ORIGINAL ARTICLE

Association between Low Vitamin D Levels and Key Characteristics of COVID-19 Patients: A Retrospective Cross-sectional Study

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ABSTRACT

Introduction: Vitamin D deficiency associated with COVID-19 patients has recently garnered interest. This is likely due to the elderly population who are most commonly affected by COVID-19. In this study, we investigated the association of vitamin D levels with the clinico-demographical and laboratory characteristics of COVID-19 patients. **Methods:** We recruited 77 COVID-19 patients who were admitted to Hospital Pengajar Universiti Putra Malaysia (HPUPM) from January 2022 until February 2023. Their clinico-demographic data were retrieved, and serum vitamin D and C-reactive protein (CRP) immunoassays were conducted. The vitamin D levels of each patient were categorized as normal (≥50 nmol/L) or low (<50 nmol/L). Statistical comparisons of the patients' clinico-demographic parameters with vitamin D levels were conducted. **Results:** In univariable analysis of categorical variables, significantly higher proportion of female COVID-19 patients presented with low serum vitamin D levels compared with male COVID-19 patients (p=0.045; 85.3% vs 65.1%). Pertaining to continuous variables, younger COVID-19 patients demonstrated significantly higher prevalence of low vitamin D levels (p=0.040; 45.58 vs 54.90 years old). COVID-19 patients with lower CRP levels also demonstrated significantly higher proportion of low vitamin D levels (p=0.046; 35.70 vs 60.92 mg/dl). These three parameters (i.e. gender, age, and CRP levels) were included in the multivariable logistic regression analysis to determine which factor(s) remained significantly associated with low vitamin D levels. All three parameters did not show significance in the multivariable analysis. **Conclusion:** Absence of statistical significance in the multivariable analysis indicates that the individual associations between age, gender, and CRP levels with low vitamin D levels are not independent of each other. These suggest underlying interactions between these factors that influence their relationships with vitamin D levels, and further studies are required to clarify such interactions in COVID-19 patients.
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Keywords: COVID-19, Vitamin D insufficiency, Vitamin D deficiency, C-Reactive Protein (CRP), Body mass index (BMI).

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INTRODUCTION

Vitamin D is a vital regulator of innate immune responses whereby it suppresses the expression of pro-inflammatory cytokines and promotes antiviral mediators production, highlighting its roles in antiviral innate defense (1). Vitamin D induces the production of antimicrobial proteins, including defensin β2 and cathelicidin, by neutrophils and macrophages (1,2). In adaptive immunity, vitamin D modulates the activities of T cells by suppressing the production of cytokines required for T cells proliferation and recruitment (3). Vitamin D also shows inhibitory effects on B cells whereby it represses proliferation and differentiation of B cells (4). Collectively, vitamin D appears to control overactive inflammatory responses in the human body.

Respiratory infections are countered by vitamin D whereby its supplementation reduces the risk of acute respiratory infections (ARI) as demonstrated by meta-analysis studies (5). Vitamin D deficiency has been

shown to be a risk factor for pneumonia (6), and vitamin D supplementation has been proposed for patients with ARI to reduce their incidence and severity (7). In terms of COVID-19, the virus mainly affects the respiratory system, thus the association between low vitamin D levels and ARI may affect both the incidence and severity of COVID-19 cases. Indeed, several lines of evidence have shown that reduced vitamin D levels is associated with increased incidence and poor prognosis of COVID-19 patients with acute respiratory failure or pneumonia attributable to the anti-inflammatory properties of vitamin D (8-11). Taken together, these findings suggest the important roles that vitamin D play against respiratory infections.

Nonetheless, there have also been reports that vitamin D levels are not associated with the outcomes or severity of COVID-19 patients (12-14). Essentially, most observational studies on vitamin D deficiency or insufficiency and COVID-19 susceptibility and severity demonstrate a high risk of bias and heterogeneity (15). Therefore, this retrospective cross-sectional study was conducted to determine the relationship between vitamin D levels and the clinico-demographical and laboratory characteristics of COVID-19 patients.

MATERIALS AND METHODS

Sample Size Calculation

According to published retrospective cross-sectional studies on COVID-19 patients and their vitamin D levels (16,17), sample size calculation was performed in accordance with the difference of means between two independent groups conducted using G*Power software (version 3.1). The α-error probability of 0.05, two-tailed hypothesis, effect size of 0.9, power (1-β error probability) of 0.95, allocation ratio (N2/N1) of 1 and dropout rate of 10% were utilized. This produced a total sample size of at least 72 participants, and any additional case was included to increase the statistical power of the study. The large effect size used in this study was comparable with other exploratory studies that also adopted the effect size of 0.9 in their sample size calculation. Specifically, these studies assessed the relationship between vitamin D levels or bone health outcomes including vitamin D levels in patients with distal radius fractures (18), bone density and geometry in post-stroke patients (19), and bone turnover in postmenopausal women (20). The aim of our study was to compare the mean vitamin D levels between two different groups of COVID-19 patients i.e., patients with normal vs low vitamin D levels. Owing to such comparison, a sample size calculation according to the difference in means was thus adopted in this study.

Patient Recruitment

In this retrospective cross-sectional study, a total of 77 COVID-19 patients admitted to Clinical Research Ward in Hospital Pengajar University Putra Malaysia (HPUPM) were included in this study. Convenience sampling method was adopted in participants' selection. Eligibility criteria include patients aged above 18 years old, those tested positive for COVID-19 by RT-PCR, and patients with duration of illness of less than 14 days. Such duration of illness was chosen due to the first two weeks following COVID-19 symptom(s) onset are the acute phase of the disease, and our study aimed to examine the risk factors associated with acute phase instead of the recovery phase of COVID-19 (21-23). Pregnant and lactating women, individuals tested negative by RT-PCR, and those with blood dyscrasias such as sickle cell anemia or thalassemia were excluded from the study.

The clinico-demographic characteristics of the patients were retrieved from the unit records as follows:
(1) Demographic data: Age, gender, ethnicity and smoking status
(2) Clinical data: Comorbidities including diabetes mellitus, hypertension, obesity, chronic obstructive pulmonary disease (COPD), bronchial asthma, cancer, chronic kidney disease (CKD), ischemic heart disease (IHD), and other comorbidity.
(3) Laboratory results : C-reactive protein (CRP) and serum vitamin D levels

Ethical clearance

This study was reviewed and approved by the Human Research Ethics Committee, Universiti Putra Malaysia (approval registration number: UPM/JEPeM/18040213).

Serum Vitamin D Immunoassay

Serum vitamin D concentrations were analyzed from venous blood samples that were collected in EDTA tube. Serum vitamin D concentrations were measured by chemiluminescence immunoassay using an automated analyzer (UniCel Dxl 800, Beckman Coulter Inc., USA).

Serum C-reactive protein Immunoassay

Serum C-reactive protein level were analyzed from venous blood samples that were collected in EDTA tube. Serum C-reactive protein were measured by chemiluminescence immunoassay .and laser nephelometry. Serum C-reactive protein was defined as normal when the level was < 10 mg/L and elevated when it was more than 10 mg/L.

Covid-19 rt-PCR

SARS CoV 2 RNA detection using real time RT PCR Allplex™ 2019-nCoV Assay (Seegene, Korea), which targets envelope gene (E) of Sarbecovirus, and RNAdependent RNA polymerase (RdRp) and nucleocapsid (N) genes of SARS-CoV-2, was used for SARS-CoV-2 RNA detection according to the manufacturer's instructions. 8 µL of extracted RNA was added to 5 µL of 5X Real-time One-step Bufer, 5 µL of 2019-nCoV MuDT Oligo Mix (2019-nCoV-MOM), 2 µL of Real-time One-step Enzyme, and 5 µL of RNase free water. The CFX-96 real-time thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for

amplifcation. The conditions consisted of 1 cycle of 20 min at 50 °C, 1 min at 95 °C and followed by 45 cycles of 15 s at 94 °C, 30 s at 58 °C. The result was analysed using Seegene Viewer (Seegene, Korea), in which a cycle threshold value (Ct value) < 40 for all three target genes was defined as positive result.

Statistical Analysis

For all analyses, categorical variables were presented as number and percentage, and continuous variables were presented as mean and standard deviation (SD). Vitamin D was categorized as normal (≥50 nmol/L), or low i.e. insufficient (25-50 nmol/L) or deficient (<25 nmol/L) grouped together in accordance with past studies (24,25). For all tests, two-tailed p<0.05 was considered statistically significant. Normally distributed data were compared through a one-way analysis of variance (ANOVA), t-test, and categorical data were compared using the Pearson's Chi-squared (χ^2) test. Variables with a significant p-value of <0.05 in univariable analysis were selected for multivariable logistic regression analysis. All analyses were performed using SPSS v24 (SPSS Inc., Chicago, IL, USA).

RESULTS

Clinico-demographical and Laboratory Characteristics

In this cohort of COVID-19 patients, the mean age of the patients was 48 years old (SD: 17.17). Majority of the patients were Malay (n=72/77; 93.5%), with a slight preponderance of males at 55.8% (n=43/77) (Table I). Nearly half of the patients were non-smoker (n=36/77; 46.8%) and in terms of BMI, approximately one-third of the patients were obese (n=27/74; 38%). For defined comorbidities, hypertension was the most frequent (n=33/77; 42.9%) followed by diabetes mellitus (n=32/77; 41.6%), obesity (n=27/74; 38%), CKD (n=11/77; 14.3%), IHD (n=4/77; 5.2%), bronchial asthma (n=2/77; 2.6%), COPD or cancer (n=1/77 each; 1.3%).

In terms of COVID-19 clinical presentation, most of the patients were symptomatic (n=43/77; 55.8%). Majority of our cohort of COVID-19 patients presented with mild disease course i.e. category 3 and below (n=70/77; 90.9%), and the remaining seven patients (9.1%) presented with category 4 while no patient presented with category 5 COVID-19. With regards to laboratory paramters, mean value of CRP for all patients was 42.18 ± 45.50 mg/dl with majority of the patients demonstrated high CRP levels (>10 mg/dl) (n=54/77; 73%). For serum vitamin D levels (mean: 40.75 nmol/L; SD: 19.56 nmol/L), majority of the patients showed low vitamin D levels (<50 nmol/L) (n=55/77; 71.4%).

Association of Low Vitamin D Levels with Clinico-demographical and Laboratory Characteristics of COVID-19 Patients

In terms of the categorical variables potentially

Table I: Clinico-demographical and laboratory characteristics of the COVID-19 patients in this study (n=77).

Variables	Mean ± SD or n (%)
Age (years)	48 ± 17.17
<45	35 (32.1)
>45	42 (61.3)
Gender	
Male	43 (55.8)
Female	34 (44.2)
Ethnicity	
Malay	72 (93.5)
Chinese	3 (3.9)
Indian	2 (2.6)
Smoking status	
Active	25 (32.4)
Non-smoker	36 (46.8)
Ex-smoker	16 (20.8)
BMI (kg/m ^{2})*	29.03 ± 7.50
Underweight	3 (4.2)
Normal	20 (28.2)
Overweight	21 (29.6)
Obese	27 (38.0)
Comorbidity	
Diabetes mellitus	32 (41.6)
Hypertension	33 (42.9)
Obesity	27 (35.1)
COPD	1 (1.3)
Bronchial asthma	2 (2.6)
Cancer	1 (1.3)
CKD	11 (14.3)
IHD	4 (5.2)
Others	37 (48.1)
COVID-19 clinical presentation	
Asymptomatic	34 (44.2)
Symptomatic	43 (55.8)
COVID-19 category	
Category 1	2 (2.6)
Category 2a	14 (18.2)
Category 2b	18 (23.4)
Category 3a	16 (20.8)
Category 3b	20 (26.0)
Category 4a	2 (2.6)
Category 4b	5 (6.5)
Category 5	0 (0)
CRP (mg/dl) #	42.18 ± 45.50
Normal: <1	2 (2.7)
Elevated: 1-10	18 (24.3)
High: >10	54 (73.0)
Vitamin D (nmol/L)	40.75 ± 19.56
Low	55 (71.4)
Normal	22 (28.6)

*A total of 71 patients had BMI data. #A total of 74 patients had CRP levels data.
Notes: The sum of some of the parameters' percentages may not total 100 due to rounding.

associated with low vitamin D levels (i.e. deficiency and insufficiency) among the COVID-19 patients, female patients were significantly associated with low vitamin D levels compared with male patients (p=0.045; 85.3% vs 65.1%) (Table 2). The rest of the examined categorical variables were not significantly associated with vitamin D levels including ethnicity, smoking status, COVID-19 clinical presentation, COVID-19 categories, and comorbidities (diabetes mellitus, hypertension, COPD, bronchial asthma, cancer, CKD, IHD, other comorbidity) (Table II).

Regarding the potential association of continuous variables with serum vitamin D levels, younger age (p=0.040; mean age: 45.58 vs 54.90 years old) and lower CRP levels (p=0.046; mean levels: 35.70 vs 60.92 mg/dl) were significantly associated with low serum vitamin D levels in this cohort of COVID-19 patients. No significant association with vitamin D levels was observed for other continuous variables i.e. height, weight, and BMI (Table III).

The abovementioned categorical (gender) and continuous (age and CRP levels) variables that showed significance in univariable analysis were subsequently selected for multivariable logistic regression analysis. BMI was also included in the multivariable regression model due to the impact of body adiposity on vitamin D status. None of these variables demonstrated significance in the multivariable analysis even though female patients yielded the highest hazard ratio (HR) with higher upper bound of 95% confidence interval (CI) (p=0.064; HR: 3.82; 95% CI: 0.93-15.76) compared with the other two variables i.e. younger age (p=0.272; HR: 1.02; 95% CI: 0.98-1.06), lower CRP levels (p=0.142; HR: 1.01; 95% CI: 1.00-1.03), and lower BMI (p=0.424; HR: 0.97; 95% CI: 0.88-1.05) (Table IV).

Table II: Comparison of categorical variables with serum vitamin D levels in COVID-19 patients.

Variables	Vitamin D levels		*p*-value
	Normal (%)	Low (%)	
Gender			
Female	5 (14.7)	29 (85.3)	**0.045**
Male	15 (34.9)	28 (65.1)	
Ethnic			
Malay	18	54	
Chinese	2	1	0.219
Indian	0	2	
Smoking status			
Active smoker	7	18	
Non-smoker	6	30	0.116
Ex-smoker	7	9	
COVID-19 clinical presentation			
Asymptomatic	9	25	0.929
Symptomatic	11	32	
COVID-19 categories			
Categories 1-2	9	25	0.929
Categories 3-4	11	32	
Diabetes mellitus			
Yes	7	25	0.489
No	13	32	
Hypertension			
Yes	10	23	0.453
No	10	34	
Chronic obstructive airway disease (COAD)			
Yes	1	0	0.260
No	19	57	
Bronchial asthma			
Yes	0	2	>0.999
No	20	55	
Cancer			
Yes	0	1	>0.999
No	20	56	
Chronic kidney disease (CKD)			
Yes	1	10	0.271
No	19	47	
Ischaemic heart disease (IHD)			
Yes	2	2	0.276
No	18	55	
Other comorbidity			
Yes	11	26	0.470
No	9	31	

DISCUSSION

In this study, we demonstrated that low vitamin D levels were significantly associated with females in univariable analysis but this was insignificant in multivariable analysis. This indicates that more women had lower vitamin D levels than men but association of lower vitamin D levels with females was not present in our cohort of patients. This observation is comparable with an independent study demonstrating that low vitamin D levels were more prevalent among female than male COVID-19 patients (26). There are several factors that may contribute to higher prevalence of female COVID-19 patients with low vitamin D levels such as reduced exposure to sunlight. Individuals with COVID-19 could be housebound, which might limit their exposure to sunshine and cause reduced vitamin D levels (8) .

As Malaysia has a significant Muslim population, many Muslim women wear fully or near-fully covered clothing that reduces direct skin exposure to sunlight. This leads to reduced vitamin D synthesis by the skin, causing a higher susceptibility to lower vitamin D levels. These observations are supported by past studies in which Muslim women wearing hijab demonstrated significantly lower levels of serum vitamin D compared with women wearing western clothes (27). Moreover, women

Table III: Association of continuous variables with serum vitamin D levels in COVID-19 patients.

Variables	Vitamin D levels		*p*-value
	Normal	Low	
Age (years)	54.90 (17.76)	45.58 (16.43)	**0.040**
Height (cm)	164.71 (8.52)	162.18 (12.31)	0.429
Weight (kg)	74.54 (17.82)	77.53 (22.22)	0.610
BMI (kg/m²)	27.57 (6.95)	29.50 (7.68)	0.358
CRP (mg/dl)	60.92 (64.18)	35.70 (35.49)	**0.046**

All values expressed as mean ± SD

Table IV: Variables associated with low vitamin D levels in COVID-19 patients according to multivariable logistic regression analysis.

Variables	B coefficient	Hazard ratio (95% confidence interval)	*p*-value
Age (younger age)	0.022	1.02 (0.98-1.06)	0.272
Gender (female)	1.341	3.82 (0.93-15.76)	0.064
CRP (lower levels)	0.011	1.01 (1.00-1.03)	0.142
BMI (lower BMI)	-0.036	0.97 (0.88-1.05)	0.424

wearing fully concealing clothes also demonstrated a higher prevalence of lower vitamin D levels compared with their non-fully concealing counterparts (28).

Vitamin D is a fat-soluble vitamin that can be stored in the body's fat cells. Multiple studies have shown that body fat content could affect the levels of vitamin D (29). In particular, vitamin D can be stored and trapped for extended periods in body's fat cells, leading to decreased serum vitamin D levels (30,31). Females usually present with a higher percentage of body fat than males (32,33). This may cause more vitamin D to be stored in body fat tissues, causing decreased serum vitamin D levels. This represents another potential explanation for the higher prevalence of female COVID-19 patients with lower vitamin D levels.

Skin color, which is linked to ethnicity, is another factor that influences vitamin D levels. Darker skin contains more melanin which functions to block ultraviolet rays, but this also leads to reduced vitamin D synthesis by the skin. Hence, individuals with darker skin present with higher risk of lower vitamin D levels (34,35). As most Malaysians have a generally darker skin tone due to the tropics location, this may present another factor that contributes to lower vitamin D levels among our cohort of COVID-19 patients. Interestingly, there have been reports that darker skin represents a risk factor for increased COVID-19 disease severity, although these reports are based on correlation instead of direct causation (36,37).

In this study, it was demonstrated that there was no significant relationship between the categories 1-2 vs categories 3-4 of COVID-19 patients and their vitamin D status. This finding could potentially be explained by factors such as viral load and other variables not investigated in this study (38). COVID-19 patients with higher viral loads tend to present poorer clinical outcomes (39). However, we were unable to measure the precise viral load of the patients during the time of our study due to the stringent COVID-19 restrictions in place made it challenging to conduct such detailed level of testing, as well as budgetary limitations.

Higher prevalence of low vitamin D levels was observed among younger COVID-19 patients in this study in univariable analysis. This may be due to lifestyle factors of the young population such as staying indoors attributable to frequent use of electronic screen technology and urban living (40), contributing to reduced likelihood of sunshine exposure and hence lower cutaneous vitamin D synthesis. Poor dietary intake of vitamin D, particularly among individuals who consume little or no fish or other dietary sources of vitamin D and for those with darker skin pigmentation, will further reduce the ability of the skin to synthesize vitamin D in response to sunlight exposure (41).

In univariable analysis, COVID-19 patients with low vitamin D levels were significantly associated with lower CRP levels. Lower COVID-19 disease severity may result in reduced systemic inflammation, thereby reduced levels of acute phase reactants such as CRP (42,43). Vast majority of the COVID-19 patients in this study demonstrated a mild (or asymptomatic) disease course (category 3 and below), and this may partially explain on the low vitamin D levels association with lower CRP levels. However, vitamin D and CRP associations are complex and more research, particularly in larger multi-cohort COVID-19 patients, are required to conclude their relationship.

We acknowledge the limitations of the study as follows: 1) This is a retrospective study and this it can only demonstrate an association rather than establishing causation; 2) Our study measured vitamin D and CRP levels at one particular point in time, rather than multiple times over the course of the disease. This may make it more challenging to observe any changes in these levels over time and to determine if they are associated with the development, severity, or remission of the disease; 3) Although multivariable analysis was conducted on variables that showed significance in

univariable analysis, these findings can be affected by variables not included in our study. We recommend for subsequent studies to include more variables such as supplementation status of the subjects, individual diets, and sun exposure duration for more comprehensive assessments; 4) The inter- and intra-assay coefficients of variation (CV) values were not determined for this study's vitamin D immunoassay. We recommend that future investigations should assess these CV values to ensure precision in the vitamin D assay.

CONCLUSION

Gender, age, and CRP levels individually showed significance with low vitamin D levels in univariable analysis but such significance was not maintained when considered together in a multivariable model. Hence, these imply the presence of underlying interactions among these factors that warrant further investigations.

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