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

Short title: Vitamin D and COVID-19 mortality

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**Conflict of interest:**  JMR with the University of Liverpool and Provexis UK, holds a patent for use of a soluble fibre preparation as maintenance therapy for Crohn’s disease plus a patent for its use in antibiotic-associated diarrhoea. Patent also held with the University of Liverpool and others in relation to use of modified heparins in cancer therapy. SS has received speaker fees from MSD, Actavis, Abbvie, Dr Falk pharmaceuticals, Janssen, Takeda, Boehringer-Ingelheim, Shire and received educational grants from MSD, Abbvie, Actavis and is an advisory board member for Abbvie, Dr Falk pharmaceuticals, Takeda, Janssen and Vifor pharmaceuticals.

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Abbreviations: 25-hydroxyvitamin D (25(OH)D); COVID-19-coronavirus disease 2019; chronic kidney disease-CKD; C-reactive protein (CRP); vitamin D binding protein-DBP; fibroblast growth factor (FGF); interleukin-IL, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), tumour necrosis factor (TNF)

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.

**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 25(OH)D status hard to interpret. Because of this, measurement of unbound “free” and albumin-bound “bioavailable” 25(OH) D has been proposed.

**Objective:** 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 co-morbidities 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.

**Findings:** Four hundred and seventy-two patients with COVID-19 were included, of whom 112 (23.7%) died within 28 days. Non-survivors were older (mean age 73, [range 34-98]) than survivors (65, [19-95]; P=0.003) and more likely male (67%; P=0.02). The frequency of vitamin D deficiency (25(OH)D <50nmol/L) was similar between non-survivors (71/112 [63.4%]) and survivors (204/360 [56.7%]; P=0.15) but, after adjustment for age, sex and co-morbidities, increased odds for mortality were present in those with severe deficiency (25(OH)D <25nmol/L), OR 2.37 (95% CI 1.17, 4.78), or high 25(OH)D (≥100nmol/L), OR 4.65 (95% CI 1.51, 14.34) compared with 25(OH)D 50-74 nmol/L (reference). Serum DBP levels were not associated with mortality after adjustment for 25(OH)D, age, sex and co-morbidities. Neither free nor bioavailable 25(OH)D were associated with mortality.

**Conclusion:**

Vitamin D deficiency as commonly defined by serum 25 (OH)D levels (<50nmol/L) is not associated with increased mortality from COVID-19 but extreme low (<25nmol/L) and high (>100nmol/L) levels may be associated with risk. Neither free nor bioavailable 25(OH) D associate with risk.

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

vitamin D

**Introduction**

COVID-19, has accounted for nearly 200 million infections and over 4.25 million deaths worldwide as of November 22nd, 2021(1). Factors associated with adverse outcomes include older age, male sex, co-morbidities 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 reduced impact of vitamin D on the immune response (3). A role for vitamin D is further supported by correlations between latitude or ultraviolet B 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 severity of respiratory disease – for example the need for intensive care in infants hospitalized with bronchiolitis(8). Vitamin D deficiency up-regulates pro-inflammatory cytokines such as tumour necrosis factor (TNF), type 1 interferon and interleukin-6 (IL-6) (9, 10). Moreover, genetically modified mice lacking vitamin D receptor show greatly increased susceptibility to lipopolysaccharide-induced experimental lung damage (11).

Various studies have examined associations between vitamin D status and 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 measurement of vitamin D, sometimes 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 (20,21).

Vitamin D status is determined by serum concentration of 25(OH)D and this is largely protein bound, approximately 85% to vitamin D binding protein (DBP) and 15% to albumin. Binding to albumin is less avid and albumin-bound vitamin D is therefore commonly designated “bioavailable”. Both serum DBP(18) and albumin(19) concentrations may fall in illness as a negative acute phase response. Only 0.03% of circulating 25(OH)D is unbound or “free” (20). 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(20). Thus, computational methods which 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(21). Both free 25(OH)D and active 1,25(OH)2D 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)2D(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 two acute hospital sites of the Liverpool University Hospitals NHS Foundation Trust. We included all patients who had surplus sera available for analysis of 25(OH)D concentrations. The population studied were unvaccinated. We collected baseline clinical information including demographic data such as age, sex, body mass index (BMI) and co-morbidities (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 (CPAP) or mechanical ventilation and death within 28 days of SARS-CoV-2 positive 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 severe acute respiratory syndrome coronavirus 2

(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 non-survivor 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.

*Primary outcome:* The primary outcome measure was mortality within 28 days of a positive SARS-CoV-2 test.

*Measurement of serum 25(OH) D and vitamin D binding protein (DBP)*

Serum samples were aliquoted and stored at -80°C until they could be analysed for DBP and total 25(OH)D. Concentrations of DBP were measured using a commercial polyclonal antibody ELISA kit (Immundiagnostik AG (Bensheim, Germany) according to the manufacturer’s protocol. The 25(OH)D2 and 25(OH)D3 concentrations were measured using established in-house liquid chromatography with tandem mass spectrometry (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 bromcresol green based method (Roche Diagnostics, Mannheim) 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 (21):

free vitamin D metabolite = total vitamin D metabolite /1+(Kaalb\*albumin)+(KaDBP\*DBP)

where Ka is the affinity constant. We used values of 7 x 108 M-1 for KaDBP and 6 x 105 M-1 for Kaalb.

Bioavailable 25(OH)D was calculated using the following affinity constants: DBP for 25(OH)D = 7 x 108 M-1, DBP for 1,25(OH)2D = 4 x 107 M-1, albumin for 25(OH)D = 6 x 105 M-1, and albumin for 1,25(OH)2D = 5.4 x 104 M-1 and the following variables: concentration of 25(OH)D, 1,25(OH)2D (assigned 0.1 nM in all cases), DBP and albumin. A MATLAB program implements a system of coupled non-linear algebraic equations for each vitamin D metabolite(21) for extracellular steady state (eSS) free-ligand modeling yielding calculated values for the bioavailable and free 25(OH)D metabolites of interest(21).

*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 interquartile range 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, 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, i.e. <25, 25-49, 50-74, 75-99 nmol/L plus a category with high values ≥100nmol/L plus a group for high values ≥100nmol/L previously shown to associate with increased FGF23 that suppresses vitamin D activation via the 1-alpha-hydroxylase(24-26), with significance determined in the univariate analysis using Chi square goodness-of-fit. Free 25(OH)D, bioavailable 25(OH)D and DBP were categorized into quintiles and similarly examined for association with mortality. All variables identified as being possibly associated with the outcome (P<0.1) were then taken forward into a multivariable model. The two clinical variables that were included in the final model (CKD and neurological diseases) have been previously associated with worse clinical outcomes(27). Two different multivariable models were fitted, one with vitamin D as a continuous variable and the other with vitamin D as a categorised variable. A further analysis was performed by logistic regression but with vitamin D as a continuous rather than categorised variable, again with adjustment 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, Armonk, NY: 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, 18 March to 2 November 2020. We only included patients who had surplus sera available for 25(OH)D analysis (for patient flowchart see online supplementary figure 1). The 28-day mortality rate was 23.7%. The mean age at death was 73 years (SD 19) and 28-day mortality rate was 28.0% and 18.5% among men and women respectively. Univariate analysis showed a significant association between increased risk of mortality by 28 days and older age, male sex, and pre-existing neurological disease (Table 1). There were no significant associations found with BMI, ethnicity, and other co-morbidities (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, 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 <50nmol/L) in the cohort was 58.1% while 25.0% were severely deficient (25(OH)D <25nmol/L). There was no significant difference between 25(OH)D concentration amongst deceased (median 39.5 nmol/L, IQR 48.5) compared with survivors (43.0 nmol/L, IQR 41.0; P=0.11) (Table 4). Unadjusted, there was also no significant difference between frequency of 25(OH)D vitamin D deficiency (25(OH)D <50nmol/L) amongst those who died (71/112 [63.4%]) compared with survivors (204/360 [56.7%]; P=0.15). Total 25(OH)D, analysed by five concentration categories according to the different definitions of sufficiency (<25, 25-49, 50-74, 75-99 nmol/L plus a group for high values ≥100nmol/L previously shown to associate with increased FGF23 (24-26)), did however suggest an association with 28-day mortality with lowest mortality for those with 25(OH)D 50-74nmol/L (P=0.04; Table 4 and Figure 1). This pattern remained after excluding patients who received prescribed vitamin D supplementation (Supplementary Figure 2). A similar analysis but with 25(OH)D as continuous variable was not significant. There was no significant association between free or bioavailable 25(OH)D and mortality (Table 4).

Multivariable analysis was therefore performed to investigate the associations between mortality and 25(OH)D concentration ranges with adjustment for age, sex and co-morbidities. This showed increased mortality in those with 25(OH)D <25nmol/L (odds ratio (OR) 2.37 (95% CI, 1.17, 4.78, p=0.016)) and in those with 25(OH)D >100 nmol/L (OR 4.65 (95% CI 1.51, 14.34, p=0.007)) compared with 25 (OH)D 50-74 nmol/L, 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 numbers with 25(OH)D >100nmol/l were small (n=18). 25(OH)D levels between 25-49 and 75-99 nmol/L were not associated with significant differences in mortality compared with 50-74 nmol/L (Table 5). Logistic regression analysis of mortality, with adjustment for age and sex, but with vitamin D set as 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 un-associated with mortality in an age, sex and co-morbidity adjusted model (Supplementary Tables 1 and 2).

*DBP and mortality*

On univariate analysis, median DBP concentrations were lower in patients who died (P=0.005, Table 4 and Figure 3). However, multivariable analysis with adjustment for 25(OH)D, age, sex, and significant co-morbidity did not show an association between DBP and mortality (Supplementary Table 3).

*Association 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 DBP concentration and free 25(OH)D concentration was significant (Spearman’s rho=-0.572) but only sizeable at very low concentrations of DBP (<500mg/L) (Figure 3). There was no significant relationship between free 25(OH)D and serum albumin concentrations (Spearman’s rho=0.048, Supplementary Figure 3).

Serum 25(OH)D concentrations correlated with baseline serum CRP (Spearman’s rho=-0.102,) in keeping with a negative acute phase response but with a modest size effect – mean 25(OH)D falling from approximately 50nmol/L at CRP <5mg/L to approximately 40nmol/L at CRP 200mg/L (Figure 4A). 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 observed for DBP association, falling from a mean 25(OH)D concentration of approximately 50nmol/L at albumin 40g/L to approximately 40nmol/L at albumin 30g/L (Figure 4D). There was also an association between serum albumin concentration and baseline CRP (Spearman’s rho=-0.212, Supplementary Figure 4). However, the difference in serum albumin concentrations between survivors (median 38 g/L, IQR-7) and non-survivors (median 35 g/L, IQR-8) although significant was only modest (Table 2).

*Vitamin D supplementation and mortality*

Prescribed vitamin D supplements were being taken by 131 (27.8%). Data were not available for use of supplements purchased “over the counter”. Mean age of death was significantly higher (80.2 years, SD 11.9) amongst those taking prescribed supplements compared with those not taking prescribed supplements (71.1, SD 12.0; P<0.001) but the average age of all people taking prescribed supplements (71.5 years, SD 16.0) was greater than those not taking prescribed supplements (62.0 years, SD 18.1; p<0.0001) and analysis of 28-day mortality did not show significant association with prescribed supplements after adjustment for age (OR 0.67, 95% CI 0.40, 1.12; P=0.12). There was an association between prescribed supplements usage and 25(OH)D concentration (supplement usage 0% in ≤25nmol/L; 23% 25-49nmol/L; 32% 50-74nmol/L; 59% 75-99nmol/L; 53% ≥100nmol/L).

**Discussion**

In this retrospective single centre cohort study, we confirm that older age and male sex are associated with increased 28-day mortality from COVID-19, consistent with other studies (30-32). We found no significant relationship between overall 25(OH)D deficiency (<50nmol/L) and mortality from COVID-19. However, when 25(OH)D values on admission were categorised according to the varying definitions of deficiency (<25, <50, <75 nmol/l) plus an additional category for “high” values >100nmol, multivariable analysis, adjusted for age, sex, and comorbidities, showed increased mortality amongst those with very low (<25nmol/l) or high (>100nmol/l) serum 25(OH)D compared with with 25 (OH)D 50-74 nmol/L, selected as reference since it approximates the typical range for 25(OH)D seen from June to December in healthy British adults (30). The increased mortality in patients with serum 25(OH)D <25nmol/L is consistent with that reported in some other studies (15, 31) but patients with high serum 25(OH)D concentration ≥100nmol/L also had an increased mortality in our cohort. This needs to be interpreted with caution though since numbers were small (n=18) in the highest (>100nmol/l) category. Moreover, 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 versus sufficient, 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 significantly increased likelihood of needing oxygen support in patients with 25(OH)D <50nmol/L(16); a Brazilian study showed a non-significant 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 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 adjustment 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, average age 51 followed over 3 years, confirmed a U-shaped association with lowest all-cause mortality again seen for those with 25(OH)D 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 ≥60ng/ml, equivalent to 150nmol/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 men at 25(OH)D >72nmol/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 standardised serum 25(OH)D measurements in 26,916 individuals from eight prospective European studies showed lowest all-cause mortality for 25(OH)D 75-100nmol/l but with no significant increase in mortality at levels >100nmol/l(36). There is a biologically plausible explanation for the U-shaped curve: the phosphaturic hormone fibroblast growth factor 23 (FGF23) is typically induced when 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: (a) 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 (b) serum concentrations of free 25(OH)D are more than 10,000-fold lower than total 25(OH)D, while bioavailable, i.e. 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, free and bioavailable 25(OH)D. Importantly, vitamin D levels were measured contemporaneously unlike 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 sample size, though considerable, is probably underpowered to detect small differences in overall serum 25(OH)D levels between the groups, thus to detect with statistical significance a difference of 3.5nmol/l 25(OH)D as found between survivors and non-survivors in this study would, according to retrospective analysis, 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 presence of co-morbidities 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 which showed a lower risk of COVID-19 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 population.

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

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**Table 1: Clinical characteristics of included subjects**

|  |  |  |  |
| --- | --- | --- | --- |
| **Variable** | **Alive** | **Deceased** | **P value** |
| N | 360 | 112 |  |
| Age Mean (SD)  Range | 65 (26)  19 - 95 | 73 (19)  34 - 98 | 0.003 |
| Sex Female  Male | 167 (46.4%)  193 (53.6%) | 37 (33.0%)  75 (67.0%) | 0.02 |
| BMI kg/m2 Mean (SD) | 27 (9) | 26 (8) | >0.999 |
| BMI Underweight  Normal  Overweight  Obese  Not recorded | 25 (6.9%)  97 (26.9%)  74 (20.6%)  110 (30.6%)  54 (15%) | 12 (10.7%)  30 (26.7%)  22 (19.6%)  33 (29.5%)  15 (13.4%) | 0.627 |
| Ethnicity Black and minority  White  Not recorded | 41 (11.3%)  302 (83.9%)  17 (4.7%) | 12 (10.7%)  94 (83.9%)  6 (5.3%) | >0.999 |
| DM No  Yes | 270 (75.0%)  90 (25.0%) | 77 (69.8%)  35 (30.2%) | 0.22 |
| Hypertension No  Yes  Not recorded | 203 (56.4%)  156 (43.6%)  1 (0.2%) | 55.(49.1%)  57 (50.9%) | 0.19 |
| CKD No  Yes  Not recorded | 301 (83.8%)  58 (16.2%)  1 (0.2%) | 77 (68.8%)  25 (31.2%) | 0.06 |
| IHD No  Yes  Not recorded | 294 (81.9%)  65 (18.1%)  1 (0.2%) | 88 (78.6%)  24 (21.4%) | 0.49 |
| Smoking status: Non-/Ex-smoker  Current smoker  Not recorded | 311 (86.4%)  39 (10.8%)  10 (2.8%) | 99 (88.4%)  5 (4.5%)  8 (7.1%) | 0.113 |
| Respiratory No  Yes | 256 (71.1%)  104 (28.9%) | 83 (74.1%)  29 (25.9%) | 0.576 |
| Neurological No  Yes  Not recorded | 291 (81.1%)  68 (18.9%)  1 (0.2%) | 76 (67.9%)  36 (32.1%) | 0.003 |
| Liver disease. No  Yes  Not recorded | 339 (94.4%)  20 (5.6%)  1 (0.2%) | 103 (92.0%)  9 (8.0%) | >0.999 |
| Active malignancy  No  Yes  Not recorded | 330 (91.9%)  29 (8.1%)  1 (0.2%) | 102 (91.1%)  10 (8.9%) | 0.84 |

BMI=Body mass index, CKD=Chronic kidney disease, DM=Diabetes mellitus, IHD=Ischemic heart disease, SD-standard deviation. Univariate analysis was performed to compare variables across alive and deceased categories using chi-squared test for categorical variables and Mann-Whitney U test for continuous variables. Categorical data are represented as counts and percentages.

**Table 2: Biochemical and radiological parameters among survivors and deceased. Figures are based on values at hospital admission.**

|  |  |  |  |
| --- | --- | --- | --- |
| **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/1.73m2)  ≤ 90  >90 | 220 (62.0%)  135 (38.0%) | 85 (77.3%)  25 (22.7%) | 0.003 |
| CXR changes No  Yes | 63 (42.6%)  85 (57.4%) | 7 (18.0%)  32 (82.0%) | 0.005 |
| CT changes No  Yes | 4 (10.8%)  33 (89.2%) | 4 (100.0%) | >0.999 |

CRP-C-reactive protein. eGFR-estimated glomerular filtration rate, CXR-Chest X-ray, CT-computed tomography, IQR-inter-quartile range, ALT-alanine aminotransferase. COVID-19 changes on chest X-ray or CT scan were considered present on any imaging during hospital admission. Univariate analysis was performed to compare variables across alive and deceased categories using chi-squared test for categorical variables and Mann-Whitney U test for continuous variables. Categorical data are represented as counts and percentages and continuous variables as median and inter-quartile range.

**Table 3: Treatments received by Covid-19 patients**

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

ICU-Intensive care unit, CPAP-continuous positive airways pressure. Univariate analysis was performed to compare variables across alive and deceased categories using chi-squared test for categorical variables and Mann-Whitney U test for continuous variables. Categorical data are represented as counts and percentages and continuous variables as median and inter-quartile range.

**Table 4: Levels of total 25(OH)D, D-binding protein (DBP), free and bioavailable 25(OH) D among Covid-19 patients.**

|  |  |  |  |
| --- | --- | --- | --- |
| **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 (<50nmol/L) (N,%) | 204 (56.7%) | 71 (63.4%) | 0.15 |
| 25(OH)D by categories: |  |  | 0.04 |
| <25 nmol/L (number of patients (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 |
| Free 25(OH)D, 0-1.55 pmol/L, N (%) | 66 (20.4%) | 19 (21.1%) |  |
| Free 25(OH)D, 1.56-2.68 pmol/L, N (%) | 68 (21.0%) | 12 (13.3%) |  |
| Free 25(OH)D, 2.69-4.10 pmol/L, N (%) | 67 (20.7%) | 18 (20.0%) |  |
| Free 25(OH)D, 4.11-6.11 pmol/L, N (%) | 64 (19.8%) | 17 (18.9%) |  |
| Free 25(OH)D, >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 |
| Bioavailable 25 (OH)D, 0-0.459 nmol/L, N (%) | 62 (19.1%) | 20 (22.2%) |  |
| Bioavailable 25 (OH)D, 0.46-0.81 nmol/L, N (%) | 70 (21.6%) | 12 (13.3%) |  |
| Bioavailable 25 (OH)D, 0.82-1.3 nmol/L, N (%) | 64 (19.8%) | 23 (25.6%) |  |
| Bioavailable 25 (OH)D, 1.31-2.037 nmol/L, N (%) | 64 (19.8%) | 16 (18.9%) |  |
| Bioavailable 25 (OH)D, >2.038 pmol/L, N (%) | 64 (19.8%) | 19 (21.1%) |  |
| D-binding protein (DBP) (mg/L), median(IQR) | 858 (707.8) | 707 (441.0) | 0.005 |
| DBP by quintile categories: |  |  | 0.003 |
| DBP (0-579.9 mg/L) | 66 (20.3%) | 21 (22.3%) |  |
| DBP (580-733.6 mg/L) | 69 (21.2%) | 16 (17.0%) |  |
| DBP (733.61-960.4 mg/L) | 51 (15.7%) | 30 (31.2%) |  |
| DBP (960.41-1615 mg/L | 74 (22.8%) | 10 (11.9%) |  |
| DBP (>1615 mg/L) | 65 (20.0%) | 17 (18.1%) |  |

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. Univariate analysis was performed using chi-squared goodness of fit test. Categorical data are represented as counts and percentages and continuous variables as median and inter-quartile range.

Levels of total 25(OH)D were categorized based on published definitions of adequacy and free 25(OH)D, bioavailable 25(OH)D and D-binding protein (DBP) were categorized as quintiles.

**Table 5: Multivariable analysis of age, sex and co-morbidities and total 25(OH)D with**

**28-day mortality**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Variable** | **Regression**  **co-efficient** | **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 (25-49nmol/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 |

OR-odds ratio, CKD-Chronic kidney disease, CI-confidence intervals. Logistic regression analysis was performed to determine associations between the variables and 28-day mortality.

**Figure legends:**

Figure 1

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 who 360 were survivors and 112 were non-survivors.

Figure 2

Log odds ratio for 28-day mortality according to serum 25(OH)D concentration on admission, compared with mean vitamin D (47.4nmol/L) as reference, adjusted for age and sex, and analysed by logistic regression with cubic spline smoothing and showing 95% confidence intervals. This analysis, performed with 25(OH)D as 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 who 360 were survivors and 112 were non-survivors.

Figure 3

Association between serum free 25(OH)D and D binding protein (DBP) concentrations. 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.

Figure 4

Associations between serum 25(OH)D and CRP serum albumin and associations between D-binding protein and serum 25(OH)D and CRP

Panel A: Association between serum 25(OH)D concentrations and baseline C-reactive protein (CRP). It can be seen that there is only a modest correlation between CRP and total 25(OH)D. 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 who 360 were survivors and 112 were non-survivors.

Panel B: Association between serum 25(OH)D concentrations and serum D-binding protein (DBP). 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.

Panel C: Association between serum DBP and CRP. 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.

Panel D: Association between serum 25(OH)D and serum albumin. 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 who 360 were survivors and 112 were non-survivors.