19, coronavirus disease 2019; CRP, C-reactive protein; DRP, vitamin D–binding protein; 25(OH)D, 25-hydroxyvitamin D.

mortality among current smokers (27), though other studies report an opposite association (41). Overall, these differences are likely explained by variations in sample size and patient populations.

In summary, we found no significant relationship between overall 25(OH)D deficiency (<50 nmol/L) and mortality from COVID-19, but there were associations noted when 25(OH)D status was assessed by 5 concentration categories, although these did not retain significance when vitamin D was analyzed as a continuous variable. There are several possible explanations: *1*) there may be no relationship between vitamin D status and COVID-19 outcome; *2*) vitamin D deficiency may impact the risk for hospitalization with COVID-19 but not its subsequent course; and *3*) the relationship between vitamin D status and outcome may be complex, with both low and high 25(OH)D levels negatively impacting outcomes. Further studies, whether interventional or observational, should consider this last possibility as being at least plausible.

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Data Availability

Data described in the manuscript will be made publicly and freely available without restriction at: https://drive.google.com/file/d/10lnd2MKQUhakKBERLpaIyxrz2Du502c2/view?usp=sharing. The code book and analytic code are available from the corresponding author on reasonable request.

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