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Association of vitamin D with the modulation of the disease severity in COVID-19

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ABSTRACT

In late 2019, SARS-CoV-2 started to spread throughout the world causing the COVID-19 that has taken a considerable number of lives. Results obtained from several investigations have explained the virus origin, pathogenicity, and transmission. Similar to SARS coronavirus, the pulmonary angiotensin converting enzyme (ACE) 2 was introduced as the virus receptor for entering the cell. An increased body of epidemiological and clinical evidences has shown modulating effects of vitamin D in lung injuries through several mechanisms. Several clinical symptoms as well as molecular factors have shown to be related to the disease transmission and severity. In this study, vitamin D, ACE concentrations, and neutrophil to lymphocyte ratio (NLR) were measured in patients with confirmed COVID-19 in comparison with control group. Results demonstrated significant alterations in vitamin D and ACE levels as well as NLR in the patients' group. Contribution of those factors with the prognosis and severity of the disease has been shown.

1. Introduction

In December 2019, several cases of pneumonia occurred in Wuhan, Hubei province, China, caused by a new type of beta-coronavirus. The disease and the causative coronavirus were originally named by the World Health Organization as COVID-19 and nCoV-2019 respectively. On February 11, 2020, Coronavirus Study Group (CSG) of the International Committee on Taxonomy of Viruses tentatively named the novel coronavirus as SARS-CoV-2 (Coronaviridae Study Group of the International Committee on Taxonomy of, V., 2020). Chinese scientists promptly identified the viral sequence isolated from patients and confirmed the human-to-human transmission of the virus. The R_0 (the basic reproduction number) of the virus was computed by scientists of various countries and declared to be about 2.2 and even higher (from 1.4 to 6.5) (D'Arienzoa and Coniglio, 2020; Muniz-Rodriguez et al., 2020). Clinically, patients with COVID-19 showed respiratory symptoms that were initially very similar to those of other respiratory viral infections. They were also characterized by ground-glass opacity in lung x-ray, which was even detectable in patients with a milder form of the disease. Recent research on the involvement of laboratory parameters in predicting of COVID-19 cases has suggested that the level of LDH, CRP, ALT and NEU can contribute to the clinical outcomes of the disease (Mardani et al., 2020). The new SARS-CoV-2 structure was recognized as a coated virus possessing an RNA genome with positive polarity, closely related to other SARS coronaviruses and more distant from common respiratory viruses circulating in humans (Coronaviridae Study Group of the International Committee on Taxonomy of, V., 2020). The new virus has 79.5 % genetic similarity with SARS-CoV (SARS agent in 2002) and 96.2 % with the RaTG13 bat coronavirus. Structural studies of the virus receptor protein sequences have revealed that SARS-CoV-2 can recognize angiotensin converting enzyme 2 (ACE2) from humans and other animal species such as ferrets, cats, and others as intermediate hosts (Wan et al., 2020). Inside the lungs, the ACE2 protein has more expression in the apical surface of the deep alveolar epithelial cells. This receptor is expressed in multiple human organs. It assists the human-to-human and cross-species transmission of the virus (Andersen et al., 2020; Hussain et al., 2020a). ACE2 is a zinc-metallopeptidase which is an antagonist of the angiotensin converting enzyme (ACE). ACE converts the angiotensin

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(Ang) I to Ang II, a vasoconstrictor, by removing a dipeptide from its C-terminal. Besides, ACE is a destroyer of bradykinin which is a vasodilator. The ACE2 acts, unlike ACE, to remove a single amino acid from the end of a protein, hence unable to convert Ang I to Ang II or inactivate bradykinin. The main role of ACE2 is to convert Ang II to Ang-(1–7) and to promote the relevant pathway. From the physiological point of view, it counteracts the activities of Ang II (pressor, proliferative effect, and pro-fibrotic effect). In fact, both enzymes appear to balance the rennin-angiotensin-aldosterone system (RAAS). Certain researches have shown that RAAS inhibitors, namely angiotensin receptor blockers (ARBs) and ACE inhibitors can increase the ACE2 expression (Zheng et al., 2020). RAAS inhibitors have heterogeneous effects by affecting different enzymes and peptides involved in the system. Various laboratory animal models have shown that ARBs and mineralocorticoid receptor blockers increase both ACE2 expression and activity. Increased ACE has a positive regulatory effect on the production of angiotensin II, which in turn results in positive feedback on the activity of the ACE2 (Tikellis and Thomas, 2012). Studies show that an increase in the level of ACE2 could activate the angiotensin II-Mas receptor axis, which acts as a cardiopulmonary protector through its anti-inflammatory and antioxidant effects (Shenoy et al., 2010). Renin is the initial hormone in the cascading process of the RAS system, on which vitamin D plays a moderating role.

There is a body of epidemiological and clinical evidence showing that vitamin D can reduce lung injuries through several mechanisms, including inducing the antimicrobial peptides, reducing the concentrations of pro-inflammatory cytokines and increasing the anti-inflammatory cytokines (Foley et al., 1998). In several observational studies, vitamin D deficiency has been shown to have an independent association with increased risk of acute viral respiratory infections. Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are among the leading causes of mortality in intensive care units. They have common characteristics, including increased permeability of the epithelium of the alveoli and endothelium of the pulmonary capillaries, edema, and pulmonary fibrosis. It is also likely that vitamin D can exert protective effects against COVID-19 through suppression of cytokine response and reduce the risk of ARDS (Martineau et al., 2017). Research on mice lacking vitamin D receptors (VDR-null mice) has shown increased renin production and hyper reninemia in those animals, suggesting the negative regulation of renin by $125(OH)_2$ D_3. Renin production in the kidney during the RAAS process breaks down the angiotensinogen and converts it to Ang I. The latter compound is converted to Ang II by ACE as mentioned. Calcitriol has been shown to reduce the risk of lung damage through the RAS system by negative regulation of the renin gene. Decrease in ACE and ACE2 in lung has been shown in animal models with increased mRNA levels of proinflammatory cytokines, and AT1R levels, associated with activation of the ACE-AngII-AT1R (angiotensin II receptor type 1) axis, pulmonary injury, and progression of cytokine storms. In this study, serum levels of vitamin D and ACE in patients with confirmed COVID-19 have been measured and results were discussed in relation to the certain possible pathways that could be involved in the progression of the disease.

2. Materials and methods

2.1. Patient participation and data collection

The study protocol was accepted by the Review Board and the Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1399.131). All individuals participated in the study voluntarily and provided signed informed consent. Individuals were consisted of 65 male and 58 female outpatients, aged between 18–78 years, referred to Behpooyan clinic (Tehran, Iran) during March 2020 with respiratory difficulties including cough and shortness of breath and/or with CT-scan showing ground glass opacity in lungs. Oropharyngeal (OP) specimens were collected and samples were transferred to the laboratory using viral transport medium (VTM). All samples were tested for SARS-CoV-2 using probe based real-time RT-PCR (PishtazTeb, IRAN). Briefly, the viral RNA extractions followed by cDNA synthesis were performed on the OP specimens. The qRT-PCR was carried out using specific probes and primers conforming to the kit instructions. Based on the RT-PCR results, samples were divided into two groups of positive or negative for COVID-19.

Patients with comorbidities such as chronic lung diseases, hematological diseases, liver disease, having undergone radiotherapy, and chemotherapy were excluded from the study.

We do not have the sample at the peak of the disease and there was no data on patients' clinical condition subsequent to this study since their samples were collected on admittance to the hospital, except knowing that one female and three male patients have deceased during the study.

2.2. Laboratory testings

Blood tests were performed using routine methods for the detection of Lymphocyte (LYM), and Neutrophil (NEU) counts. The angiotensin converting enzyme (ACE) was measured in the sera using ELISA method (Biorexfars, Iran) conforming to the instructions of the manufacturer. The serum level of vitamin D was measured using ELISA method (Monobind, USA), based on the kit instructions. In this regard, four levels for vitamin D concentrations were initially considered in the present study as deficient (<10 ng/mL), insufficient (10−30 ng/mL), sufficient (30−100 ng/mL), and potential toxic (>300 ng/mL). According to the previously reported test protocols used for the vitamin D quantification, two ranges of sufficient and insufficient vitamin D levels (>30 ng/mL and <30 ng/mL respectively) have been considered in this study (Holick, 2009).

2.3. Statistical analysis

The acquired data obtained were expressed as mean values with ± standard deviations (SD). Statistical differences between or among groups were calculated using Mann-Whitney and Kruskal-Wallis tests the GraphPad Prism Statistical Software V6.

3. Results

3.1. Laboratory findings in the COVID-19 patients and the control groups

In the present study, 123 (65 males and 58 females) individuals have participated comprised of 63 confirmed COVID-19 patients and 60 COVID-19 negative controls with an average of 42 and a median age of 39 years old. Suspected patients showed shadows or ground-glass opacities in their CT scans. However, they were undergone confirmatory diagnosis through the collection of oropharyngeal swab specimens and nucleic acid analysis tests (Xie et al., 2020). The results of two groups of positive and negative COVID-19 in terms of age, the absolute value of neutrophils, lymphocytes, nucleic acid analysis, as well as analyses of their relationships with COVID-19 are shown in Table 1 and Figs. 1 and 2, and he Supplementary data.

Table 1
The comparison between the two groups in terms of the average age, Neutrophil, Lymphocyte, ACE and vitamin D is presented here. These data showed that the differences between the two groups were statistically significant ($p < 0.0001$).

Covid-19	Average				
	Age	Neutrophil	Lymphocyte	ACE	Vitamin D
Positive	43.3	59.5	38.1	39.8	18.5
Negative	40.1	47.4	52.1	31.2	30.2

3.2. Association of vitamin D and ACE concentrations with the COVID-19 patients

The differences between COVID-19 positive and negative groups, in terms of the vitamin D and angiotensin converting enzyme (ACE) concentrations, are depicted in Fig. 1. The results show that vitamin D had an important change in the group of COVID-19 positive individuals. Four patients have unfortunately deceased during this study for whom, the mean vitamin D concentration was significantly decreased compared to the control group and other COVID-19 patients ($p < 0.0001$). Serum ACE concentration showed a significant increase in patients group, compared to the control group ($p < 0.0001$). The ACE concentration was significantly higher in deceased individuals even compared to the other COVID-19 patients ($p < 0.0001$). Looking at individuals with different vitamin D levels, the ACE concentration showed a higher quantity among individuals with insufficient vitamin D concentration ($p < 0.0039$). Those results have been depicted in Fig. 1A to D.

3.3. Association between neutrophil-to-lymphocyte ratio and the COVID-19 patients

Significant decrease in lymphocyte count and lymphopenia has been observed in this study ($p < 0.0001$). There was also a significant increase in the patients' group in terms of the neutrophil count ($p < 0.0001$). In the case of the deceased patients, the change in lymphocyte and neutrophil count had an important difference not only with the control group but also with other COVID-19 patients ($p < 0.0001$). Consequently, the ratio between neutrophils and lymphocytes (NLR) was also considerably higher in the COVID-19 group, and beyond in the case of the deceased patients ($p < 0.0001$). The results are demonstrated in the Fig. 2.

4. Discussions

By the end of 2019, COVID-19 started in Wuhan, China, and emerged rapidly as a pandemic all over the world. Quickly after its appearance, COVID-19 was detected in Iran during the winter 2020. In the present work, patients under study were individuals who had been contaminated by the SARS-CoV-2 and needed hospitalization after confirmation of the clinical COVID-19. Among them, 4 individuals (6.3 % of the patients' group and 3.2 % of the total) were deceased which was compliant with the death rate, previously reported for the COVID-19 (Epidemiology Working Group for Ncip Epidemic Response, C.C.F.D.C. and Prevention, 2020; Team, 2020). Important changes in vitamin D and ACE concentrations as well as the NLR have shown in our study to be among important parameters associated with the severity of the COVID-19. There is more than a century of evidence for the effects of vitamin D in remedying various pathogens' effects (Lang et al., 2013). Extended animal studies support the regulatory effects of vitamin D on innate and adaptive immunity (Hewison, 2011). Vitamin D deficiency has been shown to be a risk factor in more severe courses of infection among critically ill patients. It has been observed to be inversely related to infections with various pathogens of the lower, as well as the upper respiratory tract (Ginde et al., 2009). In a 3.5 month follow-up study of a healthy cohort, a 2-fold less viral respiratory tract infections in individuals with >95 nmol/L of circulating 25(OH)VitD was observed (Sabetta et al., 2010). A recent study using UK Biobank samples aimed to assess the association of blood 25(OH)VitD concentration with COVID-19 risk. The results of that study did not find important link between blood vitamin D concentrations with COVID-19 risk, nor

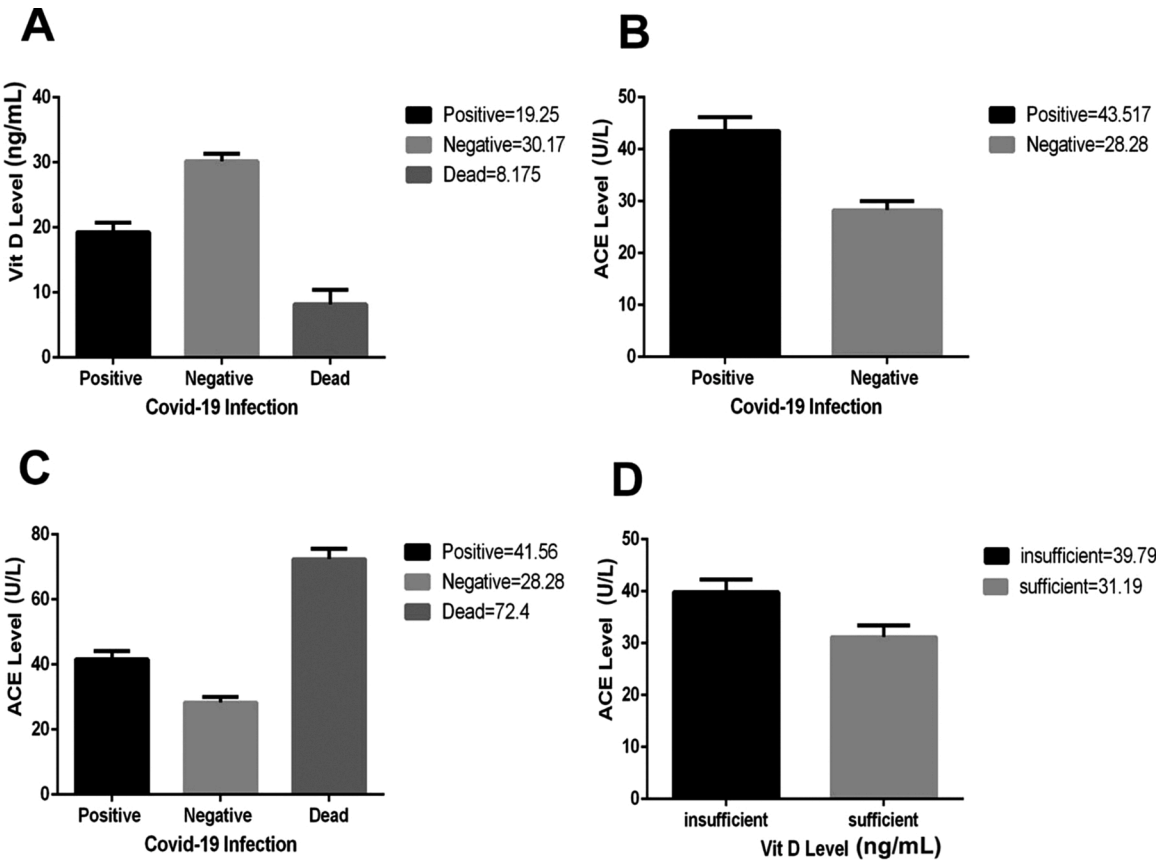

Fig. 1. Analysis of the COVID-19, in terms of correlation with vitamin D and ACE modifications, in comparison with the control group. Higher levels of Vitamin D were seen in non−COVID-19 individuals (A). Increase in the ACE was seen in COVID-19 (B) with higher quantities in dead individuals (C). Such increase in ACE showed relationship with insufficient amounts of vitamin D in patient group (D).

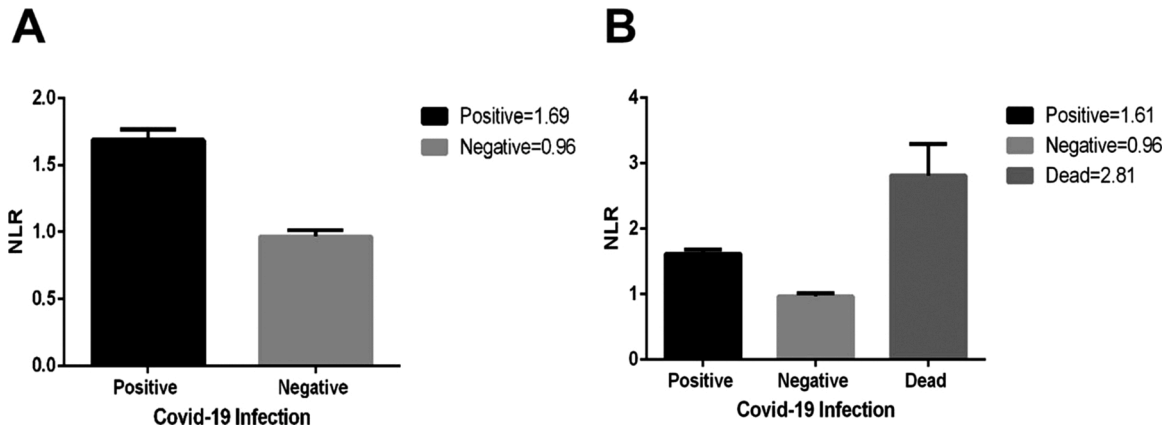

Fig. 2. Association of the NLR with the vitamin D concentrations in COVID-19 patients. (A) The Neutrophil to Lymphocyte ratio (NLR) is higher in COVID-19 than the non−COVID-19 group ($p < 0.0001$). (B) The mean value of the NLR shows a significant increase in the individuals who died from the COVID-19 ($p < 0.0001$).

suggested the usefulness of vitamin D measurement in clinical practice to assess the risk of COVID-19 infection (Hastie et al., 2020). In our study, the insufficient concentrations of vitamin D were associated with the hospitalization of COVID-19 patients (Fig. 1). Less than 16 ng/mL values of the serum vitamin D have been reported to be possibly associated with increased risk of sepsis in critically ill patients (Moromizato et al., 2014). An optimal range for 25(OH)VitD is reported as 25−80 ng/mL, and the definition of vitamin D insufficiency is sometimes reported as <30 ng/mL. Concentrations lower than 10 ng/mL for vitamin D are reported as severe vitamin D deficiency (Kennel et al., 2010). The status of vitamin D in the four individuals with COVID-19 who deceased in the course of this study was lower than 10 ng/mL. On admission, those individuals had severely low vitamin D levels, significantly less than both the control group and the patients' group, as depicted in Fig. 1a. Vitamin D is a steroid hormone that controls a broad range of metabolic and cell regulatory functions. It circulates in the blood as 25 (OH) D and its concentration defines the vitamin D status of the body. Diabetes and other comorbidities such as hypertension, obesity and ethnicity have been reported as significant predictors of morbidity and mortality in patients with COVID-19 (Garg et al., 2020). Although it's association with ethnicity was not supported by certain other results (Hastie et al., 2020). Vitamin D is associated with many diseases through manipulating the innate and adaptive immune system pathways (Prietl et al., 2013). Multiple cells in the immune system possess the vitamin D receptor (VDR) and, are capable of converting 25(OH)VitD to 1,25(OH) 2VitD. Many other cell types than kidney cells can produce 1,25(OH) 2VitD by the action of cytochrome p450 family member CYP27B1, with the assistance of TLRs or alternate PRRs. Endocrine or intracrine stimulating effect of 1,25(OH)2VitD on the expression of CYP27B1 enhances the epithelial cell expression of the antimicrobial peptide Cathelicidin LL-37 and beta-defensin (Gombart, 2009; Adams and Hewison, 2012). This mechanism has been shown to induce the chemotaxis of immune cells and prevent neutrophil apoptosis that increases their lifespan and consequently modulate the respiratory immune response to viral pathogens such as RSV and influenza (Nagaoka et al., 2012; Ahmed et al., 2019).

The NLR has been introduced as a useful indicator of systemic inflammation and tested as a guide for the prognosis of various diseases, including sepsis and cancer (Martins et al., 2019). It is a routine simple measure and not costly examination in hospitals. Association of the NLR increase has also been demonstrated in ARDS and ALI (Zhang et al., 2019). Meta-analysis investigations support that NLR and LCR (lymphocyte to C-reactive protein ratio) values can help predict clinical severity in patients with COVID-19 (Lagunas-Rangel, 2020). In the current study, significant decrease in lymphocyte along with increase in neutrophil count was demonstrated in patients (supplementary Fig. 1). Consequently, we have shown the NLR increase in COVID-19 patients compared to control group, with significantly higher values in those patients for whom the disease was fatal (Table 1 and Fig. 2). Considering that the blood samples of participants in this study was analyzed on admission, one possibility could be that the decreased lymphocyte count might be due to the IFN-I dependent transient lymphopenia which is observed in many viral infections (Kamphuis et al., 2006). It has not been demonstrated whether or not the direct viral infection through spike receptors in T-lymphocyte could contribute to lymphopenia (Wang et al., 2020). In a study with a Time-Lymphocyte percent model in patients with COVID-19, the importance of lymphopenia, with the lymphocyte count of less than 20 percent has been demonstrated as a decisive point to predict the disease severity (Tan et al., 2020). In our study, a significant lymphopenia was observed in COVID-19 patients, as previously reported by other studies (Lagunas-Rangel, 2020; Tan et al., 2020). However, the blood lymphocyte in none of those deceased individuals was lower than 20 percent on admission. Therefore, we rather suggest that the cost-effective NLR to be considered as a marker to aid complication predictions or poor prognosis in COVID-19.

Another factor demonstrated in this study was the significant increase of circulating ACE in the COVID-19 patients (Fig. 1B). The renin angiotensin system (RAAS) was primarily thought to be responsible for the regulation of blood pressure and sodium and water homeostasis. However, it has been revealed that RAAS could be closely associated with the lung injury (Chen et al., 2013). In a regular way, juxtaglomerular cells within the kidneys release renin following a blood pressure drop, which hydrolyzes circulating angiotensinogen to produce Ang I, which is then cleaved by ACE and converted to biologically active octapeptide Ang II. Ang II is the most important effector peptide of the RAAS that preferentially binds to and stimulates the Ang II type 1 receptor (AT1R), inducing vasoconstriction, inflammation, oxidative stress, and cell proliferation (Schalekamp and Danser, 2013). When metabolized by ACE2 to form Ang-(1-7), Ang II can induce the G-protein coupled MasR axis and subsequently oppose the vasoconstrictor effects of Ang II, aldosterone secretion and counteract the AT1R downstream effects. Inhibition of the Ang II signaling pathway and/or RAAS has protective effects on lung injury (Yu et al., 2016). Clear shreds of evidence show that RAAS activation contributes to pulmonary arterial hypertension through actions of Ang II and particularly aldosterone (Maron and Leopold, 2014). An increase in ACE can potentially overdrive the Ang II generation and promote the detrimental effects of the AT1R classical axis. In addition to elevated ACE, we have also observed an association between increased ACE level and vitamin D insufficiency in COVID-19 patients (Fig. 1D). These results are in line with the fact that RAAS can provide feedback to vitamin D signaling and block the act of vitamin D as a transcription factor in renin gene suppression whereby it exerts a negative endocrine regulator activity on RAAS(Shroff et al., 2012). Lung is a major source of ACE and therefore a major site of

systemic Ang II synthesis and RAAS action. It is thought that ACE2 activity is upregulated by Increased Ang II levels. SARS coronavirus has been suggested as a predisposing factor for ARDS. RAAS components including ACE, Ang II and the Ang II type 1a receptor (AT1a) exacerbate, while ACE2 can protect, from the disease outcomes including lung edema and impaired lung function (Imai et al., 2005). Pulmonary ACE2 appears to regulate the balance between the levels of circulating Ang II and Ang-(1–7). Transcriptome analysis for ACE2 expression in the lungs of patients with comorbidities has shown high expressions in patients with severe COVID-19, compared to control individuals (Pinto et al., 2020). In the case of diabetes, as the expression of ACE2 depends on the progression of the disease, adverse outcomes might be reduces through patient management strategies, rigorous glucose monitoring and careful consideration of drug interactions (Hussain et al., 2020b). Collectively, it seems that SARS-CoV-2 in the same way as SARS-CoV infection, could shift the balance of ACE/Ang II/AT1R axis over the ACE2/Ang (1–7)/MasR axis in the lung, resulting in acute lung injury (Kuba et al., 2005). High expression of VDR in the lung and interaction with vitamin D can prevent lung injury through blocking the RAAS (Kong et al., 2013). Altogether, sufficient vitamin D can have a modulating effect on the consequences of SARS-CoV-2 infection through interference with the RAAS and immune system elements functions through VDR which is a ligand-activated transcription factor.

CRediT authorship contribution statement

R. Mardani: Conceptualization, Methodology. **A. Alamdary:** Visualization, Formal analysis. **S.D. Mousavi Nasab:** Validation, Writing - review & editing. **R. Gholami:** Investigation, Writing - review & editing. **N. Ahmadi:** Writing - review & editing, Funding acquisition. **A. Gholami:** Project administration, Supervision, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2020.198148.

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Applied nutritional investigation

Cohort study to evaluate the effect of vitamin D, magnesium, and vitamin B_{12} in combination on progression to severe outcomes in older patients with coronavirus (COVID-19)

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ABSTRACT

Objectives: The aim of this study was to determine clinical outcomes of older patients with coronavirus (COVID-19) who received a combination of vitamin D, magnesium, and vitamin B_{12} (DMB) compared with those who did not. We hypothesized that fewer patients administered this combination would require oxygen therapy, intensive care support, or a combination of both than those who did not.
Methods: This was a cohort observational study of all consecutive hospitalized patients ≥50 y of age with COVID-19 in a tertiary academic hospital. Before April 6, 2020, no patients received the (DMB) combination. After this date, patients were administered 1000 IU/d oral vitamin D_3, 150 mg/d oral magnesium, and 500 mcg/d oral vitamin B_{12} upon admission if they did not require oxygen therapy. Primary outcome was deterioration leading to any form of oxygen therapy, intensive care support, or both.
Results: Between January 15 and April 15, 2020, we identified 43 consecutive patients ≥50 y of age with COVID-19. Seventeen patients received DMB before onset of primary outcome and 26 patients did not. Baseline demographic characteristics between the two groups were significantly different by age. In univariate analysis, age and hypertension had a significant influence on outcome. After adjusting for age or hypertension separately in a multivariate analysis, the intervention group retained protective significance. Fewer treated patients than controls required initiation of oxygen therapy during hospitalization (17.6 vs 61.5%, $P = 0.006$). DMB exposure was associated with odds ratios of 0.13 (95% confidence interval [CI], 0.03–0.59) and 0.20 (95% CI, 0.04–0.93) for oxygen therapy, intensive care support, or both on univariate and multivariate analyses, respectively.
Conclusions: A vitamin D / magnesium / vitamin B_{12} combination in older COVID-19 patients was associated with a significant reduction in the proportion of patients with clinical deterioration requiring oxygen

CWT and LPH contributed equally. CWT, LPH, SK, JGL, and HJN co-wrote the manuscript. CWT, LPH, JWMC, MC, SK, JGL, and HJN were involved in the design of the study. BPZC, YET, SYT, HMW, PJWT, JGL, and SK conducted the pilot study. CWT, LPH, CN, and RG were involved in data analysis. LPH formulated the supplement combination. All authors have read and agreed with the manuscript. The authors have no conflicts of interest to declare.

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support, intensive care support, or both. This study supports further larger randomized controlled trials to ascertain the full benefit of this combination in ameliorating the severity of COVID-19.

Introduction

The coronavirus (COVID)-19 pandemic, which began in late 2019, has raged across the globe with >20 million infections and 700 000 deaths recorded to date. A broad theme of immune hyperinflammation has emerged as a key determinant of patient outcome with uncontrolled immune response postulated as a pathophysiologic factor in disease severity. Intuitively, immunomodulation becomes an attractive potential treatment strategy. In addition to lung involvement, gastrointestinal symptoms are frequent and carry a worse prognosis [1]. Therefore, COVID-19 is a multiorgan phenomenon and it is becoming evident that appropriate systemic inflammatory control is necessary for overall survival benefit. Age >50 y, hypertension, diabetes, and coronary artery disease, are also known patient factors associated with increased severity and death [2].

Much of the current therapeutic effort is targeted at viral elimination instead of pre-emptively modulating hyperinflammation. A number of immunomodulatory agents may serve the latter role. Vitamin D, for instance, has a protective effect against respiratory tract infection [3]. Magnesium enhances vitamin D function in addition to being an antihypertensive, antithrombotic, and bronchodilator [4,5]. Vitamin B_{12} is an important modulator of gut microbiota [6]. Importantly, these compounds are generally safe and well tolerated by patients. A short course of vitamin D/magnesium/vitamin B_{12} supplements (DMB) upon diagnosis of COVID-19 could potentially exert synergistic effects to modulate host immune response, ameliorate COVID-19 severity, and reduce adverse outcomes. This study was conducted to evaluate the potential efficacy of DMB on progression of COVID-19 to severe disease.

Methods

Study design

This study was approved by the Institutional Ethics Committee of Singapore General Hospital with waiver of consent granted. We included all consecutive COVID-19 patients ≥50 y of age admitted to Singapore General Hospital, a tertiary academic hospital, between January 15 and April 15, 2020. Diagnosis required a positive severe acute respiratory syndrome coronavirus (SARS-CoV)-2 polymerase chain reaction (PCR) from nasopharyngeal or throat swab. Primary outcome was defined as the requirement of oxygen therapy when oxygen saturation fell <95% detected by pulse oximetry, intensive care unit (ICU) support, or both. As the COVID-19 situation evolved, we decided to start DMB beginning April 6, 2020 on all consecutive COVID-19 patients >50 y of age if they did not require oxygen therapy, ICU support, or both. These patients then served as the intervention group. All remaining patients >50 y of age during the study period who were not given DMB accordingly served as the control group. Therapy comprised a single daily oral 1000-IU dose of vitamin D_3 (colecalciferol), 150 mg of magnesium oxide, and 500 μg vitamin B_{12} (methylcobalamine) for ≤14 d. DMB could be discontinued if a patient subsequently deteriorated or was deemed to have recovered based on symptom resolution and two consecutive negative SARS CoV-2 reverse transcriptase PCR respiratory samples. All patients were followed through either to hospital discharge or day 30 from onset of symptoms, whichever was earlier.

Data collection

Clinical and laboratory data were collected from electronic health records in a standardized form with two investigators independently reviewing the data for accuracy.

Statistical analysis

Primary outcome requiring oxygen therapy was treated as binary data with categories "*yes*" or "*no*." Demographic and clinical characteristics were summarized with respect to intervention and control group. Continuous variables were expressed as mean/SD if normal distribution, or median (interquartile range [IQR]) if non-normal distribution, and categorical data was expressed as number counts and percentages as appropriate. Univariate and multivariable binary logistic regression was performed to find associated risk factors for primary outcome. Quantitative association from logistic regression was expressed as odds ratio (OR) with 95% confidence interval (CI). All tests were two-sided and $P < 0.05$ was considered statistically significant. Statistical software SPSS 25 (IBM, Armonk, NY, USA) was used for analysis.

Table 1
Baseline demographic and clinical characteristics and outcomes of the patients given DMB therapy and control patients

	DMB (n = 17)	Control (n = 26)	*P*-value
Baseline characteristics			
Age, y, mean (SD)	58.4 (7)	64.1 (7.9)	**0.021**
Male, n (%)	11 (64.7)	15 (57.7)	0.755
Female, n (%)	6 (35.3)	11 (42.3)	0.755
Comorbidities, n (%)	8 (47)	20 (76.9)	0.057
DM	0 (0)	6 (23.1)	0.066
Hypertension	6 (35.3)	18 (69.2)	0.058
Hyperlipidemia	5 (29.4)	15 (57.7)	0.118
CVD	1 (6)	6 (23)	0.376
Asthma/COPD	2 (11.7)	2 (7.7)	1.000
Stroke	0 (0)	2 (7.7)	0.511
Clinical features			
Normal CXR on admission, n (%)	7 (41.2)	7 (26.9)	0.507
Time from onset of symptoms to admission, d, median (IQR)	7 (1–9)	5 (3–8)	0.455
Time from onset of symptom to initiation of therapy, d, median (IQR)	7 (4–10)		
Time from admission to initiation of therapy, d, median (IQR)	1 (0–1)		
Duration of therapy, d, median (IQR)	5 (4–7)		
Treatment with Kaletra/remdesivir/hydroxychloroquine, n (respective numbers), %	3 (1/2/0), 17.6	16 (8/7/1), 61.5	
Outcome			
- Requiring oxygen therapy (including ICU support), n (%)	3 (17.6)	16 (61.5)	**0.006**
- Requiring oxygen therapy (but no ICU support), n (%)	2 (11.8)	8 (30.8)	
- Requiring ICU support, n (%)	1 (5.9)	8 (30.8)	
- Mortality, n (%)	0 (0)	0 (0)	

CVD, cardiovascular disease; CXR, chest x-ray; DM, diabetes mellitus; DMB, vitamin D, magnesium, vitamin B_{12}; ICU, intensive care unit; IQR, interquartile range.
P-values < 0.05 are highlighted in bold.

Results

Forty-three consecutive patients were identified, with 17 patients in the DMB arm and 26 in the control arm. Baseline demographic and clinical characteristics were significantly different for age between the two groups (Table 1). In the treatment arm, most patients received DMB within the first day of hospitalization with a median duration of therapy of 5 d (IQR = 4 to 7 d). Significantly fewer patients receiving DMB required subsequent oxygen therapy compared with controls (3 of 17 vs 16 of 26; $P = 0.006$; Table 1). On univariate analysis, increasing age and hypertension demonstrated significantly higher OR for oxygen therapy, whereas exposure to DMB therapy was associated with a significantly improved OR (Table 2). Multivariate analysis showed that DMB remained a significant protective factor against clinical deterioration after adjusting for age or hypertension separately.

Eight of the 16 patients who needed supplemental oxygen therapy in the control group also required further ICU support. Of the three

Table 2
Univariate and multivariate analyses of OR in developing primary outcome requiring oxygen therapy for clinical variables and DMB therapy

Unadjusted univariate analysis				Adjusted multivariate analysis			
Variable	OR for requiring oxygen therapy	95% CI	*P*-value	Variables	OR for requiring oxygen therapy	95% CI	*P*-value
DMB	0.134	0.031–0.586	**0.008**	DMB	0.195	0.041–0.926	**0.040**
				Age, y	1.131	1.006–1.271	**0.039**
Age, y	1.150	1.035–1.278	**0.009**				
				DMB	0.182	0.038–0.859	**0.031**
Hypertension	6.250	1.575–24.798	**0.009**	Hypertension	4.528	1.041–19.694	**0.044**
Male	2.800	0.765–10.246	0.120				
Comorbidities	3.173	0.811–12.416	0.097				
DM		*	0.999				
Hyperlipidemia	3.429	0.972–12.095	0.055				
CVD	8.214	0.868–77.739	0.066				
Asthma/COPD	1.294	0.165–10.150	0.806				
Stroke		*	0.999				

COPD, chronic obstructive pulmonary disease; CVD, cardiovascular disease; DM, diabetes mellitus; DMB, vitamin D, magnesium, vitamin B_{12}; OR, odds ratio.
P-values < 0.05 are highlighted in bold.
*Not estimable.

Table 3
Subgroup analysis removing patients <60 y of age with diabetes. Baseline demographic and clinical characteristics and outcomes of these patients given DMB therapy and control patients

	DMB (n = 8)	Control (n = 12)	*P*-value
Baseline characteristics			
Age, y, mean (SD)	65 (2.7)	66 (4.7)	0.571
Male, n (%)	4 (50)	6 (50)	1.000
Female, n (%)	4 (50)	6 (50)	1.000
Comorbidities, n (%)	6 (75)	10 (83.3)	1.000
Hypertension	5 (62.5)	9 (75)	0.642
Hyperlipidemia	4 (50)	6 (50)	1.000
CVD	1 (12.5)	2 (16.7)	1.000
Asthma/COPD	2 (25)	2 (16.7)	1.000
Stroke	0 (0)	0 (0)	Not estimable
Clinical features			
Normal CXR on admission, n (%)	2 (25)	2 (16.7)	1.000
Time from onset of symptoms to admission, d, median (IQR)	7 (1–10)	5 (3–8)	0.919
Time from onset of symptom to initiation of therapy, d, median (IQR)	7 (3–11)		
Time from admission to initiation of therapy, d, median (IQR)	1 (0–1)		
Duration of therapy, d, median (IQR)	7 (5–7)		
Treatment with Kaletra/remdesivir/hydroxychloroquine, n (respective numbers),%	2 (1/1/0), 25	7 (4/3/0), 58.3	
Outcome			
Requiring oxygen therapy (including ICU support), n (%)	2 (25)	7 (58.3)	0.197
- Requiring oxygen therapy (but no ICU support), n (%)	1 (12.5)	2 (16.7)	
- Requiring ICU support, n (%)	1 (12.5)	5 (41.7)	

COPD, chronic obstructive pulmonary disease; CVD, cardiovascular disease; DMB, vitamin D, magnesium, vitamin B_{12}; ICU, intensive care unit; IQR, interquartile range.

patients who required oxygen therapy in the DMB group, two were started on oxygen therapy within 24 h from initiation of DMB. The third patient required supplemental oxygen therapy after 3 d of DMB supplementation, but did not require ICU support. Among the nine patients given DMB within the first week of onset of symptoms, only one patient required oxygen therapy. This patient was one of the two cases who deteriorated within 24 h of DMB initiation. Of note, there were no side effects or adverse events directly attributable to DMB. There was no death in either group during the follow-up period.

Subgroup analysis was performed by removing patients with diabetes and <60 y of age, in order to better match the DMB and control groups (Table 3). Thirteen patients were excluded and the baseline characteristics did not differ between the two groups. One-fourth of the patients in the DMB group required oxygen therapy or ICU support compared with more than half (58%) of the controls.

Discussion

COVID-19 is now understood to be potentially life-threatening in ≤20% of patients. As the world awaits an effective vaccine, the effectiveness of various antivirals are largely muted by lack of survival benefit. Targeted therapies against cytokines and antithrombotic agents may only address the terminal events in severe cases with limited benefits. At the point of giving DMB to older patients, it became obvious that preemptive downregulation of hyperinflammation with relatively safe agents was an attractive alternate strategy. This combination was chosen based on substantial, albeit indirect evidence of their role in tempering the inflammatory response to viral infections. Vitamin D, through its effect on nuclear factor-κB and other pathways, can attenuate various proinflammatory cytokines mediating the uncontrolled cytokine storm seen in severe COVID-19 with deficiency associated with severe COVID-19 [7]. Magnesium is critical in the synthesis and activation of vitamin D, acting as a cofactor in many of the enzymes involved in vitamin D metabolism. Vitamin B_{12} is essential in supporting a healthy gut microbiome, which has an important role in the development and function of both innate and adaptive immune systems [8]. This could be pivotal in preventing excessive immune reaction [9], especially in COVID-19 patients with microbiota dysbiosis, which has been associated with severe disease [10].

Our results provide early positive evidence of an immunomodulatory approach to ameliorating severe outcomes in COVID-19. DMB-treated patients were significantly less likely to require oxygen therapy than controls. Among three DMB-treated patients with clinical deterioration, two likely deteriorated within 24 h from their underlying infection but were included in an intention-to-treat analysis. Had they been excluded on the basis of inadequate time of DMB exposure, the demonstrated benefits would have been more profound. The last patient who deteriorated was started on DMB 7 d after onset of symptoms. To benefit from its preemptive effects, patients may need to be started earlier in the infective course. The ease of administration of DMB should allow for early initiation in the primary care setting at first onset of symptoms, or as prophylaxis among high-risk contacts during outbreaks in identified clusters.

As all agents in this combination are readily available, safe, and inexpensive, DMB can benefit a large swath of the world population, especially in economically challenged countries with limited or late access to vaccines and other therapies. DMB may also exhibit a generic efficacy against other viral infections with similar pathologic mechanism.

The limitations of this study included its retrospective cohort design with the control arm being older and showing a trend of having more comorbidities, especially diabetes. However, subgroup analysis excluding these factors still demonstrated a lower proportion of the DMB group requiring oxygen therapy or ICU support than the controls (25 versus 58%, respectively). The benefits of DMB were retained, although statistical significance could not be demonstrated, considering the small number of patients in this subgroup analysis. Post hoc estimation of necessary sample size was performed using two-sided Fisher's exact test with assumed proportion of primary outcomes of requirement of oxygen therapy, ICU support, or both in the DMB and control groups to be 40% and 80%, respectively and the significance level of the test set as 5%. The necessary sample size was estimated to be 28 patients for both the DMB and control groups, requiring 56 patients. This study was conducted under difficult dynamic circumstances and is thus limited by the small sample size, which was smaller than our post hoc estimation. Additionally, we were not able to include systematic biological measures to support our findings.

Nonetheless, this proof-of-principle effort has yielded promising results supporting the benefit of the vitamin D / magnesium / vitamin B_{12} combination in preventing clinical deterioration in patients at high risk. Our findings will need to be further validated in a well-designed randomized controlled trial.

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"Effect of calcifediol treatment and best available therapy versus best available therapy on intensive care unit admission and mortality among patients hospitalized for COVID-19: A pilot randomized clinical study"

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Cytokine/Chemokine storm
Renin-angiotensin system
Hypercoagulability
Hydroxychloroquine
Chloroquine
Covidiol
Neutrophil activity
Vitamin D endocrine system
Cuboidal alveolar coating cells type II
Cathelicidin peptide
Defensins
TLR co-receptor CD14
Vitamin D receptor

ABSTRACT

Objective: The vitamin D endocrine system may have a variety of actions on cells and tissues involved in COVID-19 progression especially by decreasing the Acute Respiratory Distress Syndrome. Calcifediol can rapidly increase serum 25OHD concentration. We therefore evaluated the effect of calcifediol treatment, on Intensive Care Unit Admission and Mortality rate among Spanish patients hospitalized for COVID-19.

Design: Parallel pilot randomized open label, double-masked clinical trial.

Setting: University hospital setting (Reina Sofia University Hospital, Córdoba Spain.)

Participants: 76 consecutive patients hospitalized with COVID-19 infection, clinical picture of acute respiratory infection, confirmed by a radiographic pattern of viral pneumonia and by a positive SARS-CoV-2 PCR with CURB65 severity scale (recommending hospital admission in case of total score > 1).

Procedures: All hospitalized patients received as best available therapy the same standard care, (per hospital protocol), of a combination of hydroxychloroquine (400 mg every 12 h on the first day, and 200 mg every 12 h for the following 5 days), azithromycin (500 mg orally for 5 days. Eligible patients were allocated at a 2 calcifediol:1 no calcifediol ratio through electronic randomization on the day of admission to take oral calcifediol (0.532 mg), or not. Patients in the calcifediol treatment group continued with oral calcifediol (0.266 mg) on day 3 and 7, and then weekly until discharge or ICU admission. Outcomes of effectiveness included rate of ICU admission and deaths.

Results: Of 50 patients treated with calcifediol, one required admission to the ICU (2%), while of 26 untreated patients, 13 required admission (50 %) p value X^2 Fischer test $p < 0.001$. Univariate Risk Estimate Odds Ratio for ICU in patients with Calcifediol treatment versus without Calcifediol treatment: 0.02 (95 %CI 0.002−0.17). Multivariate Risk Estimate Odds Ratio for ICU in patients with Calcifediol treatment vs Without Calcifediol treatment ICU (adjusting by Hypertension and T2DM): 0.03 (95 %CI: 0.003-0.25). Of the patients treated with calcifediol, none died, and all were discharged, without complications. The 13 patients not treated with calcifediol, who were not admitted to the ICU, were discharged. Of the 13 patients admitted to the ICU, two died and the remaining 11 were discharged.

Conclusion: Our pilot study demonstrated that administration of a high dose of Calcifediol or 25-hydroxyvitamin D, a main metabolite of vitamin D endocrine system, significantly reduced the need for ICU treatment of patients requiring hospitalization due to proven COVID-19. Calcifediol seems to be able to reduce severity of the disease, but larger trials with groups properly matched will be required to show a definitive answer.

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1. Introduction

A new coronavirus-induced pneumonia was called coronavirus disease 2019 (COVID-19) by the World Health Organization (WHO) on the February 11, 2020, at the same time the international virus classification commission announced that the new coronavirus was named coronavirus 2 severe acute respiratory syndrome (SARS-CoV-2) [1]. Its epidemic spread has increased since it appeared. On the 31 st of January 2020, the WHO announced that COVID-19 was labeled as Public Health Emergency of International Concern (PHEIC).

Patients with COVID-19 show clinical clusters of severe respiratory illness manifestations including fever, nonproductive cough, dyspnea, myalgia, fatigue, abnormal leukocyte counts, and radiographic evidence of pneumonia, which are similar to the symptoms of previous SARS-CoV and MERS-CoV infections [2].

SARS-CoV-2 infection can remain asymptomatic or cause modest symptoms. Severely sick patients require hospital admission and about 20 % of hospitalized patients will developed Acute Respiratory Distress Syndrome (ARDS) and require intensive care unit (ICU) treatment [3]. ARDS, also in patients with Coronavirus Disease 2019 (COVID-19) is a life-threatening condition [4,5]. Although frequencies vary according to series, more than 40 % of patients hospitalized because of COVID-19 pneumonia developed ARDS of which more than 50 % ultimately died [6]. ARDS onset is often rapidly progressive and appears approximately nine days after the onset of severe COVID-19 [2]. The epidemiologic, morbidity and mortality patterns of ARDS are similar regardless of the trigger [7]. Moreover, ARDS is a pivotal component in the development of multiple organ dysfunction and mortality risk [8]. In the absence well documented effective treatments [4], there is a strong interest in identifying a strategy [9] to taper down the severity of COVID-19, as it would reduce the morbidity and maybe mortality and lower the need for the limited ICU health care resources [10].

It has been proposed that the activation of the vitamin D receptor (VDR) signaling pathway may generate beneficial effects in ARDS [11] by decreasing the cytokine/chemokine storm, regulating the renin-angiotensin system, modulating neutrophil activity and by maintaining the integrity of the pulmonary epithelial barrier, stimulating epithelial repair and tapering down the increased coagulability [12–16]. Recently, two ecological studies have reported inverse correlations between national estimates of vitamin D status and the incidence and mortality of IDOC-19 in European countries [17,18]; lower concentrations of circulating 25 (OH) D have also been reported to be associated with susceptibility to SARS-CoV-2 infection [19] and the severity of the evolution of COVID-19 [20]. Vitamin D deficiency is frequent in wintertime even in Southern Spain [21] and even more so in patients requiring ICU treatment [22].

Therefore, considering the number of deaths associated to COVID-19, especially the speed with which ARDS is established in a significant number of patients, we performed a pilot study to assess the clinical effectiveness of treatment of patients hospitalized for COVID-19 with calcifediol (25-hydroxyvitamin D_3) in early stages to evaluate whether such treatment can reduce the need for admission to ICU and consequently the derived potential risk of death, as a preliminary step to a more extensive randomized clinical trial.

2. Methods

The study protocol was approved by the Pharmacy Committee, and by Ethics committee for the Treatment of COVID-19 of the Reina Sofía University Hospital, Córdoba, Spain EU. (Act-29/2020). The study was conducted in accordance with the principles of the Declaration of Helsinki and the Good Clinical Practice guidelines of the International Conference on Harmonization. All patients and/or legal representatives were verbally informed about the objectives of the trial and their participation, by formally obtaining their consent, and its acceptance recorded in the electronic medical record of the Hospital.

2.1. Study design site and participants

Pilot Covidiol was a parallel pilot randomized open label, double-masked clinical study aiming to assess whether calcifediol can reduce the need for admission to ICU, and related death, as a previous part of the clinical trial Covidiol (Prevention and treatment with Calcifediol of Coronavirus induced acute respiratory syndrome (SARS) COVID-19 (COVIDIOL)" (NCT04366908)) and facilitate the sample calculation. This pilot trial was conducted at Reina Sofia University Hospital, Cordoba Spain.

Were included in the study seventy-sixth consecutive patients hospitalized with COVID-19 infection clinical [23,24] picture of acute respiratory infection, confirmed by a radiographic pattern of viral pneumonia and by a positive SARS-CoV-2 PCR with CURB65 severity scale (recommending hospital admission in case of total score > 1) [25]. Patients younger than 18 years and pregnant women were not included (Fig. 1).

All hospitalized patients received as best available therapy the same standard care, (per hospital protocol), of a combination of hydroxychloroquine (400 mg every 12 h on the first day, and 200 mg every 12 h for the following 5 days), azithromycin (500 mg orally for 5 days) and for patients with pneumonia and NEWS score$\geq$5, a broad spectrum antibiotic (ceftriaxone2 g intravenously every 24 h for 5 days) was added to hydroxychloroquine and azithromycin.

Hydroxychloroquine (EC50 = 0.72 μM) was chosen because it was in vitro more potent than chloroquine (EC50 = 5.47 μM). Based on physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) models results, a loading dose of 400 mg twice daily of hydroxychloroquine sulfate given orally, followed by a maintenance dose of 200 mg given twice daily for 4 days is recommended for SARS-CoV-2 infection, as it reached 3 times the potency of chloroquine phosphate when given 500 mg twice daily 5 days in advance [26].

The patients were admitted to the ICU by applying the rigorous protocol of the Reina Sofia University Hospital (see supplementary material). Several fundamental aspects were considered when evaluating admission to the ICU: Presence of comorbidities, either individually or quantified in the modified age Charlson Comorbidity Index; Barthel's Index for functional assessment. It establishes the level of dependence of a patient according to his or her needs and clinical criteria: CURB-65 and SOFA scale and ATS/IDSA criteria [27]. A multidisciplinary Selection Committee was created, made up of intensivists, pulmonologists, internists and members of the ethics committee who decided on admission to the ICU.

Sample Size Calculation was carried out for a pilot study with 75 patients randomized in the proportion of 2:1 to carry out the definitive trial (COVIDIOL) (NCT04366908). The sample size calculation is based on the proportion of a participant treated with Calcifediol could meet the criteria for admission to the Intensive Care Unit which is estimated as 5% (with 90 % confidence intervals) and the proportion of a participant not treated with Calcifediol which could be 10 %. According to these assumptions the estimated final sample size for our pilot clinical study was 50 patients in the arm of patients treated with Calcifediol and 25 patients in the group of patients not treated with Calcifediol [28]. The attrition rate is assumed to be 12 %.

2.2. Procedures

Eligible patients were allocated at a 2 calcifediol:1 no calcifediol ratio through electronic randomization performed by hospital statisticians (Fig. 1) on the day of admission to take oral Calcifediol (Faes-Farma, Lejona, Spain), in soft capsules (0.532 mg), or not. Patients in the calcifediol treatment group continued with oral calcifediol (0.266 mg) on day 3 and 7, and then weekly until discharge or ICU admission [22,29]. Patients were followed-up until admission to ICU, hospital discharge or death.

2.2.1. Randomization and masking

An electronically generated randomization 2:1 list was prepared by independent statisticians. The list was accessible only to nonmasked specialists in the study in an attempt to minimize observation bias. The patients' data were recorded in the hospital's electronic medical record, with blind access by the technical data collectors and the statistician who carried out the study.

2.2.2. Outcomes

Outcomes of effectiveness included rate of ICU admission and deaths. The working hypothesis of this pilot trial was that calcifediol treatment would decrease the need for ICU admissions and the potential risk of death associated with these admissions.

2.3. Laboratory Analysis and respiratory function test

Clinical samples for SARS-CoV-2 diagnostic testing were obtained according to WHO guidelines [30]. For each patient, a sampling strategy was implemented in which samples were obtained on admission. Upper respiratory tract samples were obtained by nasopharyngeal exudate sampling. Procedures for RNA extraction and real-time RT-PCR (rtRT-PCR) were undertaken in the local Central Microbiology Laboratory (Code 202 MagCore® Viral Nucleic Acid Extraction Kit and Allplex™ 2019-nCoV Assay by Seegene or VIASURE SARS-CoV-2 Real Time PCR Detection Kit).

Hematology analyses included blood count (Flow cytometry on ADVIA 2120i, Siemens Healthineers, Erlangen, Germany) and coagulation study including D-Dimer (clotting and immunoturbidimetric assay on ACL TOP 700, Instrumentation Laboratory/Werfen). Biochemical tests including renal function, liver function, lactate dehydrogenase (spectrophotometric assay on Advia chemistry 2400 XPT, Siemens Healthineers, Erlangen, Germany), ferritin and C-reactive protein (immunoturbidimetric assay on Advia chemistry 2400 XPT, Siemens Healthineers, Erlangen, Germany. IL-6 (chemiluminescent immuno assay on Advia Centaur XPT, Siemens Healthineers, Erlangen, Germany)

Respiratory function was assessed by PaO2/FiO2 index [5]. A chest X-ray was taken in all patients on admission All X-ray tests were evaluated by an expert team of chest radiologist.

2.4. Statistical analysis

Descriptive statistics were used for demographic, laboratory, and clinical prognostic factors related to COVID-19 for each treatment arm.

The comparison between groups of quantitative variables were performed by using *t*-test for qualitative variables, $\chi 2$ tests and Fisher exact tests (with frequencies <5) were used.

Univariate and multivariate logistic regressions were used to estimate Odds ratio and 95 % CIs for the probability of admission to ICU. Significant p-value was considered when $p < 0.05$.

All the analysis has been done using IBM SPSS Statistics software (SPSS).

The pilot trial was reported according to the Consolidated Standards of Reporting Trials (CONSORT) reporting guideline [31].

3. Results

Table 1 shows demographic characteristics of the patients in both groups. Seventy-six patients (45 men (59 %) and 31 women) were enrolled in the study and randomized: 26 without calcifediol treatment, 50 with calcifediol treatment (Fig. 1). Mean age was 53 ± 10 (mean ± SD) years, being 54 ± 9 years for men and 51 ± 11 years for women. There was no significant gender difference in age between patients in each group ($p = 0.09$).

Baseline factors associated with bad prognosis of COVID-19 are listed

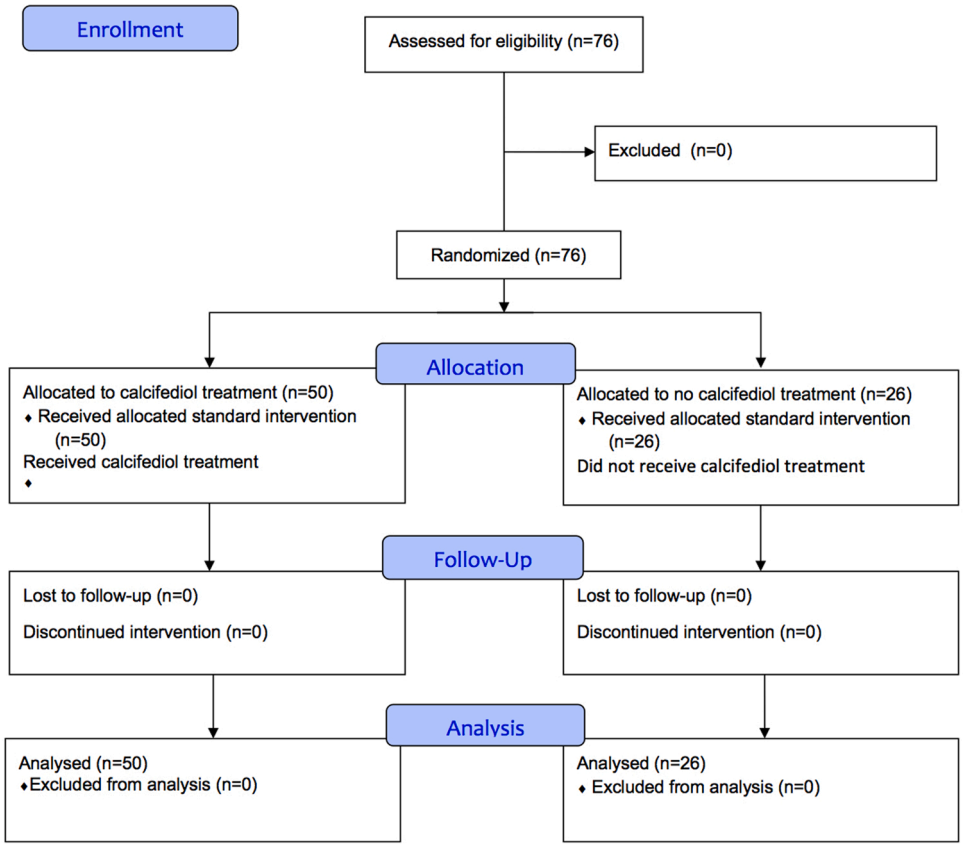

Fig. 1. Patients Flow Diagram.

in Table 2 as absolute and relative frequencies for categorical variables, and as median plus standard deviation for numerical variables. In addition, both groups were compared for homogeneity at baseline.

At baseline, there was no significant difference in number of subjects with at least one risk factor. Patients assigned to calcifediol were slightly (not significantly) older, whereas the control group had a higher percentage of hypertension (Table 2).

Among 26 patients not treated with calcifediol, thirteen required ICU admission (50 %), while out of fifty patients treated with calcifediol only one required admission to the ICU, whereas the other patients remained in conventional hospitalization

Although at baseline, there was no significant difference in number of subjects with at least one risk factor, the randomization did not achieve a homogeneous distribution of all the variables investigated between the two comparison groups (with and without calcifediol (Table2). A statistically significant difference was identified for the variable hypertension (26 had a history of hypertension of which 11 (42 %) received Calcifediol and 15 (58 %) not (CI: −0.58 to −0.13; p: 0.002) and close to statistical significance for diabetes 3 (6%) versus 5 (19 %). Therefore, a multivariate logistic regression analysis was performed to adjust the model by possible confounding variables such as hypertension and type 2 diabetes mellitus for the probability of the admission to the Intensive Care Unit in patients with Calcifediol treatment vs Without Calcifediol treatment (odds ratio: 0.03 (95 %CI: 0.003−0.25) (Table 3). The dependent variable considered was the need to be treated or not in ICU (dichotomous variable).) CI:-0.30−0.03 p:0.08.

Of the patients treated with calcifediol, none died, and all were discharged, without complications. The 13 patients not treated with calcifediol, who were not admitted to the ICU, were discharged. Of the 13 patients admitted to the ICU, two died and the remaining 11 were discharged.

4. Discussion

In line with our hypothesis on a possible link between VDR activation and the severity of ARDS or COVID-19 [11], our pilot study suggests that administration of a high dose of calcifediol or 25-hydroxyvitamin D_3, a main metabolite of vitamin D endocrine system, significantly reduced the need for ICU treatment of patients requiring hospitalization due to proven COVID-19.

The best available treatment that at the beginning of the outbreak in our hospital, included the use of hydroxychloroquine/azithromycin therapy [23,24,26]. However, taking into consideration more recent data on the safety and efficacy of chloroquine and hydroxychloroquine in small randomized clinical trials, case series, and observational studies this treatment is no longer considered effective [32] in treating COVID-19.

Randomization generated groups with comparable percentage of unfavorable risk factors as there was no significant difference in subjects with at least one risk factor, except for high blood pressure and diabetes mellitus, known risk factors for unfavorable disease progression [2], which were more frequent in patients not treated with calcifediol.

However, even considering these factors, calcifediol significantly decreased the need for ICU admission in COVID-19 patients in a way not previously reported in this process until now [4]. From a mechanistic perspective there are good reasons to postulate that vitamin D endocrine system favorably modulates host responses to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), both in the later hyper-inflammatory and early viraemic phases of COVID-19. as outlined in our

Table 1
Demographic characteristics.

	Group receiving Calcifediol (n = 50)	Group without Calcifediol (n = 26)	IC 95 %	P
Age (years)	53.14 +/- 10.77	52.77 +/- 9.35	−0.34 – 9.60	0.07
Males [n (%)]	27 (54 %)	18 (69 %)	−0.38 – 0.07	0.20
Females [n (%)]	23 (46 %)	8 (31 %)	−0.07 – 0.38	0.20
Male's age (years)	56.30 +/ 8.29	52.13 +/- 10.05	−9.67 – 1.41	0.14
Female's age (years)	49.43 +/- 12.28	54.13+/- 7.99	−4.87 – 14.25	0.32

Results are expressed as mean +/- Standard Deviation.

Table 2
Prognostic factors for COVID-19 at baseline.

Poor prognosis risk factor	Group receiving Calcifediol (n = 50)	Group without Calcifediol (n = 26)	IC 95 %	P
≥ 60 years	14 (28 %)	5 (19.23 %)	−0.11 – 0.28	0.40
Previous lung disease	4 (8%)	2 (7.69 %)	−0.12 – 0.13	0.96
Previous Chronic kidney disease	0	0	–	–
Previous Diabetes mellitus	3 (6%)	5 (19.23 %)	−0.30 – 0.03	0.08
Previous High blood pressure	11 (24.19 %)	15 (57.69 %)	−0.58 – −0.13	0.002
Previous Cardiovascular disease	2 (4%)	1 (3.85 %)	−0.09 – 0.09	0.97
Immunosuppressed & transplanted	6 (12 %)	1 (3.85 %)	−0.03 – 0.20	0.24
At least one prognostic bad risk factor[a]	24 (48 %)	16 (61.54 %)	−0.37 – 010	0.26
PaO2/FiO2 (mean +/-SD)	346.57 +/- 73.38	334.62 +/- 66.33	−22.29 – 46.19	0.49
C-reactive protein (mg/L) (mean +/-SD)	82.93 +/- 62.74	94.71 +/- 63.64	−42.15 – 18.59	0.44
LDH (U/L)(mean +/-SD)	308.12 +/- 83.83	345.81 +/- 108.57	−82.46 – 7.08	0.10
D-Dimer (ng/mL) (mean +/-SD)	650.92 +/- 405.61	1333.54 +/- 2570.50	−360.29 – 1725.53	0.19
Lymphocytes < 800/μL	10 (20 %)	6 (23.08 %)	−0.16 – 0.23	0.75
Ferritin (ng/mL) (mean +/-SD)	691.04 +/- 603.54	825.16 +/- 613.95	−166.31 – 434.55	0.36
IL-6 (22/48) (pg/mL) (mean +/-SD)	28.88 +/- 75.05	19.54 +/- 19.45	−41.88 – 23.19	0.41

SD: Standard Deviation.

[a] Patients with at least one of the following risk factors (age >60, previous lung disease, chronic kidney disease, diabetes mellitus, hypertension, cardiovascular disease or Immunosuppressed and transplanted patients).

Table 3
Requirements for admission to the Intensive Care Unit, in patients hospitalized with COVID-19 (treated or not with calcifediol).

	Without Calcifediol Treatment (n = 26)	With Calcifediol Treatment (n = 50)	p value (1d712;2) Fischer Test
Need for ICU			<0.001
Not requiring ICU, n (%)	13 (50)	49 (98)	
Requiring ICU, n (%)	13 (50)	1 (2)	

* Univariate Risk Estimate Odds Ratio for ICU in patients with Calcifediol treatment vs Without Calcifediol treatment: 0.02 (95 %CI 0.002−0.17).

** Multivariate Risk Estimate Odds Ratio for ICU in patients with Calcifediol treatment vs Without Calcifediol treatment ICU (adjusting by Hypertension and T2DM): 0.03 (95 %CI: 0.003−0.25).

previous review [11].

It is important to highlight that the cuboidal alveolar coating cells type II (ACII), like the cells of the immune system express all the enzymatic endowment (see above), to use calcifediol as substrate synthesize 1.25 (OH)2D3 or calcitriol [33]. With high basal expression of 1α-hydroxylase activation and low expression of inactivating enzyme (24-hydroxylase).The result is that ACII constitutively convert calcifediol to 125-dihydroxyvitamin D3, the hormonal form of the endocrine system of vitamin D. The calcitriol generated by the ACII acting on themselves and cells of the immune system may then lead to increased expression of genes with important innate immune functions (the antimicrobial cathelicidin peptide, defensins and the TLR co-receptor CD14 etc…). In addition, in a viral infection model, dsRNA leads to increased regulation of 1α-hydroxylase and synergizes with calcifediol and calcitriol sequentially to induce cathelicidin [34].

It should be noted that the role of calcifediol and calcitriol in the animal model and ACII cells [12,34] and the immune system [35,36] were about equipotent suggesting that ACII cells actively converted calcifediol. Interestingly, when ACII cells are treated with a concentration of calcifediol $\geq 10^{-7}$ M (or ≥40 ng / ml) the same effects are achieved as when calcitriol is used, which is a guide to the serum levels of 25OHD3 to be achieved in our trial [12,34].

This pilot study has several limitations as it is not double-blind placebo controlled. On the other hand, in the first studies evaluating risk factors for severe disease and/or death from COVID-19, the possible role of obesity was not considered. Therefore, given the isolation characteristics of the patients, we did not collect the BMI, which would have allowed us to add obesity as a risk factor for severe evolution of COVID-19 [37] It is striking to consider that obesity shares with aging and black or asian ethnicity a surprising overlap as risk factors for severe COVID-19 and vitamin D deficiency [38].

Serum 25OHD concentrations at baseline or during treatment are not available [39,40]. Overall, adults living in the Córdoba area are relatively vitamin D deficient (16 ng/mL on average) in late winter and early spring [17]. Patients with severe ARDS [28,29] or requiring ICU [30] are [17] frequently severely vitamin D deficient. In addition, low serum 25-hydroxyvitamin D (25[OH]D) levels in patients hospitalized with COVID-19 are associated with greater disease severity [20].

Furthermore, to correct vitamin D deficiency in severely sick patients much higher doses of vitamin D than usual are needed. Our study does not include a comparison with cholecalciferol, the native vitamin D3 form and nutritional substrate for calcifediol, so that we cannot conclude that calcifediol is superior to vitamin D itself. Nevertheless, calcifediol may have some advantages over native vitamin D. It has a more reliable intestinal absorption (close to 100 %) and can rapidly restore serum concentrations of 25OHD as it does not require hepatic 25-hydroxylation. This is especially relevant in clinical situations whereby rapid restoration of serum 25OHD is desirable and *CYP2R1* expression is compromised. Such impaired *CYP2R1* activity has been well demonstrated in several animal models [41] and has also been observed in patients with COPD or asthma [42]. In addition, calcifediol is more potent when compared to oral vitamin D3 [43]. In subjects with a deficient state of vitamin D, and administering physiological doses (up to 25 μg or 1000 IU daily, approximately 1 in 3 molecules of vitamin D appears as 25OHD; the efficacy of conversion is lower (about 1 in 10 molecules) when pharmacological doses of vitamin D/25OHD are used. [42]

The tissue effects of restoring the activation of the vitamin D receptor (VDR) signaling pathway may be due to circulating endocrine 125(OH) 2D or, more likely, on the local conversion (para/autocrine) of 25OHD into the active hormone in pulmonary alveolar cells, immune cells or other potential target tissues [33].

5. Conclusions

In conclusion, our pilot study demonstrated that administration of calcifediol may improve the clinical outcome of subjects requiring hospitalization for COVID-19. Whether that would also apply to patients with an earlier stage of the disease and whether baseline vitamin D status modifies these results is unknown. Therefore, a multicenter randomized controlled trial using calcifediol, properly matched (Prevention and Treatment With Calcifediol of COVID-19 Induced Acute Respiratory Syndrome (COVIDIOL)), in 15 Spanish hospitals, funded by Clinical Research Program at COVID-19 "Progreso y Salud" Foundation and Foundation for Biomedical Research of Córdoba (FIBICO), Spain, (registered as NCT04366908 in NIH Trialnet database) will be carried out with the number of patients recalculated from the data provided by this study.

An interesting perspective of the new COVIDIOL trial with the recently available information, could be to evaluate calcifediol associated to dexamethasone or other corticoid vs. dexamethasone or other corticosteroid, since dexamethasone, which has potent anti-inflammatory actions, has recently been shown to reduce mortality in hospitalized patients on Covid-19 who are on respiratory assistance [44]; so that treatment guidelines have been updated to recommend the use of glucocorticoids (including dexametasone) [45], now proposed as the best available treatment in many hospitals around the world

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Vitamin D deficiency and co-morbidities in COVID-19 patients – A fatal relationship?

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1. Introduction

Infections of the respiratory tract are more frequent in the winter months and especially in the northern latitudes than they are in summer [1]. This obviously also applies to the COVID-19 infectious disease that briefly spread all over the world in the winter months and became a pandemic [2,3]. A common feature of the winter months and the inhabitants of all countries north of the 42nd parallel is a hypovitaminosis D that frequently occurs during this period [4]. In addition during cold temperature the virus will be more easily transmitted. This raises the question of whether an inadequate vitamin D supply has an influence on the progression and severity of COVID-19 disease.

A low vitamin D status, measured as the plasma level of the transport form of vitamin D, 25(OH)D,is widespread worldwide and is mainly found in regions of northern latitudes, but also in southern countries [5]. In Europe, vitamin D deficiency is widely prevalent during the winter months and affects mainly elderly people and migrants. In Scandinavia only 5% of the population is affected by a low vitamin D status, in Germany, France and Italy more than 25%, particularly older people e.g. in Austria up to 90% of senior citizens [6,7]. In Scandinavian countries, the low incidence of vitamin D deficiency may be due to the traditional consumption of cod liver oil rich in vitamin D and A or to genetic factors resulting in higher synthesis of vitamin D in the epidermal layer [8]. Taken together, low vitamin D status is common in Europe with the exception of the Scandinavian countries. The calculated COVID-19 mortality rate from 12 European countries shows a significant ($P = .046$) inverse correlation with the mean 25(OH)D plasma concentration [9].

This raises the question whether insufficient vitamin D supply has an influence on the course of COVID-19 disease? An analysis of the distribution of Covid-19 infections showed a correlation between geographical location (30–50° N+), mean temperature between 5–11 °C and low humidity [10]. In a retrospective cohort study (1382 hospitalized patients) 326 died, Among them 70.6% were black patients. However, black race was not independently associated with higher mortality [11]. An excess mortality (2 to sixfold have been described in African-Americans with average latitudes of their state of residence in higher latitudes (> 40) [12]. The mortality of COVID-19 (cases/ million population) shows a clear dependence on latitude. Below latitude 35, mortality decreases markedly [13]. Indeed, there are exceptions e.g. Brazil (tenfold higher than all other latin American countries – except mexico), however, the management of the pandemic may increase infection risk.

1.1. Vitamin D effects

The skeletal and extra skeletal effects of vitamin D have recently been described in an extensive review [14]. Vitamin D exerts a genomic and non-genomic effect on gene expression. The genomic effect is mediated by the nuclear vitamin D receptor (VDR), which acts as a ligand activated transcription factor. The active form $1,25(OH)_2D$ binds to the VDR and in most cases heterodimerizes with the retinoid X receptor (RXR), whose ligand is one of the active metabolites of vitamin A, 9-cis retinoic acid. The interaction of this complex with the vitamin D responsive element can regulate the expression of target genes either positively or negatively [15]. The non-genomic effects involve the activation of a variety of signaling molecules that interact with Vitamin D responsive element (VDRE) in the promoter regions of vitamin D dependent genes [16]. Vitamins A and D are also of particlular importance for the barrier function of mucous membranes in the respiratory tract [17,18].

1.2. Vitamin D and immune system

Vitamin D plays an essential role in the immune system [19]. Vitamin D interferes with the majority of the immune systems cells such as macrophages, B and T lymphocytes, neutrophils and dendritic cells, which express VDR (for details [20] and Fig. 3). Cathelicidin, a peptide formed by vitamin D stimulated expression, has shown antimicrobial activity against bacteria, fungi and enveloped viruses, such as corona viruses [21,22]. Furthermore Vitamin D inhibits the production of pro-inflammatory cytokines and increases the production of anti-inflammatory cytokines [23].

The active metabolite of vitamin D in macrophages and dendritic cells, derived from the precursor 25(OH)D, leads to the activation of VDR, which, after RXR heterodimerization, results in the expression of various proteins of the innate and adaptive immune system (Treg cells, cytokines, defensins, pattern recognition receptors etc.) [24]. Vitamin D

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exerts opposite effects on the adaptive (inhibition) and innate (promotion) immunsystem This correlates with an anti-inflammatory response and balances the immune response [25].

The active metabolite of vitamin D, 1,25(OH)2D3 can be formed in T and B lymphocytes and inhibits T cell proliferation and activation [26]. This way, vitamin D may suppress T-cell mediated inflammation and stimulate Treg cells proliferation, by increasing IL-10 formation in DC cells, and thus enhance their suppressive effect [27,28].

1.3. Food sources

There are only few dietary sources of vitamin D (cod liver oil, fat fish) that could satisfy the recommended daily allowance (15–20 µg/day for adults). To reach such amount besides availability of dietary sources, vitamin D skin synthesis, which contributes to 80% in healthy individuals up to the age of 65, is important.

With the exception of mushrooms there are no plant sources of vitamin D. In particular wild mushrooms, which are grown in light. Sun-dried but not fresh mushrooms can contain between 7 and 25 µg/100 g of vitamin D2 [29], which is an important source [30] with a good shelf life [31] and comparable bioavailability to vitamin D3 [32]. Vitamin D status can be significantly improved by fortified foods, as was shown in a meta-analysis [33].

1.4. Vitamin D deficiency

Insufficient levels of vitamin D are caused by two main physiological causes: Low UVB exposure, especially in northern regions during the winter season [34] and in case of strong pigmentation, as well as decreased vitamin synthesis in the skin with aging [35]. In addition a poor diet, low in fish and fortified food (if available) are the major reason for deficiency in old age and people living in poverty. Major risk groups [36], besides pregnant women and children under 5, include elderly, over 65 years, those with little or no sun exposure (full body coverage, little contact with the outside world) as well as people with dark skin, especially in Europe and the USA.

The vitamin D deficiency is a worldwide problem, which is not only observed in the northern countries, but increasingly also in the south. While in Europe, for example, deficits (< 30 nmol) are between 20 and 60% in all age groups, in Asia the figure for children is 61% (Pakistan, India) and 86% (Iran) [37,38].

Particularly critical is the number of migrants from Southern countries with insufficient vitamin D status (< 25 nmol/L) [39]: e.g. Netherlands 51%, Germany 44% (in summer), UK 31% (end of summer) and 34% (autumn). In India, the number of adults with values < 25 nmol/L ranges from 20% to 96% depending on the region.

The half-life of 25(OH)D_3 is about 15 days and that of 25(OH)D_2 is between 13 and 15 days, due to the weaker affinity to the vitamin D binding protein [40]. Consequently, longer periods of time indoor, e.g. in care homes or longer time in quarantine, pose risk for developing vitamin D deficiency.

1.5. Risk factors for severe courses of COVID-19

Older age and co-morbidities are linked to an insufficient vitamin D supply. Over 60 years of age, a reduction in the synthesis of vitamin D in the skin becomes apparent, which further increases getting older [41]. The precursor of vitamin D, 7-dehydrocholesterol in the skin declines about 50% from age 20 to 80 [42], and the elevation of cholecalciferol levels in serum following UVB radiation of the skin shows more than a 4-fold difference in individuals aged 62–80 yrs. compared with controls (20–30 yrs) [43]. This explains the high number of older individuals with an inadequate vitamin D status.

Based on a meta-analysis including 30 studies with 53.000 COVID-19 patients, co-morbidities are risk factors for disease severity:

Risk factor	Odds ratio	95% CI
Old age > 50 yrs	2.61	2.29–2.98
Male	1.38	1.195–1.521
Smoking	1.734	1.146–2.626
Any co-morbidity	2.635	2.098–3.309
Chronic kidney disease	6.017	2.192–16.514
COPD	5.323	2.613–10.847
Cerebrovascular disease	3.219	1.486–6.972

Independent prognostic factors for COVID-19 related death:

Risk factor	Relative risk	95% CI
Old age > 60	9.45	8.09–11.04
CVD	6.75	5.40–8.43
Hypertension	4.48	3.69–5.45
Diabetes	4.43	3.49–5.61

Co-morbidities and old age show a relationship with Renin-Angiotensin-Aldosteron-System (RAS), vitamin D status and COVID-19 infection.

1.6. The renin-angiotensin-system (RAS)

RAS plays an important role in maintaining vascular resistance and extracellular fluid homoeostasis. Fig. 1 summarizes the essential steps of this system.

Mainly in the juxtaglomerular apparatus of the kidney, but also in other tissues and cells, renin is formed, which cleaves the angiotensinogen secreted from the liver very selectively to the inactive form angiotensin I (Ang I). This decapeptide is then cleaved by a further protease the angiotensin-converting-enzyme (ACE) on the surface of the endothelial cells to the active angiotensin II (Ang II), which can bind to two different receptors AT1R or AT2R. Synthesis and secretion of renin in the kidney, as rate limiting enzyme of RAS, is stimulated by fluid volume, reduction of the perfusion pressure or salt concentration and by the sympathetic nervous system activity.

Renin synthesis and secretion is inhibited with increasing Ang II via an AT1R mediated effect and stimulated with decreasing Ang II [44]. The stimulating effect on renin synthesis and secretion due to either low levels of Ang II or Ang II converting inhibitors (ACEI) or Ang II receptor blockers (ARB) is mediated through ligands that activate cAMP/PKA (Protein Kinase A) pathways (e.g. catecholamines, prostaglandins and nitric oxide) [45,46].

Ang II leads to the release of catecholamines and vasoconstriction. Via AT1R, Ang II increases aldosterone release and sodium reabsorption. Furthermore, binding to AT1R has pro-inflammatory and pro-oxidative effects and inhibits the action of insulin in endothelial and muscle cells. The latter can lead to a decrease in NO production in endothelial cells and thus will further increase vasoconstriction [47].

With the discovery of ACE2, a novel homologue of ACE, a transmembrane metallopeptidase with an extracellular ectodomain, the understanding of RAS manifold regulatory function was deepened (Review [48]). ACE2, a monocarboxypeptidase has been shown to cleave Ang I to Ang 1–9, and Ang II to Ang 1–7. This degradation can weaken the effect of Ang II at AT1R and thus counteract the pathological changes. While Ang 1–9 exerts a cardioprotective effect via AT2R [49], Ang 1–7 acts via the Mas Oncogene receptor. This counterbalances the effect of ANG II at AT1R and subsequently the “overstimulation” of the RAS and its pathological consequences [50]. ACE2 is expressed in many organs, especially kidney and lung, and in the cardiovascular system in cardiomyocytes, cardiac fibroblasts, vascular smooth muscle and endothelial cells. It can counteract the effects of RAS, such as inflammation, vasoconstriction, hypertrophy and fibrosis,

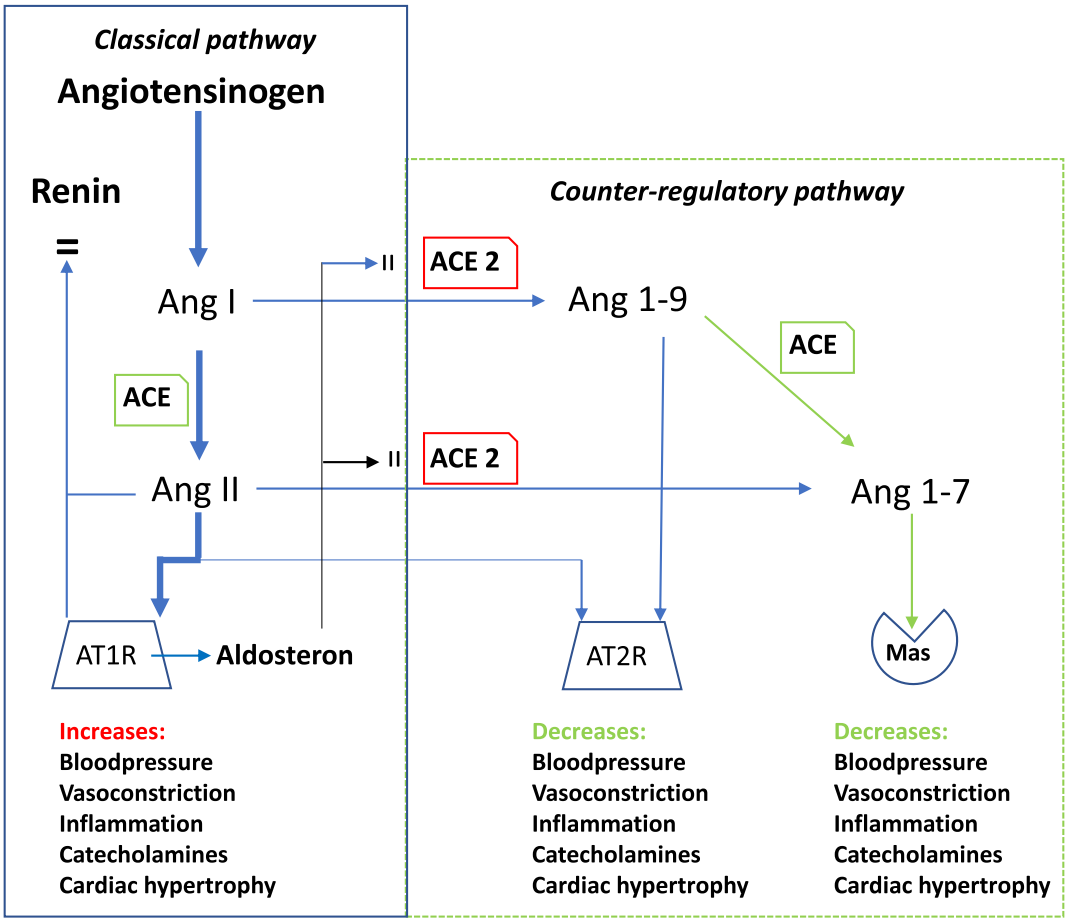

Fig. 1. In the classical RAS pathway Renin, expressed from the renin gene induces cleavage of Angiotensinogen to Angiotensin I which is converted to Angiotensin II via Angiotensin converting enzyme (ACE). Ang II activates the Angiotensin 1 receptor which results in an increase of blood pressure and further effects on the vascular system. In addition, Ang II suppresses renin synthesis via AT1R. To keep the system in balance a counter regulatory pathway exists. This pathway is activated through cleavage of Ang I to Ang1–9 via ACE2 or AT2R activation or Ang II to Ang1–7 which counter regulates via Mas receptor. This helps the system to stay within a homoeostatic balance, as long as the RAS activity is controlled.

by degrading Ang I and Ang II, thus making them less available for the ACE/AngII/AT1 axis. At the same time ACE2 can strengthen the ACE2/Ang 1–7/Mas axis which attenuates the proinflammatory RAS activation.

1.7. RAS and SARS-CoV-2

Infection with SARS-CoV-2 causes the virus spike protein to come into contact with ACE2 on the cell surface and thus to be transported into the cell. This endocytosis causes upregulation of a metallopeptidase (ADAM17), which releases ACE2 from the membrane, resulting in a loss of the counter regulatory activity to RAS [51]. As a result, proinflammatory cytokines are released extensively into the circulation. This leads to a series of vascular changes, especially in the case of preexisting lesions, which can promote further progression of cardiovascular pathologies.

SARS-CoV-2 not only reduces the ACE2 expression, but also leads to further limitation of the ACE2/Ang 1–7/Mas axis via ADAM17 activation, which in turn promotes the absorption of the virus. This results in an increase in Ang II, which further upregulates ADAM 17. Thus a vicious circle is established turning into a constantly self-generating and progressive process. This process may contribute not only to lung damage (Acute respiratory distress syndrome - ARDS), but also to heart injury and vessels damage, observed in COVID-19 patients. Thus, previous lesions of the cardiovascular system represent a risk factor, since coexisting pathologies can progress as a result of the virus infection [52,53].

1.8. RAS and vitamin D deficiency

Several studies have shown increased plasma renin activity, higher Ang II concentrations and higher RAS activity as a consequence of low vitamin D status [54,55]. The same applies to the decreasing Renin activity with increasing vitamin D levels [56]. There is an inverse relationship between circulating 25(OH)D and renin, which is explained by the fact that vitamin D is a negative regulator of renin expression and reduces renin expression by suppressing transcriptional activity in the renin gene promoter, thus acting as a negative RAS regulator to prevent overreaction In VDR knock out mice [57,58]. The 1,25(OH)2D induced repression of the renin gene expression is independent from Ang II feedback regulation.

Permanent increase of the renin levels with an increased Ang II formation has been described, suggesting that in vitamin D deficiency the expression and secretion of renin is increased at an early stage [59,60]. This results in increased fluid and salt intake and rise in blood pressure, that has been explained by an increase in renin and consecutive upregulation of the RAS in the brain [61].

Fig. 2 gives a short description of the impact of vitamin D on RAS.

In a small (open-label, blinded endpoint) study with 101 participants who received 2000 IU vitamin D3 or placebo over 6 weeks, a significant decrease in plasma renin activity and concentration was described [62].

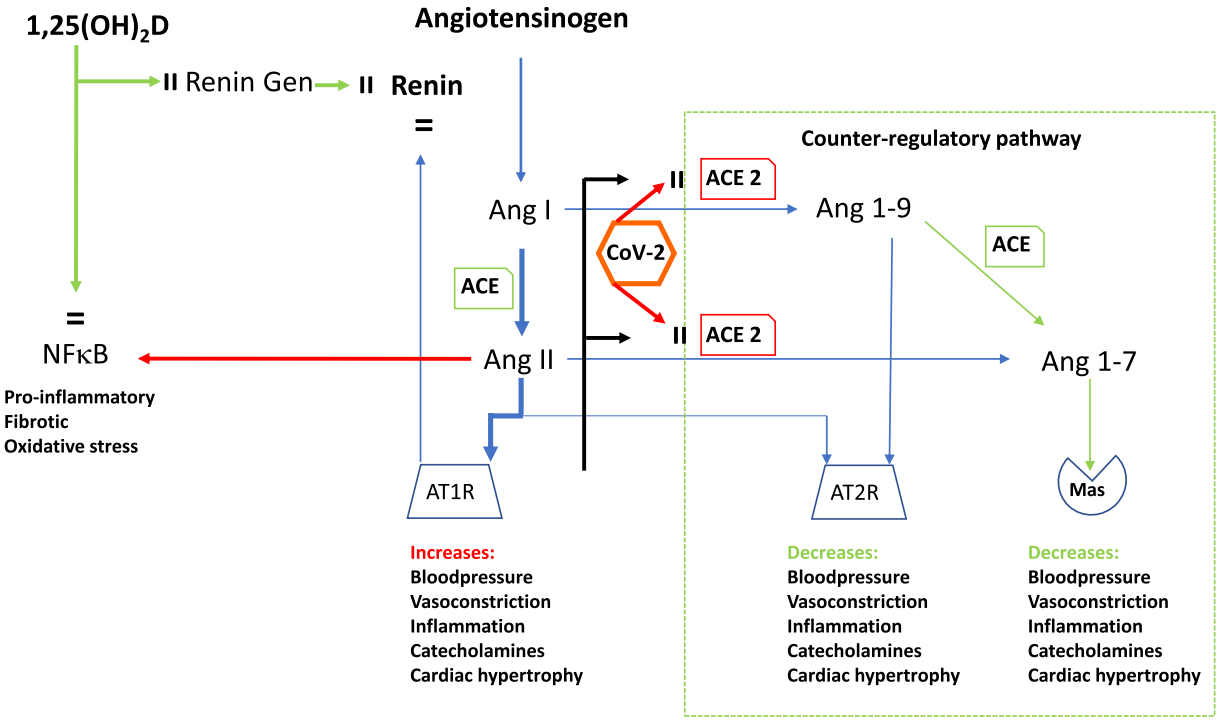

Fig. 2. If the system is dysbalanced this may result in a rising formation of Ang II and a higher renin synthesis which at least increases inflammatory responses. This is important in cases of a poor vitamin D status because vitamin D ($1,25(OH)_2D$) can counteract the disbalance via negative expression of the renin gen which results in lower renin synthesis independent from Ang II. An increase of aldosterone will block the activities of the ACE2 and as a consequence attenuate the counter regulatory balance. If the counter regulatory circle is disrupted via ACE2 dysfunction due to SARS-CoV2 infection an uncontrolled classical pathway will run out of control and increase proinflammatory reactions and blood pressure and contribute to a couple of problems (e.g. cardiovascular, ARDS, Kawasaki disease). Ang II activates NFκB through AT1 receptors [194]. This and further interactions of the RAS with inflammatory stimuli results in an increasing and less controlled inflammatory reaction. Beside its effect on renin expression vitamin D can effectively inhibit NFκB activation [195]. This is especially efficient when the VDR is upregulated, which also plays an important role in other processes in the immune system through vitamin D activity.

The EVITA study examined the effect of vitamin D supplementation (4000 IU/day) over 36 months [63]. No relationship was found between blood levels of 1,25(OH)2D and various parameters of the RAS (renin, aldosterone) and vitamin D plasma levels increase. Rather, vitamin D supplementation led to an increase in renin in a subgroup that initially had a mild deficiency of vitamin D. The 25(OH)D value in these subgroups increased from 20.4 nmol/L to 83.7 nmol/L after 36 months. Renin from 859 mIU/L to 1656mIU/L. It cannot be excluded that these were rather toxic effects of a dose in the upper level range. However, the fact that blood levels increase naturally reduced the renin concentration become clear when looking at the placebo group with initial hypovitaminosis D (21.3 nmol/L) with a strong increase after 36 months (45.6 nmol/L). Renin decreases from the initial value of 507 to 430mIU/L after 36 months. According to this, a moderate suppressive effect of vitamin D is conceivable under physiological conditions and in particular in participants with a compensated vitamin D deficiency. The plasma level of renin and 1,25(OH)2D show a significant inverse correlation in hypertensive individuals [64]. In a study on 184 normotensive participants, higher circulating Ang II levels were associated with decreasing 25(OH)D blood levels. After infusion of Ang II there was a blunted renal blood flow, both effects were considered RAS activation in the setting of lower plasma 25(OH)D [65].

1.9. Vitamin D, blood pressure, and COVID-19 mortality

Vitamin D supplementation leads to a reduction in blood pressure in patients with essential hypertension [66,67], and to a reduction in blood pressure, plasma renin activity and angiotensin II levels in patients with hyperparathyroidism [68,69]. Low vitamin D status may contribute to increased activity of the RAS and subsequent higher blood pressure. An inverse relationship between the concentration of the active metabolite 1,25(OH)2D3 and blood pressure has been described in hypertensive as well as normotensive individuals [70,71]. In a study using the mendelian randomization approach in 35 trials (146,581 participants) with four SNPs (Single Nucleotid Polymorphism), a causal relationship was shown between increasing 25(OH)D levels and decreased risk of hypertension in individuals with genetic variants leading to low Vitamin D plasma levels [72].

Depending on the study, the number of COVID-19 patients affected with hypertension was between 20 and 30% and the proportion of diabetics between 15 and 22% [73]. Data from 5 studies in Wuhan (n:1458) reported 55.3% and 30.6% cases respectively of hypertension and of diabetes [74]. 49% of the 1591 patients in ICUs in Italy (Lombardy), 1287 of whom needed respirators, had hypertension and were older than the normotensive ones [75].,

Hypertension, followed by diabetes (16.2%), was the most frequent concomitant morbidity in patients with severe course disease [76,77,78].

1.10. Vitamin D and cardiovascular diseases

Vitamin D has multiple functions in the cardiovascular system and thus represents an important protective factor of endothelial, vascular muscle, and cardiac muscle cells [79]. In a meta-analysis of 65,994 participants an inverse relationship between 25(OH)D vitamin D plasma levels (below 60 nmol/L) and cardiovascular events was shown [80]. These findings have been confirmed by the Framingham and NHANES data [81,82]. As for the positive effects on respiratory diseases shown by vitamin D supplementation, also for cardiovascular disease positive effect was reported only if there was a vitamin D-deficit before supplementation.

In a large cohort of patients ($n = 3296$) referred to coronary

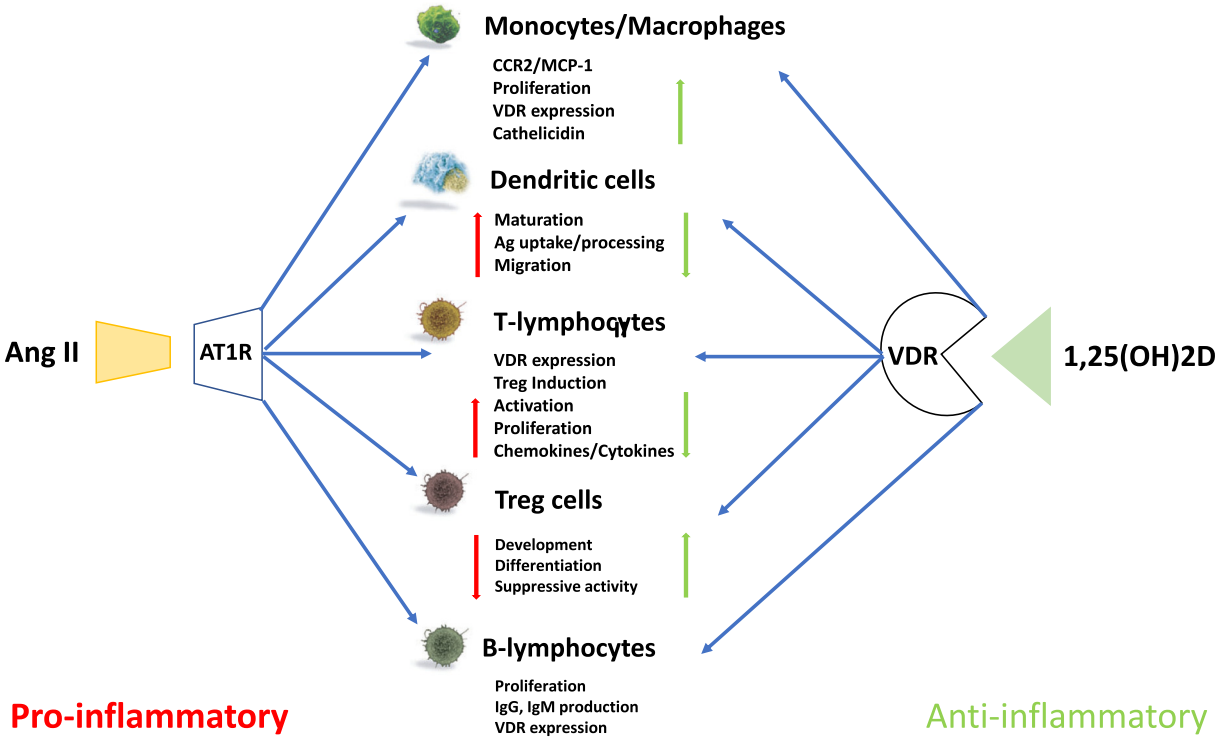

Fig. 3. Ang II leads to a series of pro-inflammatory stimuli in the immune system via the activation of AT1R. These include an increase in the expression of MCP-1 as well as the chemokine receptor CCR2, which lead to a massive infiltration of the endothelium with macrophages. The same applies to the activation, migration and maturation of dendritic cells (DC) and the antigen (Ag) presentation. The negative effect on T lymphocytes as well as on T regulatory cells further promotes a pro-inflammatory state. A number of other proinflammatory processes are triggered by AT1R and favor the development of inflammation, hypertension and diabetes. Vitamin D is considered to counteract this reaction by contributing to a normalization of immune function through a variety of processes. However, it should not be overlooked that most processes in the immune system initiated by vitamin D occur together with vitamin A [196].

angiography, a significant increase in plasma renin and angiotensin II was observed with decreased 25(OH)D and 1,25(OH)2D levels, but not with circulating aldosterone levels [83]. Vitamin D plasma levels are an independent risk factor for CVD mortality. 92% of 1801 patients with metabolic syndrome, had a low vitamin D status (22.2% were severely deficient (25(OH)D < 25 nmol). CVD mortality and total mortality were reduced respectively by 69% and 75% in those with highest 25(OH)D levels (> 75 nmol/L) [84].

CVD is considered an independent risk factor for fatal outcome in COVID-19 patients. The proportion of survivors with CVD was 10.8%, among non-survivors 20% [85]. Disturbed coagulation, endothelial dysfunction and proinflammatory stimuli described as a result of a viral infection are considered to be among the major causes [86].

1.11. Vitamin D, obesity and type II diabetes

Obesity (BMI > 30 kg/m2) is often associated with low 25(OH)D plasma level [87,88]. Using a bi-directional genetic approach, 26 studies (42,024 participants - Caucasians from Northern Europe and America), including 12 SNPs, showed that higher BMI (Body Mass Index) leads to lower 25(OH)D plasma levels. The repeatedly discussed hypothesis that low 25(OH)D level leads to increased BMI could not be verified [89]. Obesity is therefore another risk factor for an insufficient vitamin D status independent from age [90].

Low 25(OH)D plasma values are also found in diabetes II [91,92]. This is often associated with an increased risk of metabolic syndrome, hypertension and cardiovascular diseases [93,94]. One of the main causes could be insulin resistance, often found in connection with low vitamin D levels [95]. This is well documented by the evaluation of observational and intervention studies using metabolic indicators. 10 out of 14 intervention studies showed a positive effect of Vitamin D on metabolic indicators [96]. Vitamin D deficiency is therefore also considered to be a potential link between obesity and diabetes type II [97].

Via a short-loop feedback Ang II inhibits the further release of renin via AT1R.

If the renin secretion is not sufficiently inhibited, an overreaction of the RAS can lead to a further increase in blood pressure, increased sodium reabsorption, increased aldosterone secretion and thus increased insulin resistance [98]. This overreaction is considered to be a major cause of the development of hypertension, diabetes and cardiovascular disease, especially in people with high BMI, since adipose tissue contributes to an overreaction of the RAS [99]. Adiponectin synthesis in adipocytes counteracts most of these effects, however circulating levels are inversely related to BMI [100,101]. Vitamin D can regulate the formation and release of adiponectin [102,103]. Obese people often have low adiponectin and vitamin D levels and an inverse relationship between fat mass and vitamin D levels has been described [104]. Therefore, vitamin D deficiency might explain RAS overreaction and following consequences [105].

In a small study on 124 IUC patients with SARS-CoV-2 it was found that obesity (BMI > 35 kg/m^2) occurred in 47.6% of the cases and severe obesity (BMI > 35 kg/m^2) in 28.2% [106]. In the latter case, 85.7% had to be mechanically ventilated invasively, 60 patients (50%) had hypertension, 48 of these (80%) had to be ventilated invasively. A study from Shenzhen, China also confirmed that obesity is a risk factor for severe course of disease. In a cohort of 383 patients with COVID-19, overweight patients (BMI 24–27.9) had 86% higher risk of developing pneumonia and obese patients (BMI > 28) had 142% higher risk of developing pneumonia compared to normal weight patients [107].

1.12. Vitamin D and ARDS (adult respiratory distress syndrome)

The main cause of death in COVID-19 patients is ARDS. Patients (without COVID-19) (mean age 62 Y) with ARDS (n:52) and those at high risk of ARDS (n:57) (esophagectomy) had low (27.6 nmol/L) to very low (13.7 nmol/L) 25(OH)D blood levels as a sign for severe vitamin D deficiency [108].

ACE2 exerts a counter-regulation of the harmful effect of ACE. Ultimately, it would then be the balance between ACE and ACE2 that explains the reaction of the RAS. The ACE2 effect on the RAS is shown in experimental studies in which ACE2 knock out mice developed severe lung disease with increased vascular permeability and pulmonary edema [109]. Over-expression or the use of recombinant ACE2 improves blood flow and oxygenation and inhibits the development of ARDS after LPS-induced lung damage [110,111].

The development of ARDS shows typical changes in membrane permeability of the alveolar capillary, progressive edema, severe arterial hypoxemia and pulmonary hypertension [112]. The same changes can be achieved in animal experiments by injection of lipopolysaccharides (LPS) [113]. Vitamin D significantly attenuates the lung damage caused by LPS. LPS exposure leads to a significant increase in the pulmonary expression of renin and ANGII. This promotes the pro-inflammatory effects of the conversion of AngII via AT1R and suppresses ACE2 expression. The administration of vitamin D was able to reduce the increased renin and AngII expression and thus significantly lower the lung damage. The authors conclude that this may have been due to the reduction of the renin and ACE/AngII/AT1R cascade and the promotion of ACE2/Ang1–7 activity by vitamin D through its influence on renin synthesis.

Increased ACE and ANGII expression and reduced ACE2/Ang1–7 expression in lung tissue favors lung damage induced by ischemia reperfusion in mice [114]. The ACE/Ang1–7 expression and the amount of circulating Ang 1–7 was increased at the onset of ischemia and then decreased rapidly in contrast to the tissue concentration, while AngII increased. This suggests a dysregulation of local and systemic RAS. The application of recombinant ACE2 was able to correct the dysregulation and attenuate the lung damage, while ACE2 knock out increased the imbalance and was associated with more severe damage. Inhibition of the ACE/AngII/AT1R pathway or activation of the ACE2/Ang1–7 pathway have therefore been proposed as therapeutic options.

In rats with LPS-induced acute lung injury (ALI), the administration of vitamin D (calcitriol) was associated with a significant reduction in clinical symptoms of ALI. Calcitriol treatment led to a significant increase in the expression of VDR mRNA and ACE2 mRNA. VDR expression may have resulted in a reduction of angiotensin II, ACE2 expression in increased anti-inflammatory effects [115].

VDR is not only a negative regulator of renin, but also of NFkB [116], leading both to an increase in Ang II formation [117], which in turn promotes pro-inflammatory cascades. Furthermore SARS-CoV-2 infects T-lymphocytes [118] and the Covid-19 disease severity seems to be related to lymphopenia [119], which occurs in 83,2% of COVID-19 patients at hospital admission [120]. Indeed, in a recent meta-analysis on 53.000 COVID-19 patients decreased lymphocyte count and increased CRP were highly associated with severity [121].

Regulatory T cells (Treg) play an important role in the development of ARDS [122]. They can attenuate the pro-inflammatory effects of the activated immune system. Vitamin D increases the expression of Treg cells and supplementation of healthy volunteers results in a significant increase in Tregs [123]. Vitamin D causes a reduction in pro-inflammatory cytokines by inhibiting B- and T-cell proliferation [124,125]. Inflammatory processes also play an important role in the development of hypertension and CVD [126,127]. Here, an interesting but so far not proven connection between vitamin D and RAS is found. T-cells have a RAS system, which contributes to the generation of reactive oxygen species (ROS) and the development of high blood pressure through the formation of Ang II [128]. To what extent vitamin D in T cells is also a negative regulator of renin is not known, but could be one of the reasons for the anti-inflammatory effect [129].

1.13. Cytokine storm: Vitamin D, SARS-CoV-2, and ACE2

In patients with a severe disease course (ARDS) a cytokine storm is assumed to be the underlying cause [130]. SARS CoV-2 can lead to a downregulation of ACE2 in the lungs and to a shedding of the ectodomain of ACE2. This soluble sACE2 shows enzymatic activity, but the biological role is unclear. The soluble form is believed to exert systemic influence on angiotensin II [131]; since SARS-CoV-2 induces shedding, it is assumed that sACE2 is directly related to the virus- induced inflammatory response [132].

Downregulation of ACE2 expression by SARS-CoV infection is associated with acute lung damage (edema, increased vascular permeability, reduced lung function) [133] and with RAS dysregulation leading to increased inflammation and vascular permeability. Inflammatory cytokines such as TACE (TNF-a-converting enzyme) induce increase shedding [134], which in turn can be also caused by spike protein of the virus, promoting virus uptake by ACE2 [135]. Comparative studies on mortality rates in different countries and analysis of the relationship between vitamin D and CRP (as a marker of cytokine storm) plasma levels, concluded that.

risk factors for severity of the clinical course, predicted by high CRP and low vitamin D (< 25 nmol) levels, were reduced by by 15.6% following vitamin D status normalization (> 75 nmol) [136]. It is interesting to note that calmodulin kinase IV (CaMK IV) stimulates vitamin D receptor (VDR) transcription and interaction with co-activator SRC (steroid receptor coactivator) [137]. According to the authors, this would explain the linkage of the genomic and non-genomic membrane pathways of vitamin D. The calmodulin binding domain at ACE2 [138] may explain why calmodulin inhibits the shedding of the ectodomain of ACE2 [139]. It is also conceivable that vitamin D may show significant effects either by stimulating VDR-mediated transcription, or by mediating 1,25(OH)D calcium-dependent activity through CaMK II and phospholipase A [140].

1.14. Kawasaki syndrome

Children and adolescents rarely show severe disease courses. A meta-analysis comprising 18 studies with 444 children under 10 years of age and 553 between 10 and 19 years of age, reported only one case of severe complication in a 13-year-old child. In North America, 48 cases of children (4.2–16.6 yrs) have been described with severe disease course. Independently of this, COVID-19 children have a clinical picture that has not been associated with usual acute clinical manifestations of SARS-CoV-2 infection, showing an unusually high proportion of children with gastrointestinal involvement, Kawasaki disease (KD) like syndrome, until now [141].

KD is an acute vasculitis which can lead to aneurysms of the coronary arteries and is considered the leading cause of acquired heart disease in children [142]. A number of cases have been observed in recent weeks suggesting a relationship between Kawasaki syndrome and COVID-19 [143].

One reason probably relies upon ACE gene polymorphisms [144]. In these polymorphisms there is a strong increase in ACE without affecting AngII plasma levels [145]. There is a direct relationship between ACE polymorphism (with high ACE plasma levels) and the occurrence of KD, according to a recent meta-analysis [146].

Irrespective of this, the disease occurs seasonally during the winter months in extratropical northern atmosphere and is often associated to respiratory tract infections [147]. A KD associated Antigen was found in proximal bronchial epithelium in 10 out of 13 patients with acute KD and in a subset of macrophages of inflamed tissues [148]. That strengthens the hypothesis that an infectious agent entering the respiratory tract, might be the cause of KD. Indeed, it was reported that

children with KD were affected by respiratory diseases with HCoV: New Haven coronavirus [149]. The authors concluded that there was a significant association between KD and HCoV-NH infection.

Just like current evidence suggest that vitamin D-deficiency is associated with increased risk of CVD, including hypertension, heart failure, and ischemic heart disease, patients with KD also show very low vitamin D levels. Children with KD (79) had significantly lower 25(OH) D levels (9.17 vs 23.3 ng/ml) compared to healthy children of the same age [150].

Intravenous immunoglobulin (IVIG) has become the standard therapy for KD [151], with a good therapeutic response from young patients, of which only 10–20% need additional anti-inflammatory medication [152]. In a study on 91 KD children, 39 of them with very low plasma vitamin D levels (< 20 ng/ml), showed immunoglobulin resistance compared to the rest of the children (n = 52) children with higher levels (> 20 ng/ml) [153]. Children with immunoglobulin resistance also have a higher incidence of coronary artery complications [154,155].

The relationship between ACE polymorphism and peripheral vascular disease is observed in Asians but not in Caucasians [156,157]. Furthermore the prevalence of KD in Japan (240/100,000) is 10 times higher than in North America (20/100,000) [158,159]. During February and April 2020, 10 cases of COVID-19 and KD were reported in Bergamo, Italy, corresponding to 30 times higher rate than the last 5 years incidence [160]. The higher incidence of KD in Asian children (35.3 cases/100,000) as reported in California, may indeed indicate a more frequent ACE polymorphism in Asian population, followed by African-Americans (24.6/100,000) probably due to the fact that pigmentation reduces vitamin D production in the skin [161] compared to white children (14.7/100.000). From 189 children hospitalized between 1991 and 1998 136 (72%) of the children were African-American and 43 (23%) were white [162]. It is conceivable that Vitamin D deficiency which activates the RAS, promotes the development and course of KD.

1.15. Therapeutic aspects

1.15.1. Vitamin D status

The aim of a therapy with vitamin D should be a normalization of the vitamin D status, preferably > 75 nmol/L. Basically, it can be assumed that a vitamin in physiological doses can do little more than remedy the symptoms or secondary manifestations of a deficiency. Vitamin D is a prohormone. Therefore, the question of correcting the status should be treated in the same way as for other hormones (e.g. thyroid hormone). Before starting therapy, the plasma level should be determined. This allows a dosage and therapy to be initiated that corresponds to the respective status. The analysis should be carried out especially in risk groups (Table 1) in order to be able to react adequately, especially in acute cases. The general recommendation to supplement with a recommended daily dose (800 IU) may apply to people who do not belong to a risk group, are healthy.

The vitamin D status is the basis for treatment with vitamin D. There are indeed, risk groups were a poor status can be expected.

As it is known that the amount of 25(OH)D circulating in the blood and less the active metabolite 1,25(OH)2D is a better indicator for a deficit, threshold values have been set here (Table 2).

Table 2
Threshold levels to calculate deficiency ranges (25(OH)D).

Severe	< 12.5 nmol/L	< 5 ng/ml
Moderate	12.5–29 nmol/L	5–11.6 ng/ml
Mild	30.0–49 nmol/L	12–19.6 ng/ml
Sufficient	> 50 nmol/L	> 20 ng/ml[165]
	> 75 nmol/L	> 30 ng/ml[166]
Toxicity	> 250 nmol/L	> 100 ng/ml

A vitamin D status below 20 ng/ml or < 50 nmol/L should be treated to achieve a minimum level of 30 ng/ml (75 nmol/L). Values around 75 nmol/L are considered optimal, with respect to the skeletal activities [167]. Particularly in countries where vitamin D fortified foods are not available, the importance of an adequate supply should be emphasized. A sufficient vitamin D status can be achieved in the healthy populations following the recommendations and the thresholds of the plasma levels. In case of comorbidities related to the clinical development of COVID-19 there might be a higher need and therefore it is discussed to choose other recommendations for the adequate care of persons with chronic diseases [168,169].

A recent meta analysis related to vitamin D and respiratory tract infections showed that a daily or weekly Vitamin D dose between 20µg and 50µg resulted in a significant reduction of infections [170]. An isolated or added bolus with high doses (2.5 mg once or monthly) did not reduce risk. One study supplemented adults with high risk for ARDS with a 100µg/daily for one year [171]. The overall infection score was significantly reduced in the treated group. Those with an initial vitamin D deficit showed the greatest benefit of the supplementation. With respect to COVID-19 a recommendation for primary prevention of vitamin D deficiency seems meaningful. Whether this will be prevention against COVID related diseases remains speculative. If a patient belonging to a risk group is delivered to the hospital, vitamin D status should be immediately assessed and in case of insufficiency (< 50 nmol/L) or deficiency (< 25 nmol/L) higher doses might be needed as recommended by the NHS [172].

The recommendations of the National Health Service UK are based on those of various professional associations. It should be noted that vitamin D therapy is contraindicated for patients with hypercalcemia or metastatic calcification. Suggested therapy should be used when low plasma levels and the following symptoms are present:

- muscle pain
- Proximal muscle weakness
- Rib, hip, pelvis, thigh and foot pain (typical)
- Fractures.

So far, there is no experience on the use of vitamin D in COVID-19. The observation that a normal vitamin D status is important for the immune system as well as for the regulation of the RAS should, however, lead to a correction of the Vitamin D status if a deficiency is detected. Nevertheless, it should be borne in mind that high doses of

Table 1
Risk factors for deficiency (NHS) [163].

Inadequate skin synthesis	Poor oral supply	Co-Morbidities
Air pollution	Vegetarian or fish	Reduced synthesis
Northern latitude/Winter	Free diet	Increased breakdown
Occlusive garments	Malabsorption	Drugs: rifampicin, HAART-
Pigmented skin	Short bowel	Therapy, ketoconazole
Habitual sunscreen use	Cholestatic jaundice	Anticonvulsants
Institutionalized/housebound and people with poor mobility	Pancreatitis	Glucocorticoids
Age > 65	Celiac disease	CKD (eGFR < 60) [164]

vitamin D also carry risks, as they can contribute to changes in VDR competence and thus have n inhibitory effect on immune function (Ref: Mangin M, Sinha R, Fincher K. Inflammation and vitamin D: the infection connection. Inflkamm Res 2014; 63: 803-811)

The importance of a vitamin D deficiency is shown by a recently published analysis of the COVID-19 deaths of 780 COVID-19 patients in Indonesia [173].

table 3 data of patients with COVID-19 related to vitamin D levels and disease outcome

	Vitamin D: < 20 ng/ml	20-30 ng/ml	> 30 ng/ml
Overall, N	179	213	388
Mean age	66.9 ± 13.8	62.9 ± 14.7	46.6 ± 12.6
Comorbidity, %	80.0	73.8	18.8
Death, %	98.9	87.8	4.1
Active, %	1.1	12.2	95.9
Odds ratio	10.12 ($p < .001$)	7.63 ($p < .001$)	
Adjusted for age, sex and comorbidity			

The table illustrates thate old age, comorbidities and vitamin D deficiency or insufficiency contributed to outcome of the disase. Based on thes data Vitamin D plasma level is an independent precitor of mortality.

1.15.2. VDR agonists (VDRA)

VDRA are discussed to counteract the effect of imbalanced immune response and have suppressant effects on the RAS. Since VDRA have been observed to contribute to a significant reduction of inflammatory processes, they are increasingly used in immunosuppressive therapy to control TH1-related overreactions via interaction of VDRA with the chemokine CXCL10, a T cell chemoattractant chemokine [174]. The induction of CXCL10 is an important step against bacterial and virus infections. However, sustained CXCL10 induction leads to amplified neuroinflammation in Coronavirus (JHMV) induced neurologic infection [175]. CXCL10 is also considered a critical factor in ARDS. H5N1 influenza infection in mice resulted in increased CXCL10 secretion with a consequent inflamed neutrophils massive chemotaxis and a subsequent pulmonary inflammation [176]. Following SARS-CoV-2 infection, CXCL10 and other chemo- and cytokines are upregulated [177]. Anti CXCL10 antibodies have shown ARDS improvement following LPS induced lung injury with high CXCL10 levels [178].

Additionally evidence from animal models (diabetic nephropathy) has shown that VDRA block TGFß system in the glomerulus and thus abolish interstitial fibrosis [179]. It is assumed that VDRA modulates increased RAS activity. Indeed, a clinical study on 281 patients (type II diabetes with albuminuria) revealed that VDR activator paricalcitol (19-nor-1,15-dihydroxyvitamin D_2) led to a significant albuminuria reduction as well as a decrease in blood pressure despite increased salt intake, as a sign of decreased RAS activity [180]; effect that could not be achieved with losartan (ANG II receptor antagonist) [181].

1.15.3. Morphine

Morphine medication is an essential part of treatment for COVID patients with severe ARDS. it is used early for dyspnea or pain and for shivers [182]. Morphine, at doses similar to those used in humans, can lead to downregulation of VDR in human T cells and activation of RAS with renin upregulation and a threefold increase in Ang II production, resulting in increased reactive oxygen species (ROS) responsible for DNA damage and T cells apoptosis .

VDR agonist (EB1089) inhibits VDR downregulation, leading to RAS decreased activity, inhibition of morphine induced ANG II production, reduced ROS formation and lower DNA damage, thus inhibiting T-cell apoptosis [183]. In addition, if Jurkat cells were pretreated with EB 1089 and Losartan, an Angiotensin II receptor antagonist (ARB) before incubation with morphine. The combination of the Vitamin D Receptor agonist and Losartan attenuated the morphine-induced ROS formation. Indeed, as an example ARB increase ACE2 expression [184] and Ang 1–7/Mas axis activation reduced ROS formation [185].

1.15.4. Autophagy, spermidine and vitamin D

Spermidine is a metabolite of polyamines which are delivered through the diet and partially metabolized by colon bacteria from undigested proteins. Polyamines can influence macrophages development into pro-inflammatory or anti-inflammatory type by altering cellular metabolism and triggering mito- and autophagy [186]. The capacity of spermidine to ensure proteostasis through the stimulation of the cytoprotective autophagy is acknowledged as one of its main features.

Recently, the effect of spermidine on autophagy in SARS-CoV-2 infected cells which results in inhibition of autophagy has been described [187]. Since spermidine promotes autophagy, spermidine and other agents may be a therapeutic approach to SARS-CoV-2 infection.

With regard to the specific risk of elderly to develop severe course of SARS-CoV-2 infection, it is interesting to note that spermidine concentrations in organs and cells decline with age and resulting in a decrease of autophagy [188]. Consumption of LKM512 yogurt increases spermidine synthesis in the gut in elderly [189]. Whether that has any impact on supply of spermidine to enterocytes or other tissues remains to be elucidated. Spermin and spermidine but not putrescine another polyamine metabolite can activate VDR in vitro within their physiological intracellular concentrations [190]. Vitamin D and VDR play an important role in autophagy. Vitamin D can induce autophagy similar to spermidine by inhibiting mTORC1 complex activation [191] and by increasing Beclin-1 expression, similar to spermidine [192].

2. Limitations

A major limitation of al studies dealing with low levels of vitamin D and disease is the fact that there are only few studies, which show a causal relationship. Most studies show associations and data regarding the influence of COVID-19 on vitamin D status are missing. Furthermore, it should not be overlooked that many of the effects of vitamin D on genexpression in the immune system occur together with vitamin A. The effect of vitamin A deficiency in COVID-19 has not yet been investigated. However, vitamin A deficiency or combined deficiencies with vitamin D or other micronutrients exists not only in low income countries. .

3. Conclusion

An inadequate supply of vitamin D has a variety of skeletal and non-skeletal effects. There is ample evidence that various non-communicable diseases (hypertension, diabetes, CVD, metabolic syndrome) are associated with low vitamin D plasma levels. These comorbidities, together with the often concomitant vitamin D deficiency, increase the risk of severe COVID-19 events. Much more attention should be paid to the importance of vitamin D status for the development and course of the disease. Particularly in the methods used to control the pandemic (lockdown), the skin's natural vitamin D synthesis is reduced when people have few opportunities to be exposed to the sun. The short half-lives of the vitamin therefore make an increasing vitamin D deficiency more likely. Specific dietary advice, moderate supplementation or fortified foods can help prevent this deficiency. In the event of hospitalisation, the status should be urgently reviewed and, if possible, improved.

In the meantime, 8 studies have started to test the effect of supplementing vitamin D in different dosages (up to 200,000 IU) on the course of the COVID-19 disease. The aim is to clarify whether supplementation with vitamin D in different dosages has an influence on the course of the disease or, in particular, on the immune response, or

whether it can prevent the development of ARDS or thromboses [193].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Responsibility for vitamin D supplementation of elderly care home residents in England: falling through the gap between medicine and food

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ABSTRACT

Introduction Daily vitamin D supplements are recommended for elderly care home residents; however, they are rarely given and vitamin D deficiency in care homes is widespread. This study aimed to understand the determinants of current practice and perceived responsibility for the vitamin D status of residents.

Methods Thirteen semi-structured interviews were conducted with key informants in two areas of Southern England including care home managers, general practitioners (GPs) and public health professionals. Interviews were audio recorded and transcribed verbatim.

Results Inductive thematic analysis identified four themes: framing of vitamin D supplements as medicines; professional and sector boundaries whereby GPs are perceived as responsible for the vitamin D status of residents and care home managers felt unable to administer over-the-counter vitamin tablets; low awareness of national guidance; and ethical and practical issues. This results in vitamin D supplements requiring prescription by medical professionals and few residents receiving them.

Conclusion The medical framing of vitamin D supplements in care homes is a practical barrier to implementation of longstanding nutrition guidelines. A paradigm shift is needed so that vitamin D is understood as a protective nutrient as well as a medicine, and a public health as well as a medical responsibility. Vitamin D is important for musculoskeletal health. Possible links with COVID-19 are still being investigated. The pandemic has drawn attention to conditions in care homes and there is an opportunity to revise current guidance on vitamin D supplementation which will have lasting benefit for this vulnerable group.

What this paper adds

- This study suggests that the vitamin D status of elderly care home residents is perceived as the responsibility of GPs, and care home staff would feel vulnerable to suggestions of malpractice in administering over-the-counter vitamin D supplements.
- Vitamin D supplements are regulated as foods. This study found that in the elderly residential care sector they are framed as medicines and only given when prescribed. Their limited use puts residents at risk of vitamin D deficiency and poor musculoskeletal health.
- Nutrition recommendations for vitamin D supplementation rely on personal responsibility: this is not effective in a population constrained by institutional living. A review of guidelines and regulations in England is required to establish responsibility for implementing public health recommendations on vitamin D supplementation in elderly residential care homes.

INTRODUCTION

Public health is classically defined as "the art and science of preventing disease, prolonging life and promoting health through the organised efforts of society".[1] Arguably societies organise themselves more readily to improve the health of those in view. Those out of sight, including older adults in residential care settings, can often be forgotten. The coronavirus (COVID-19) pandemic has brought the health and vulnerability of those in elderly care homes to the fore.[2] Vitamin D has also received attention due to possible links with COVID-19.[3 4] Our investigation into how vitamin D nutrition supplementation is addressed in care homes reveals both a failure to implement evidence-based recommendations and a social injustice in urgent need of public health advocacy and resolution.

Vitamin D is a prohormone rather than a nutrient and the main source is endogenous synthesis in the skin. Synthesis occurs when skin is exposed to ultraviolet B (UVB) radiation in sunlight. In winter months at latitudes with shorter day lengths, the UVB is insufficient due to the low angle of the sun, thus vitamin D deficiency can be viewed as sunshine deficiency.[3 5] There are few significant food sources and it is usually not possible to meet vitamin D needs from diet alone.[6]

Vitamin D is required for the regulation of calcium and phosphorus metabolism and there is strong evidence that insufficient

vitamin D affects musculoskeletal health and development.[7] Vitamin D has also been cited as having a potential role in numerous other aspects of health, including immunity, cardiovascular health, neurological conditions, respiratory infections, lung function and cancer.[7] Most recently, there has been interest in the potential role of vitamin D status in the susceptibility to COVID-19 and the striking overlap between risk factors for severe COVID-19 and vitamin D deficiency.[4 8–10] Older people and Black, Asian and minority ethnic groups are at increased risk of vitamin D insufficiency due to spending less time outside, covering their skin, or having darker skin which needs longer exposure for synthesis to occur.[6 11] Rapid reviews in the UK concluded that there was currently insufficient evidence to support taking vitamin D supplements to prevent or treat COVID-19, but reiterated recommendations made in April 2020 advising everyone to consider taking 400 IU (10 µg) of vitamin D a day if they were indoors most of the day due to COVID-19 lockdown measures.[12–14]

Elderly residents in residential care homes/nursing homes (hereafter referred to as care homes), particularly those with limited mobility, are likely to spend more time indoors and have limited sun exposure. For nearly 30 years they have been recognised in the UK as a group vulnerable to vitamin D deficiency and requiring routine supplementation without pre-screening .[7 15 16] In 2016, recommendations for daily vitamin D supplements were extended to the entire population in the winter months, and throughout the year for those living in care homes.[17] National bodies from countries around the world have issued similar recommendations for care home residents including Australia, Canada, France, Norway, New Zealand and the USA.[18 19]

Despite the decades of recommendations for supplementation in this population, vitamin D deficiency in care home residents is widespread throughout Europe, Asia and the Americas.[18] Studies in Austria,[20] Belgium,[21] Germany[22] and Sweden[23] found that almost all residents of care homes were vitamin D deficient. A notable exception is New Zealand where a publicly funded universal vitamin D supplementation programme for care homes has been in operation since 2011. An evaluation in 2014 found that 75% of care home residents took supplements, and almost all of those receiving the supplements had healthy serum levels of 25-hydroxyvitamin D (25(OH)D), the marker for vitamin D status.[24]

Whether vitamin supplements are considered as a medicine or a food has significant implications. In most countries vitamin and mineral supplements are regulated and sold as foodstuffs rather than medicines.[25–27] Supplementation with vitamin D as a food is considered a personal responsibility which, in a care home setting, could be passed on to family members or care home staff. However, if vitamin D supplements are considered as medicines, responsibility is deferred to medical professionals. The duality of vitamin D as both a medicine and a food is evident in contradictory health and care regulations and guidance in England. Care homes are required to assess resident's nutritional needs and provide food to meet those needs, including "dietary supplements when prescribed by a healthcare professional".[28] Guidance to NHS clinical commissioning groups expressly advises GPs against routine prescription of vitamins in primary care due to "limited evidence of clinical effectiveness" and because "vitamin D supplements can be bought cheaply and easily".[29] The guidance makes exceptions for medically diagnosed vitamin D deficiency or for osteoporosis but not for maintenance or preventative treatment.

The aim of this study was to better understand current practice in the implementation of public health guidance on vitamin D supplementation in the care home setting. The purpose was to generate insights which could be used to inform and advocate for a policy and practice review as a first step towards increasing vitamin D supplement uptake and reducing vitamin D deficiency in elderly care home residents.

METHODS

The research was carried out using a pragmatic interpretive methodology. Semi-structured interviews were used to explore current practice around vitamin D supplementation, perceived responsibility for vitamin D status and supplementation, and barriers to supplementation. Interviews were conducted by telephone with key stakeholders identified as having a role in the care of elderly care home residents. The study used purposive opportunistic sampling to recruit participants, including: senior members of care home staff; general practitioners (GPs); members of local authority public health departments; and other relevant professionals (as identified by participants) with shared constituencies of care home residents. Eligible participants were approached by email and invited to participate. Informed consent was obtained from all participants before commencing the interview. Recruitment was continued until data saturation was reached.

Data collection and analysis

Interviews were conducted between 22 April 2018 and 31 August 2018, audio recorded and transcribed verbatim. The data were approached inductively using a six-stage process as described by Braun and Clarke.[30] JW read and re-read the transcripts for immersion in the data and developed initial codes and themes. All transcripts were independently read and coded by CW, using qualitative data software (NVivo) to support the process. Codes and themes were elaborated using an iterative process, with the two researchers referring back to the raw data to substantiate emerging ideas.

RESULTS

Thirteen interviews, lasting approximately 15 minutes, were conducted with employees of 13 different

Table 1 Participant demographics

Areas	Coding	Position	Organisation
A	GP1, GP2	Two general practitioners	Two general practices
	CHM1, CHM2	Two care home managers	Two care homes
	PubH1	Senior public health practitioner	Local authority
B	GP3, GP4	Two general practitioners	Two general practices
	CHM3, CHM4	Two care home managers	Two care homes
	FallSP	Falls specialist practitioner	NHS community trust
	EConsP	Consultant elderly care physician	NHS hospital trust
	PCDiet	Primary care dietitian	NHS clinical commissioning group
	PubH2	Public health programme manager	Local authority

organisations operating in two districts of non-neighbouring areas of South East England. Participant roles and employing organisations are outlined in table 1. Four broad themes and five sub-themes were identified; these are summarised in box 1 and described in detail below.

Vitamin D understood as a medical issue

All the stakeholders in elderly care we spoke to referred to vitamin D as something which residents took on an individual basis, usually because it had been prescribed by a GP. None knew of any care home where vitamin D supplements were given routinely to residents as part of a protective public health measure; *"So there's no kind of protocol or carpet/universal plan to prescribe vitamin D supplements to patients of ours at care homes"* (GP3). GP prescribing was in response to falls or fractures or following diagnosis of vitamin D deficiency or osteoporosis. (Quotations in box 2 illustrate this theme.)

Professional and sector boundaries

Perceived responsibility and roles

There was a strong agreement, including from the GP participants, that the vitamin D status of elderly care home residents was the responsibility of the GP (see box 3). Only the dietitian suggested that perhaps the primary responsibility should lie with the care home since the home had a "*responsibility to provide a diet that includes all the nutrients they need*" (PCDiet). One of the GPs touched on the issue of medicine versus diet that is central to this subject "*…if you're looking at it from a medical point of view, then I, the GP, would be responsible. If you're looking at this from a 'are the patients getting a well-balanced diet that would include vitamin D? …' I would see that as the home's responsibility*" (GP1). Several of the non-medical participants felt the responsibility was shared between the care home and the GP, but here the GP was the decision maker and their role was to alert GPs about individual residents. From the interviews it was clear that there was an implicit hierarchy and deferment to the medical profession which meant care home staff felt they would not be able to make decisions about supplements: "*we can only ask the doctor if they will prescribe it*" (CHM1). Participants from the medical profession acknowledged and understood why care home staff could feel constrained or vulnerable in this area.

Cost implications—who pays and provides?

There was considerable ambiguity about who should be responsible for providing and paying for supplements. Again, most participants felt this was a GP responsibility because of their role as prescriber, and any change from this arrangement would have financial consequences for the care home or the residents, as noted in the quotes

Box 1 Summary of themes and sub-themes

Themes and sub-themes

1. Medical framing: vitamin D understood as a medical issue
2. Professional and sector boundaries
   - Perceived responsibility and roles: authority and vulnerability
   - Cost implications: who pays and provides
   - Policy, regulations, guidance and compliance
3. Low awareness of recommendations and need for vitamin D supplements.
4. Ethical and practical considerations
   - Ethics of consent and life stage
   - Resistance to polypharmacy

Box 2 Representative quotations for theme 1: vitamin D understood as medical issue

"Quite a lot of the vitamin D stuff that we tend to prescribe is mixed in with calcium as well… Usually that will be in people who are either known to be osteoporotic or may have had a previous low trauma fracture before." (GP1)

"We don't have anything in place in terms of a protocol for specifically checking it with each patient. It tends to come up in those where it's more obvious because of their history. And if we happened to think about it, which probably means missing quite a few patients who (would) benefit from it." (GP2)

"No. Literally since I've been here, there's only been two occasions where residents have gone on to vitamin D. And that's because they've had routine blood tests done, and it's identified their levels were low." (CHM4)

Box 3 Representative quotations for theme 2: professional and sector boundaries

Sub theme: perceived responsibility and roles: authority and vulnerability

"*I would say it's us as their GPs. I guess. Because we've got all their notes. And while, so some of them are nursing homes and got trained nurses. But some of them aren't, some of them just have carers there, so we wouldn't expect it to have to come from them.*" (GP2)

"*I think its probably meant to be a mix of all of us. But I think it probably falls through the gaps.*" (GP4)

"I mean, if I go in and, as part of my assessment I find that somebody is no longer going out, their diet's quite poor, they're at risk of falls. I may, I will write to the GP. And may suggest that they consider a supplement." (FallSP)

"*It's really, really difficult to know for us because we're only carers, if somebody needs vitamin D or not. How would I know that somebody needs vitamin D?*" (CHM3)

"*The trouble is we're very highly regulated against… we can't just decide to give people a supplement.*" (CHM1)

"…if the nurses or care staff are giving out these medications then they run into risks about whether or not they've assessed properly that people should be having them. If then there's going to be any interaction between that over-the-counter medication and the prescribed medications. So, the practicalities of dispensing over-the-counter medications in care homes are very difficult." (PCDiet)

Subtheme: cost implications—who pays and provides?

"*I mean, I don't think necessarily we would expect the types of patients who are in care homes or rest homes to necessarily be purchasing it themselves.*" (GP3)

"*And then of course it takes cost out of the issue for the home and for the individual if I prescribe… But for the whole system as a whole, prescribing vitamin D is a lot more expensive than buying it over the counter.*" (GP1)

"We're not going to start prescribing supplements for everybody. The cost would be prohibitive." (PCDiet)

Subtheme: policy, regulation, guidance and compliance

"*There's no kind of local practice guidance on that* [prescribing vitamin D to all residents]. *So, there's nothing saying, you know, we should or shouldn't be doing it. So, it's just not done. As a blanket. Within our practice… I think if there was guidance to do it then we would probably do it.*" (GP3)

"*How do we get over the concept of people wanting to take something that doesn't have to be prescribed and then we get into a care home or a rest home situation that the system can allow that without putting the home at risk of being accused of doing something wrong. And equally, allowing the individual in the bed to have the right to say 'actually, I want some over-the-counter vitamins'.*" (GP1)

"*If a resident wants to take it privately, then that's fine. They can do it as a homely remedy. For a certain amount of time. But it has to be agreed by the GP if they want to take it on a long-term basis.*" (CHM1)

in box 3. The theoretical responsibility of individual residents paying for their own over-the-counter (OTC) supplements and the impractical reality of this for residents with mobility or cognitive constraints was acknowledged. One GP participant considered the financial aspects more broadly, contrasting the cost of testing serum vitamin D levels which is far higher than the cost of a year's supply of vitamin D supplements.

Box 4 Representative quotations for theme 3: low awareness of recommendations and need for vitamin D supplements

"I mean some of the patients will be more well than others, and I guess if they're outside getting a bit of sunlight and their diet is pretty good, they wouldn't necessarily need to be on it." (GP2)

"So I would never even think about a resident having vitamin D or why they haven't got vitamin D." (CHM3)

"*…the message it's not been very clear, about vitamin D, especially for falls prevention… So, we haven't taken it forward because we're not clear, you know, what the message would be. Is it that every older person should be taking vitamin D?… I'll see one thing saying* 'yes' *and then another saying* 'well, not really'." (PubH2).

Policy guidance and compliance

Each stakeholder we spoke to was adhering to the regulations and operating guidance for their sector. In the absence of any specific guidance about how to implement public health advice on vitamin D supplementation, these restricted homes to only dispensing vitamin D on prescription: "*If somebody said to us that everybody needs to have vitamin D we would make sure that everybody had vitamin D*" (CHM3). Three of the four care homes had a 'homely remedy' policy that can provide a mechanism for care homes to give OTC products to residents.[31] However, they explained that the GP needs to be involved for the supplementation to continue in the long-term. (Quotations in box 3 illustrate this theme.)

Low awareness of national guidance and need for vitamin D supplements

Apart from the dietitian, none of the participants seemed familiar with the recommendation that all elderly care home residents should receive vitamin D supplements without the need for pre-assessment, as illustrated by quotes in box 4. Some participants highlighted the role of diet and sunlight in vitamin D status but seemed unaware that these sources cannot provide adequate levels even for the general population during winter months. Several of the GPs referred to care homes needing to ensure that residents got out into the sun, which could be effective in the summer months, but mobility issues mean this is impractical for many residents.

Ethical and practical considerations

When asked about universal supplementation, several participants raised ethical issues, both around the population versus the individual approach and the limited ability of some residents to give consent: "*We have a lot of people with dementia who can't make that decision for themselves*" (CHM1). Others questioned the value of introducing another tablet given the age of residents.

Care home staff are responsible for the administration of medicines and supplements to residents and highlighted practical issues which would be barriers to wider supplement use. This included the number of medicines residents already take and how introducing more

Box 5 Representative quotations for theme 4: ethical and practical considerations

Sub-theme: ethics of consent and life stage

"*To supplement everybody without it being their individual choice. Or you know maybe, they could offer it …without people having to take it.*" (GP4)

"I don't know how much difference they make at their age, if I'm being perfectly honest." (CHM2)

Sub-theme: resistance to polypharmacy

"*It's OK for a GP to come out and say* 'yes, vitamin D, they need to boost their levels*'. It's actually trying to support and encourage the resident to actually understand why they need to take that tablet. Because, as far as they're concerned, they've reached a certain age and have never needed it. So why do they now need it?*" (CHM4)

"*Also, the other issue with vitamin D supplements. Quite often it comes with the calcium. So, it's normally a calcium and vitamin D supplement… Some older people just don't like taking it… It's not particularly nice. It's chalky.*" (CHM1)

tablets would add to the pill burden for both residents and staff. Another common point related to the formulation of vitamin D with calcium and how it was difficult to take and was *"one of our most destroyed medicines"* (CHM2). (Quotations in box 5 illustrate this theme.)

DISCUSSION

This study aimed to understand perceived responsibility for the vitamin D status of care home residents and explore factors influencing supplementation practice. The study found an overwhelmingly medical rather than public health conception of vitamin D by those involved in the welfare of elderly care home residents. This stems from regulatory and professional boundaries which treat vitamin D as a medicine and denies residents recommended dietary supplements to protect their vitamin D levels. These findings may be used to inform advocacy for policy and practice change in England and other countries where vitamin D supplementation in elderly care homes tends to be by prescription-only.

Medicine or food

The participants of this study all saw vitamin D supplementation as the responsibility of the GP. This medical framing means care home staff fear overstepping their role and defer to medical professionals to diagnose vitamin D deficiency or osteoporosis as a medical condition and then prescribe vitamin D as a "medicine" in response. In this respect, both care home staff and GPs are complying with the respective regulations and guidance governing their sector: care home staff to administer supplements only when they are prescribed; and GPs not to prescribe supplements for prevention or maintenance. As a result, few elderly care home residents receive vitamin D supplements.

The medicalisation of this nutrient is at odds with the UK's nutrition advisory committee Scientific Advisory Committee on Nutrition (SACN), and other nutrition expert panels, who consider vitamin D supplements, along with natural food sources and fortified foods, as a dietary source.[7] It is also out of step with public health recommendations which identify a population-wide need for vitamin D. Low awareness of the public health need for supplements is not unique to the elderly residential care sector; others have noted that many health professionals may be not be aware of issues around vitamin D, including synthesis, diet and the importance of supplements.[32] Furthermore, national guidance on healthy catering for older people in residential care makes a general statement on the need for vitamin D supplements without addressing how this happens in practice or who is responsible.[33]

Our findings suggest that in the elderly residential care sector, the narrative of vitamin D as a treatment is at the expense of any parallel narrative of vitamin D as a protective and preventative public health measure. Regulations and guidance must ensure both approaches are permitted. In time, a reliable preventative supplementation strategy should lessen the need for treatment of deficiency.

Social justice perspective

The recommendations for vitamin D rely on personal responsibility and it is questionable whether this is appropriate in populations with limited autonomy. Community-dwelling elderly and independent care home residents can purchase their own vitamin D supplements for personal use. However, people often move into care homes because they no longer have mobility or the mental capacity to live independently, and accordingly have more limited control over lifestyle decisions. Most elderly care home residents have some form of dementia.[34] This results in a two-tier system, discriminating against those with the least cognitive independence. Even if residents or their family purchase OTC vitamin D supplements and request that these are administered by care staff, the GP still needs to approve their use. They would not be covered by homely remedy policies since these are limited to OTC products for short term treatment of minor ailments.[31 35] The regulatory environment would make it difficult for elderly care homes to unilaterally decide to offer all residents daily vitamin D supplements, not least because residents are frequently registered with different GPs.

Finance

Even in a universal healthcare system such as the NHS, there are significant and often competing financial considerations for the different agencies involved in elderly care. The care home sector in England is fragmented with more than 15 000 residential and nursing care homes operated by thousands of different providers.[36] Depending on resident's circumstances, funding for places comes directly from residents and their families, local authorities, the NHS or charities. As highlighted by one of the participants, the involvement of GPs means

care homes, residents or other funders do not need to purchase supplements. However, GP time is scarce and costly and using NHS prescribing as a vehicle for getting vitamin supplements to a sizeable at-risk population is a poor use of NHS resources. One year's supply of OTC vitamin D costs approximately £15.00 per person at typical high street prices. These costs could be absorbed into existing care home fees or covered by local authorities. A contrast is made with the provision of free vitamin D through the NHS Healthy Start scheme for children under 5 years in low income families on benefits, which some local authorities fund for all children.[37] We believe equivalent arrangements should be in place for the frail elderly. This would enable care staff to be confident in the administration of such supplements and remove the need for medical prescribing and the associated cost.

Formulation of vitamin D

Vitamin D supplements can be formulated as tiny tablets, and are also available to be administered as drops, chewable and dispersible formulations which are easier to take than when combined with calcium. However, the additional burden to care staff of being required to administer a supplement in any form needs to be acknowledged. Using an appropriately fortified food stuff would shift the provisioning of vitamin D firmly to care homes as part of their food service. This would also eliminate the need for revising existing controls on medicines in care homes. However, the literature on the use of vitamin D fortified foods in the elderly is limited and the feasibility needs further research.[38 39]

Nutritional well-being and the ethics of inaction

Many frail elderly in the community suffer undernourishment.[40] The move into a care home can improve nutrition as meals are provided and residents receive support with eating. Vitamin D needs to be part of this care. Other commentators in this journal have noted that policies and recommendations on vitamin D do not seem to be "taken seriously enough".[4] The COVID-19 pandemic has brought conditions in care homes into the public eye and on to the political agenda. While practices in care homes are in the spotlight there is an urgent need for action to ensure vitamin D recommendations can be applied in care homes.

Limitations of the study

While this study provides insight into some of the underlying reasons why care home residents tend not to receive preventative vitamin D supplements, it is a small study and practices may be different in other areas. To improve the generalisability, we used purposive sampling in two non-neighbouring areas of southern England, and included stakeholders employed by a range of organisations. The fact that participants referred to national policy drivers as key determinants of current practice suggests the finding may be generalisable more widely in England. The literature suggests that the core issue of medicalisation of vitamin D may be relevant across the care sector in other countries.[18]

CONCLUSION

Daily vitamin D supplements are recommended for the entire UK population and the public are expected to obtain them as OTC supplements from pharmacies and other retailers.[7] Residents of elderly care homes are particularly at risk of vitamin D deficiency but have limited ability to make lifestyle decisions of this kind. This study highlights that a gap exists between public health guidance and practice around vitamin D supplementation in the care sector: the professionals involved in the care of elderly residents perceive vitamin D as a medicine rather than a food. This means not enough elderly care home residents receive vitamin D supplements. As a result, their vitamin D levels remain low and they are at increased risk of falls and poor muscle strength and function.[7] Evidence on possible links with vitamin D status and COVID-19 is currently inconclusive,[12] but this should not detract from addressing a system which neglects the nutritional well-being of a group vulnerable to COVID-19.

Vitamin D in care homes needs further attention with mechanisms developed for the administration of low-risk dietary supplements or fortified foods by care staff. This will help us to progress from a situation where care staff feel constrained and vulnerable, to one where they are supported to improve the health of those in their care. For a universal, population-based approach, vitamin D supplementation at protective levels needs a professional separation from medicine and reframing as a matter of public health nutrition.

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Clinical Research Article

Clinical Research Article

Vitamin D Status in Hospitalized Patients with SARS-CoV-2 Infection

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Abbreviations: 25OHD, 25-hydroxyvitamin D; ARDS, acute respiratory distress syndrome; CV, coefficient of variation; GFR, glomerular filtration rate; ICU, intensive care unit; IL, interleukin; IMID, immune-mediated inflammatory disease; IQR, interquartile range; PTH, parathyroid hormone; RAS, renin–angiotensin system; SD, standard deviation.

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Abstract

Background: The role of vitamin D status in COVID-19 patients is a matter of debate.
Objectives: To assess serum 25-hydroxyvitamin D (25OHD) levels in hospitalized patients with COVID-19 and to analyze the possible influence of vitamin D status on disease severity.
Methods: Retrospective case–control study of 216 COVID-19 patients and 197 population-based controls. Serum 25OHD levels were measured in both groups. The association of serum 25OHD levels with COVID-19 severity (admission to the intensive care unit, requirements for mechanical ventilation, or mortality) was also evaluated.
Results: Of the 216 patients, 19 were on vitamin D supplements and were analyzed separately. In COVID-19 patients, mean ± standard deviation 25OHD levels were 13.8 ± 7.2 ng/mL, compared with 20.9 ± 7.4 ng/mL in controls ($P < .0001$). 25OHD values were lower in men than in women. Vitamin D deficiency was found in 82.2% of COVID-19 cases and 47.2% of population-based controls ($P < .0001$). 25OHD inversely correlates with serum ferritin ($P = .013$) and D-dimer levels ($P = .027$). Vitamin D-deficient COVID-19 patients had a greater prevalence of hypertension and cardiovascular diseases, raised

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serum ferritin and troponin levels, as well as a longer length of hospital stay than those with serum 25OHD levels ≥20 ng/mL. No causal relationship was found between vitamin D deficiency and COVID-19 severity as a combined endpoint or as its separate components. **Conclusions:** 25OHD levels are lower in hospitalized COVID-19 patients than in population-based controls and these patients had a higher prevalence of deficiency. We did not find any relationship between vitamin D concentrations or vitamin deficiency and the severity of the disease.

Freeform/Key Words: 25OHD, PTH, SARS-CoV-2 infection, COVID-19

There are several lines of evidence that might support a role for vitamin D status in SARS-CoV-2 infection. Firstly, vitamin D deficiency is a common condition all around the world, and serum 25-hydroxyvitamin D (25OHD) levels follow a well-known seasonal and geographical pattern. Spain, located in temperate zones of the Northern Hemisphere, but with a higher prevalence of vitamin D deficiency (1), has reached very high rates of SARS-CoV-2 infection and lethality (2). Secondly, vitamin D is a steroid hormone involved in the modulation of the innate and acquired immune system and also in the production of antimicrobial peptides, such as cathelicidin and human β-defensin-2, as well as in the expression of genes involved in the intracellular destruction of pathogens (3-5). Thirdly, low serum 25OHD levels are frequently found in elderly individuals or in those with chronic conditions, such as hypertension, diabetes, cancer, or cardiovascular diseases, which have also been reported as poor prognostic factors for COVID-19 (6-11). Finally, the downregulation of ACE2 by SARS-CoV-2 leads to a dysregulation of the renin–angiotensin system (RAS), which contributes to the "cytokine storm" that precedes the acute respiratory distress syndrome (ARDS) characteristic of the severe form of COVID-19. In this sense, vitamin D can inhibit proinflammatory cytokine production in human monocytes/macrophages (12), and chronic vitamin D deficiency may induce RAS activation, leading to the production of fibrotic factors and, therefore, lung damage (13).

Taking into account the above considerations, we aimed to assess the serum 25OHD levels in hospitalized patients with COVID-19 compared with population-based controls. The possible association between serum 25OHD concentrations and COVID-19 severity and mortality was also analyzed.

Patients and Methods

Study design and participants

The study consists of 2 parts. Firstly, we have designed a retrospective case–control study including 216 patients aged ≥18 years with confirmed COVID-19 admitted to the University Hospital Marqués de Valdecilla in Santander, northern Spain, from March 10 to March 31, 2020, and 197 sex-matched population-based controls recruited from the Camargo Cohort (14, 15) during their last follow-up visit in January to March of the past year. From the present study, we have excluded patients or controls with malabsorption disorders, liver cirrhosis, serum creatinine levels >1.9 mg/dL, or previous treatment with anticonvulsants. Nineteen COVID-19 patients on oral vitamin D supplements for more than 3 months at admission were analyzed as a separate group, and controls who receive these supplements were also excluded from the study. Secondly, we have assessed only the group of COVID-19 patients to evaluate the possible influence of vitamin D deficiency on the outcome of the disease. Participants from the Camargo Cohort gave their informed written consent and the study was approved by the Cantabria Clinical Research Ethics Committee (internal code 2016.003). The present study was approved by the Ethics Committee of Cantabria (internal code 2020.55). Serum samples from Covid-19 patients were provided by the IDIVAL Biobank samples collection (internal code 2020-126).

Data collection

Demographic, clinical, and outcome data of COVID-19 patients were gathered from hospital records, stored in a computerized database, and independently reviewed by 2 researchers. Missing data were not imputed. Smoking status was coded as current or nonsmoker. Immunosuppression included prolonged use (≥3 months) of corticoids (>10 mg/day of prednisone or equivalent) or immunomodulatory agents, and bone marrow or organ transplantation. Chest X-ray and/or computed tomography scans were performed in all COVID-19 patients. Concerning immunomodulatory therapy, patients were selected for tocilizumab according to our institutional protocol. Thus, tocilizumab was indicated if there was clinical worsening with PaO_2/FIO_2 ratio <300 and high serum acute-phase reactant levels when no contraindication for its use was present. The endpoint variable for COVID-19 severity has been defined as the

composite of admission to the intensive care unit (ICU), requirement for mechanical ventilation, or in-hospital mortality. Clinical outcomes were monitored up to May 20, 2020. Overall, the criteria for ICU admission were those of the guidelines by the American Thoracic Society and Infectious Diseases Society of America (16) and the critical care ethic recommendations for the SARS-CoV-2 pandemic by the Intensive Medicine Spanish Society (17). ARDS was the main cause of ICU admission and a case-by-case assessment was carried out by the medical COVID team, including intensivists.

Laboratory measurements

Qualitative detection of RNA from the SARS-CoV-2 was performed by using real-time polymerase chain reaction. Blood samples from the controls were obtained from an antecubital vein in the morning after a requested 12-hour overnight fast. The serum was divided into 0.5-mL aliquots and stored at –40°C. Routine biochemical parameters were measured by standard automated methods in a Technicon Dax autoanalyzer (Technicon Instruments, CO, USA). Human interleukin (IL)-6 was measured by enzyme-linked immunosorbent assay (Enzo Life Sciences, Inc. Farmingdale, NY) following the manufacturer instructions. The sensitivity for serum IL-6 levels was 0.057 pg/mL. Intra- and interassay precision was 4.38% and 9.6%, respectively. Serum 25OHD concentrations were determined in controls by a fully automated electrochemiluminescence system (Elecsys 2010, Roche Diagnostics, GmbH, Mannheim, Germany). The detection limit of serum 25OHD was 4 ng/mL. The intra-assay coefficient of variation (CV) was 5% and interassay was 7.5%. In COVID-19 patients, serum 25OHD levels were obtained at admission and assessed by automated competitive chemiluminescence assay (Liaison XL, DiaSorin Inc, Stillwater MN, USA). Our laboratory is DEQAS (Vitamin D External Quality Assessment Scheme) certified for this parameter. The detection limit of serum 25OHD was 4 ng/mL. The intra-assay and interassay CV were 2.58% and 7.83%, respectively. We have previously found a correlation between both techniques of 0.926 ($P < .0001$) with a random sample of 52 subjects from the Camargo Cohort.

Statistical analysis

Continuous variables were expressed as mean ± standard deviation (SD) or median and interquartile range and compared with the Student's t-test or Mann–Whitney U test according to the distribution of data. Categorical variables were presented as numbers and percentages and compared using the chi-squared test or the Fisher exact test as appropriate. Spearman rho was used to assess the relationships between serum 25OHD levels and several clinical and laboratory parameters. Serum 25OHD levels were stratified into 4 categories: below 10 ng/mL, between 10 and 20 ng/mL, between 20 and 30 ng/mL, and above 30 ng/mL. Vitamin D deficiency was defined as serum 25OHD levels <20 ng/mL (50 nmol/L) following a recent position paper by the European Calcified Tissue Society Working Group (18). A multivariable general linear model was set up to compare serum 25OHD levels between COVID-19 patients and controls (Bonferroni test), adjusting for confounding variables. In the group of COVID-19 patients, univariable and multivariable binary logistic regression analyses were used to assess the association between vitamin D (as a continuous variable, or expressed as vitamin D deficiency or as quintiles) and the dependent variable of severity of the disease. A 2-sided *P*-value less than .05 was considered statistically significant in all the calculations. A post hoc power analysis with the present sample size and the obtained difference in serum 25OHD levels between cases and controls yields a power of 100% to detect this difference. In fact, a difference of 2.1 ng/mL between groups already yields a potency of 89.8%. Nevertheless, due to the sample size and the lower number of events (especially mortality) in COVID-19 patients with and without vitamin D deficiency, the post hoc power analysis for the severity endpoints was lower than 40%.

Results

We included 216 adult COVID-19 patients, of whom 19 were on vitamin D supplementation (11 patients were taking cholecalciferol, 25 000 IU/monthly in 10 cases, and 5600 IU/weekly in 1, and 8 patients were on calcifediol, 0.266 mg/monthly). The main demographic, epidemiological, and clinical characteristics of the 3 groups included in the study are summarized in Table 1. COVID-19 patients on vitamin D supplements were mainly women and had a greater prevalence of hypertension and immunosuppression than the other 2 groups analyzed. Population-based controls included more smokers and had a lower glomerular filtration rate and greater serum parathormone levels than COVID-19 patients.

Table 2 summarizes the demographic, clinical, and laboratory data of COVID-19 patients (excluding those on vitamin supplements) according to the presence of vitamin D deficiency (serum 25OHD levels <20 ng/mL. Vitamin D-deficient COVID-19 patients had a greater prevalence of hypertension and cardiovascular diseases, raised serum ferritin and troponin levels, as well as a longer length of hospital stay than those with serum 25OHD levels ≥20 ng/mL.

The features of COVID-19 patients according to the active use of vitamin D supplements are shown in Table 3.

Table 1. Main baseline features of COVID-19 patients and controls

Variable	COVID-19 N = 197	COVID-19_D N = 19	Controls N = 197	P^a	P^b	P^c
Age (years), median (IQR)	61.0 (47.5-70.0)	60.0 (59.0-75.0)	61.0 (56.0-66.0)	.082	.153	.182
Sex (male), n (%)	123 (62.4)	7 (36.8)	123 (62.4)	**.030**	.999	**.030**
BMI (kg/m^2), mean ± SD	29.2 ± 4.7	30.9 ± 6.3	28.9 ± 4.0	.134	.557	**.035**
Current smoker, n (%)	14 (7.1)	2 (10.5)	34 (17.3)	.638	**.002**	.747
Hypertension, n (%)	76 (38.6)	12 (63.2)	87 (44.2)	**.037**	.260	.113
Diabetes, n (%)	34 (17.3)	0 (0.0)	31 (15.7)	**.049**	.684	.083
Cardiovascular disease, n (%)	21 (10.7)	3 (15.8)	22 (11.2)	.451	.872	.468
COPD, n (%)	15 (7.6)	2 (10.5)	9 (4.6)	.650	.206	.250
Active cancer, n (%)	7 (3.6)	0 (0.0)	8 (4.1)	.999	.792	.999
Immunosuppression, n (%)	16 (8.1)	6 (31.6)	2 (1.0)	**.006**	**.001**	**<.0001**
ACEI / ARA2 agents, n (%)	58 (29.4)	7 (36.8)	47 (23.9)	.502	.210	.265
GFR-MDRD-4 (mL/min/1.73 m^2), median (IQR)	92.2 (73.9-113.4)	85.9 (69.9-104.9)	71.9 (63.3-91.5)	.213	**<.0001**	.053
C-reactive protein (mg/dl), median (IQR)	5.60 (2.63-11.85)	7.30 (2.90-15.10)	0.25 (0.10-0.50)	.756	**<.0001**	**<.0001**
Corrected calcium (mg/dL), median (IQR)	8.5 (8.3-9.0)	8.7 (8.4-9.0)	9.1 (8.9-9.3)	.175	**<.0001**	**<.0001**
25OHD (ng/mL), mean ± SD	13.8 ± 7.2	21.1 ± 5.9	20.9 ± 7.4	**<.0001**	**<.0001**	.914
PTH (pg/mL), median (IQR)	42.6 (32.3-62.6)	53.7 (28.8-67.4)	51.6 (42.5-65.2)	.389	**<.0001**	.719

Abbreviations: BMI, body mass index; COPD, chronic obstructive pulmonary disease; ACEI, angiotensin-converting enzyme inhibitors; ARA2, angiotensin-receptor 2 antagonists; GFR, glomerular filtration rate; MDRD, modification of diet in renal disease; 25OHD, 25-hydroxyvitamin D; PTH, parathyroid hormone.
[a]COVID-19 group vs COVID19_D group.
[b]COVID-19 group vs Controls.
[c]COVID-19_D group vs Controls.

Patients on supplements had a significantly lower PaO_2/FIO_2 ratio <300 prevalence, lower serum ferritin levels, and received less frequently tocilizumab than COVID-19 patients who did not take vitamin D supplements. They also had an overall lower percentage of the combined severity endpoint and ICU admissions, as well as a shorter length of hospital stay, although these data did not reach statistical significance.

Furthermore, when we pooled together patients with 25OHD levels ≥20 ng/mL (both at basal levels and with vitamin D supplements) and compared with patients with vitamin D deficiency, those with higher levels had a slightly better outcome expressed as a lower PaO_2/FIO_2 ratio <300 (12.8% vs. 27.8%; $P = .034$), lower requirements for tocilizumab (17% vs. 33.1%; $P = .032$), less frequent radiological progression (14.9% vs. 30.2%; $P = .037$), lower ICU admissions (12.8% vs. 26.6%; $P = .048$), and also a shorter hospital stay (12.0 [8.0-17.0] vs. 8.0 [6.0-14.0] days; $P = .002$). No difference was found regarding the composite severity endpoint (21.3% vs. 30.8%; $P = .203$) nor mortality (12.9% vs. 9.8%; $P = .590$).

In COVID-19 patients, mean ± SD 25OHD levels were 13.8 ± 7.2 ng/mL, compared with 20.9 ± 7.4 ng/mL in controls ($P < .0001$). The distribution of serum 25OHD levels in hospitalized COVID-19 patients with or without vitamin D supplements and controls, grouped by gender, is shown in Fig. 1. Serum 25OHD values were lower in men than in women. Fig. 2 shows the percentage of COVID-19 cases (without vitamin D supplementation) and controls within the different intervals of serum 25OHD levels. Vitamin D deficiency was found in 82.2% of COVID-19 cases and 47.2% of population-based controls ($P < .0001$). 25OHD inversely and significantly correlated with serum ferritin and D-dimer, and there was a trend with C-reactive protein levels (Fig. 3). We did not find any statistical relationship between serum vitamin D and IL-6 levels in patients with COVID-19 (rho –0.032; $P = .67$), although levels of this cytokine were lower, albeit nonsignificant, in patients with serum vitamin D levels ≥20 ng/mL and those on vitamin D supplements (Table 3).

In the multivariable general linear model, mean serum 25OHD levels were significantly lower in COVID-19 patients (excluding those on vitamin D supplements) than in population-based controls after adjusting for age, smoking, hypertension, diabetes mellitus, history of cardiovascular events, immunosuppression, body mass index, serum corrected calcium, glomerular filtration rate, and the month of vitamin D determination: 11.9 (95% CI 9.6-14.3) ng/mL versus 21.2 (95% CI 19.7-22.7) ng/mL ($P < .0001$).

In COVID-19 patients (once those on vitamin D supplements at admission had been excluded), no relationship was found between serum vitamin D levels (as a continuous variable or expressed as vitamin D deficiency or as quintiles), and the composite severity endpoint or its separate components, in crude or adjusted logistic regression models (combine severity endpoint: unadjusted OR 1.55, 95% CI 0.66-3.65; $P = .315$; adjusted OR 1.13, 95% CI 0.27-4.77; $P = .865$; for vitamin D deficiency).

Table 2. Main characteristics of COVID-19 patients according to the presence of vitamin D deficiency

Variable	25OHD < 20 ng/mL N = 162	25OHD ≥ 20 ng/mL N = 35	*P*
Baseline characteristics			
Age (years), median (IQR)	62.0 (48.0-70.3)	58.0 (45.0-69.0)	.292
Sex (male), n (%)	106 (65.4)	17 (48.6)	.062
BMI (kg/m2), mean ± SD	29.0 ± 4.9	29.8 ± 4.1	.428
Current smoker, n (%)	13 (8.0)	1 (2.9)	.471
Hypertension, n (%)	68 (42.0)	8 (22.9)	.035
Diabetes, n (%)	28 (17.3)	6 (17.1)	.984
Cardiovascular disease, n (%)	21 (13.0)	0 (0.0)	.029
COPD, n (%)	13 (8.0)	2 (5.7)	.999
Active cancer, n (%)	7 (4.3)	0 (0.0)	.357
Immunosuppression, n (%)	11 (6.8)	5 (14.3)	.169
ACEI/ARA2 agents, n (%)	52 (32.1)	6 (17.1)	.078
Clinical and laboratory data			
Pneumonia, n (%)	155 (95.7)	33 (94.3)	.662
Respiratory rate >22, n (%)	36 (22.2)	4 (11.4)	.150
CURB-65 score, median (IQR)	1 (1, 2)	1 (1)	.229
SBP < 100 mmHg, n (%)	4 (2.5)	1 (2.9)	.999
PaO_2/FIO_2 ratio, median (IQR)	444 (424-452)	444 (436-452)	.168
PaO_2/FIO_2 ratio < 300, n (%)	46 (28.4)	6 (17.1)	.171
Lymphocytes (mm^3), median (IQR)	900 (600-1200)	1100 (700-1250)	.255
Neutrophils (mm^3), median (IQR)	3900 (2875-6125)	3700 (2900-4600)	.207
Neutrophil/Lymphocyte ratio, median (IQR)	4.85 (3.00-7.52)	3.63 (3.00-6.67)	.422
Platelet count (×109/L), median (IQR)	167 (138-217)	169 (143-211)	.833
D-dimer (ng/mL), median (IQR)	710.5 (469.0-1021.0)	575.0 (434.0-693.0)	.057
Ferritin (ng/mL), median (IQR)	833.0 (330.8-1488.3)	310.0 (137.3-764.0)	<.0001
hs-Troponin I (ng/L), median (IQR)	6.0 (3.0-12.0)	3.0 (3.0-6.0)	.015
C-reactive protein (mg/dL), median (IQR)	6.10 (3.10-13.60)	3.20 (2.30-8.70)	.064
IL-6 (pg/mL), median (IQR)	58.9 (19.1-124.0)	45.6 (20.5-119.0)	.63
GFR-MDRD-4 (mL/min/1.72 m^2), median (IQR)	91.4 (73.5-114.7)	98.0 (82.4-113.1)	.323
Corrected calcium (mg/dL), median (IQR) Corrected calcium (mg/dl), median (IQR)	8.5 (8.3-9.0)	8.7 (8.4-9.0)	.289
25OHD (ng/mL), mean ± SD	11.2 ± 4.3	25.8 ± 5.6	<.0001
PTH (pg/mL), median (IQR)	44.2 (32.3-64.8)	35.6 (30.9-46.4)	.092
Therapeutic scheme			
Hydroxychloroquine, n (%)	156 (96.3)	35 (100)	.593
Lopinavir/ritonavir, n (%)	122 (75.3)	31 (88.6)	.088
Azithromycin, n (%)	117 (72.2)	30 (85.7)	.096
Corticosteroids, n (%)	40 (24.7)	7 (20.0)	.555
β-Interferon, n (%)	37 (22.8)	7 (20.0)	.715
Tocilizumab, n (%)	55 (34.0)	8 (22.9)	.202
Anakinra, n (%)	12 (7.4)	1 (2.9)	.471
Noninvasive ventilation, n (%)	12 (7.4)	1 (2.9)	.471
Outcome			
ICU admission, n (%)	44 (27.2)	6 (17.1)	.217
Mechanical ventilation[a], n (%)	37 (84.1)	6 (100)	.576
Radiological worsening, n (%)	50 (30.9)	6 (17.1)	.103
Secondary infection, n (%)	38 (23.5)	6 (17.1)	.416
Thrombotic events[b], n (%)	10 (6.2)	0 (0.0)	.214
Death, n (%)	16 (10.2)	4 (11.4)	.765
Composite severity endpoint, n (%)	111 (68.5)	27 (77.1)	.312
Length of stay (days), median (IQR)	12.0 (8.0-17.0)	8.0 (6.0-14.0)	.013

Abbreviations: BMI, body mass index; COPD, chronic obstructive pulmonary disease; ACEI, angiotensin-converting enzyme inhibitors; ARA2, angiotensin-receptor 2 antagonists; hs, high-sensitivity; GFR, glomerular filtration rate; MDRD, modification of diet in renal disease; 25OHD, 25-hydroxyvitamin D; PTH, parathyroid hormone.
[a]Refers only to the number of patients admitted to ICU.
[b]Included pulmonary embolism, deep venous thrombosis, acute coronary syndrome, and cerebrovascular disease.

Table 3. Main features in COVID-19 patients with or without oral vitamin D supplements at admission

Variable	COVID-19 N = 197	COVID-19_D N = 19	*P*
Clinical and laboratory data			
Pneumonia, n (%)	188 (95.4)	18 (94.7)	.999
Respiratory rate >22, n (%)	40 (20.3)	3 (15.8)	.772
CURB-65 score, median (IQR)	1 (1, 2)	1 (1, 2)	.353
SBP < 100 mmHg, n (%)	5 (2.5)	0 (0.0)	.999
PaO_2/FIO_2 ratio, median (IQR)	444 (428-452)	444 (432-452)	.524
PaO_2/FIO_2 ratio < 300, n (%)	52 (26.4)	1 (5.3)	.049
Lymphocytes (mm^3), median (IQR)	900 (700-1200)	900 (500-1400)	.890
Neutrophils (mm^3), median (IQR)	3900 (2900-5600)	4000 (2200-5100)	.624
Neutrophil/Lymphocyte ratio, median (IQR)	4.75 (3.00-7.38)	4.58 (2.81-7.82)	.891
Platelet count ($\times 10^9$/L), median (IQR)	167 (138-214)	168 (142-236)	.478
D-dimer (ng/mL), median (IQR)	735.5 (254.3-1367.3)	599 (431-1336)	.731
Ferritin (ng/mL), median (IQR)	861 (330-1418)	315 (147.0-743.0)	.012
hs-Troponin I (ng/L), median (IQR)	6.0 (3.5-125.0)	7.0 (3.5-17.0)	.979
C-reactive protein (mg/dl), median (IQR)	5.55 (2.60-11.85)	7.30 (2.90-15.10)	.73
IL-6 (pg/mL), median (IQR)	57.6 (21.6-125.0)	48.8 (13.0-129.8)	.80
Therapeutic scheme			
Hydroxychloroquine, n (%)	191 (97.0)	19 (100)	.999
Lopinavir/ritonavir, n (%)	153 (77.7)	12 (63.2)	.164
Azithromycin, n (%)	147 (74.6)	14 (73.7)	.999
Corticosteroids, n (%)	47 (23.9)	5 (26.3)	.783
β-interferon, n (%)	44 (22.3)	2 (10.5)	.378
Tocilizumab, n (%)	63 (32.0)	1 (5.3)	.015
Anakinra, n (%)	13 (6.6)	1 (5.3)	.999
Non-invasive ventilation, n (%)	13 (6.6)	2 (10.5)	.627
Outcome			
ICU admission, n (%)	50 (25.4)	1 (5.3)	.05
Mechanical ventilation[a], n (%)	43 (86.0)	1 (100)	.999
Radiological worsening, n (%)	56 (28.4)	2 (10.5)	.093
Secondary infection, n (%)	44 (22.3)	2 (10.5)	.378
Thrombotic events[b], n (%)	10 (5.1)	1 (5.3)	.999
Death, n (%)	20 (10.4)	2 (10.5)	.999
Composite severity endpoint, n (%)	59 (29.9)	3 (15.8)	.193
Length of stay (days), median (IQR)	12.0 (8.0-16.0)	8.0 (6.0-14.0)	.107

Abbreviations: BMI, body mass index; COPD, chronic obstructive pulmonary disease; ACEI, angiotensin-converting enzyme inhibitors; ARA2, angiotensin-receptor 2 antagonists; hs, high-sensitivity; GFR, glomerular filtration rate; MDRD, modification of diet in renal disease; 25OHD, 25-hydroxyvitamin D; PTH, parathormone.

[a]Refers only to the number of patients admitted to ICU.

[b]Included pulmonary embolism, deep venous thrombosis, acute coronary syndrome, and cerebrovascular disease.

Discussion

We have found that serum 25OHD levels are significantly lower in hospitalized COVID-19 patients than in population-based controls of similar age and sex, and that these differences remain significant even after adjusting for the main confounding factors. These levels were especially lower in the group of men with COVID-19. Despite the high frequency of vitamin D deficiency in patients hospitalized for COVID-19, we did not find an association between circulating levels of 25OHD and the severity of SARS-CoV-2 infection.

Vitamin D is a hormone with a pleiotropic role and there is compelling evidence for an epidemiological association between low serum 25OHD levels and human infections such as influenza, HIV, and hepatitis C virus infection (19). The interplay between vitamin D and viral infection is an area of growing interest, and interaction with host and viral factors, immunomodulatory effects, induction of autophagy and apoptosis, and even genetic and epigenetic factors have been reported as antiviral effects of this hormone (20). In this scenario, the SARS-CoV-2 virus pandemic has rapidly spread during winter with extreme virulence through southern European countries such as Italy and Spain. Although there was a considerable variation in the prevalence of vitamin D deficiency across countries, mainly dependent on age and the use of

vitamin D supplements or food fortification, vitamin D deficiency (25OHD levels <20 ng/mL) is found in 40% of European citizens irrespective of age group, ethnic mix, and latitude (18). The population with more severe COVID-19, such as elderly people and patients with comorbidities with the highest case fatality rates (21), are also those with lower serum 25OHD levels according to published data (18). Thus, the Seneca study showed a mean 25OHD concentration of 10.4 ng/mL (26 nmol/L) in elderly subjects aged 70 to 75 years in Spain (22). Recently, Ilie et al. (23) found significant crude associations between serum vitamin D levels and the number of COVID-19 cases and mortality when they analyzed, in some European countries, the mean 25OHD levels reported in some population studies.

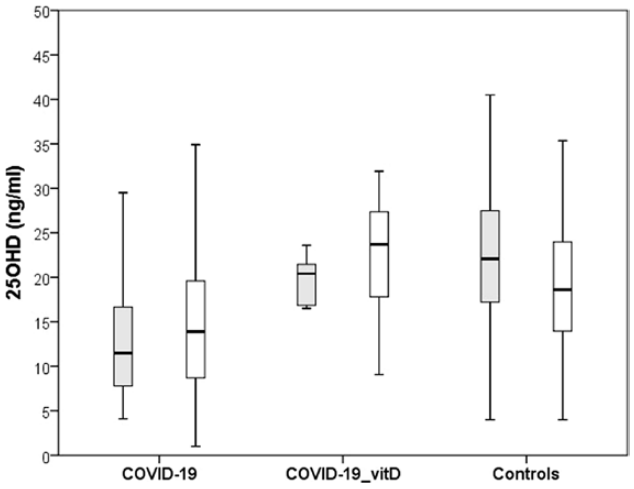

Figure 1. Serum vitamin D levels in hospitalized COVID-19 patients with and without active oral vitamin D supplements and population-based controls, according to gender. Grey bars represent men and white bars represent women.

Moreover, SARS-CoV-2 downregulates ACE2 expression, the main receptor for the virus to enter human cells, and thereby induces high angiotensin II production leading to myocardial and mainly lung inflammation and ARDS (24). In experimental models, vitamin D deficiency induces chronic RAS activation leading to impaired lung function and overexpression of profibrotic factors (13). The key pathogenic mechanism for SARS-CoV-2 to develop severe complications and lethality is the hyperinflammatory state ("cytokine storm") that occurs over the first week of the onset of the symptoms. This cytokine storm may lead to severe COVID-19 complications, such as ARDS, myocarditis, and acute heart and renal failure, causing increased mortality, especially in elderly people or in patients with previous cardiovascular comorbidity (25). The intrinsic mechanism of the anti-inflammatory effect of vitamin D remains uncertain, although its role on both, innate and adaptive immunity, has been suggested (26). In this regard, experimental evidence indicates that vitamin D may inhibit IL-6 and tumor necrosis factor-α by attenuating p38 MAP kinase activation in human monocytes/macrophages, Moreover, $1,25OH2D_3$ promotes the induction of T regulatory cells, thereby inhibiting production of proinflammatory cytokines, including IL-17, IL-21, and γ-interferon (27).

In this scenario, a recent study using 350 000 UK Biobank samples obtained between 2006 and 2010 did not find an association between serum 25OHD concentrations (or vitamin D deficiency, defined as <25 mmol/L; or insufficiency <50 mmol/L) and COVID-19 risk (assessed in 449

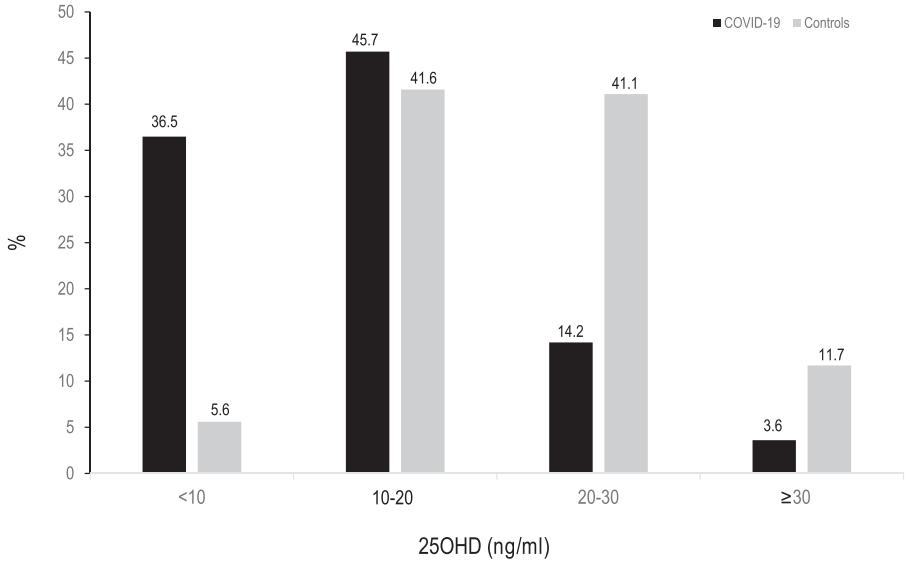

Figure 2. Percentage of COVID-19 cases (excluding those on vitamin D at admission) and controls according to different intervals of serum 25OHD levels.

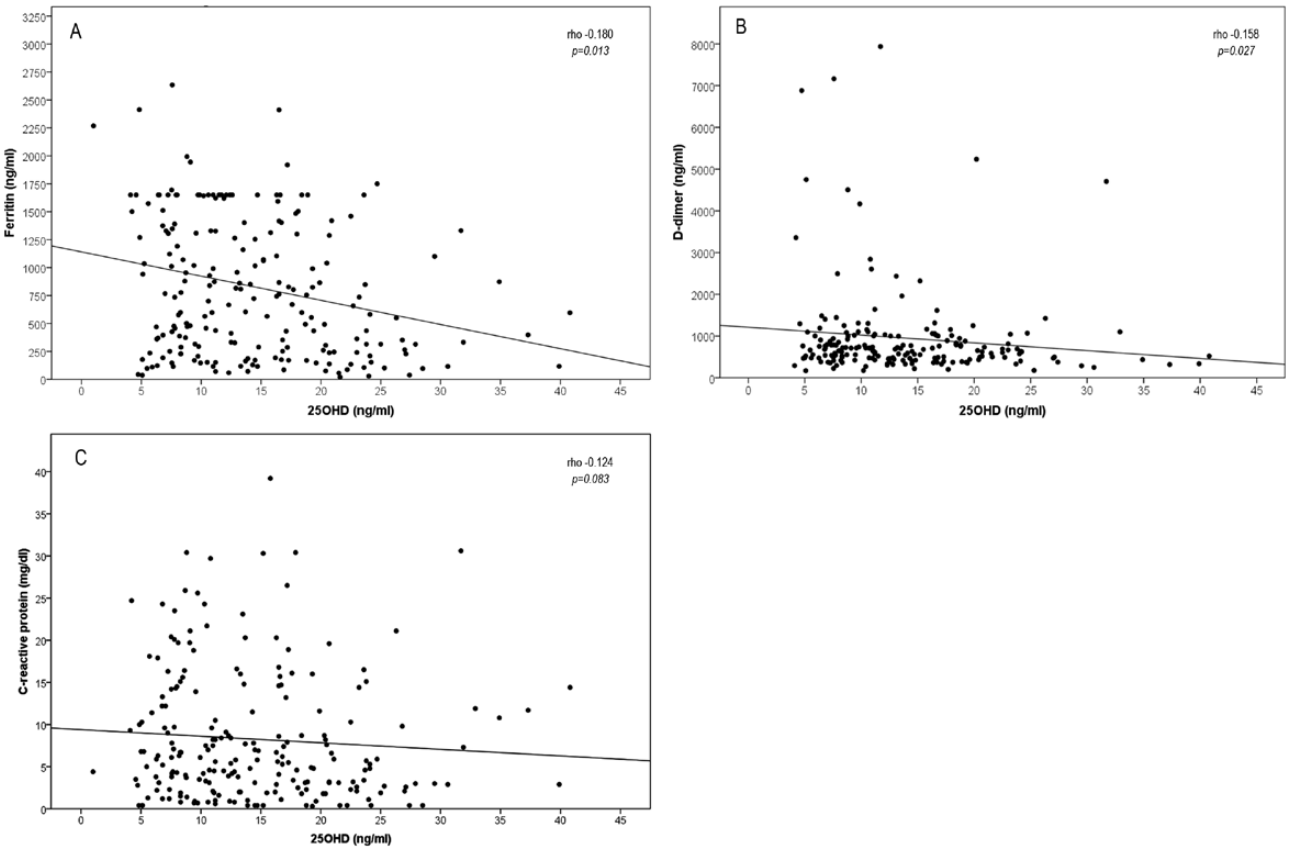

Figure 3. Correlation between serum 25OHD and inflammatory markers (ferritin, A; D-dimer, B; and C-reactive protein, C).

COVID-19 patients with complete data), after adjusting for potential confounders. Besides, their results did not support that vitamin D might play a role in the reported ethnic variations in COVID-19 incidence (28). Baseline 25OHD levels were obtained a decade ago; information on the severity of COVID-19 was also lacking and the study included all positive tests regardless of clinical outcome. D'Avolio et al. (29) retrospectively analyzed 107 patients who underwent SARS-CoV-2 polymerase chain reaction testing (80 with a negative and only 27 with a positive result) and simultaneous 25OHD measurement, in a Swiss hospital, from March 1 to April 14, 2020. A control cohort that included 1377 patients with serum 25OHD levels obtained in the same period of the year 2019 was also analyzed. The authors found that SARS-CoV-2-infected patients had median 25OHD levels of 11.1 ng/mL, compared with 24.6 ng/mL in SARS-CoV-2-negative subjects and controls.

Our study was carried out in a hospitalized population, and, in this sense, it is worth mentioning that serum 25OHD has been considered as a negative acute-phase reactant, and its values have been reported to be decreased during acute inflammatory diseases (30). Thus, our COVID-19 patients had a high prevalence of vitamin D deficiency, and serum 25OHD levels significantly and negatively correlated with ferritin and D-dimer values, indicating that vitamin D might have a beneficial role on the systemic inflammatory state of this viral disease. Interestingly, 25OHD concentrations in COVID-19 patients on previous hormone supplements were lower than expected, supporting its behavior as a negative acute-phase reactant. Therefore, 25OHD levels should be interpreted with caution in this scenario, although the population at risk for a more severe SARS-CoV-2 infection is probably the same as that at risk for vitamin D deficiency, especially elderly individuals with comorbidities.

We did not find any relationship between serum 25OHD levels and the parameters of COVID-19 severity, such as ICU admission, the need for mechanical ventilation, or mortality, assessed as a combined endpoint or separately. In contrast to other studies (31, 32), we did not find an association between serum 25OHD levels and the severity of the disease. However, it cannot be completely ruled out due to the small number of events and the statistical power of the present study. Nevertheless, we had the opportunity to assess a group of 19 COVID-19 patients who were on oral vitamin D supplements at hospital admission. We observed that they had a slightly less unfavorable outcome than COVID-19 patients who did not take vitamin D supplements, with a significantly more favorable PaO_2/FIO_2

ratio, lower ferritin levels, and decreased requirements for tocilizumab, and even a trend for lower ICU admissions.

Interestingly enough, 6 out of 19 COVID-19 patients on vitamin D supplements also received chronic corticosteroids or immunosuppressant agents at least during the previous 3 months because of immune-mediated inflammatory diseases (IMIDs) or suprarenal insufficiency. This is an interesting matter of debate since it has been recently suggested that the use of anticytokine and other immunosuppressive therapies is not associated with worse COVID-19 outcomes (33). In this sense, IMID patients on chronic corticosteroids usually received vitamin D supplements as prophylaxis or treatment of bone disease. Furthermore, they are under tight control of their comorbidities, and vitamin D deficiency is more frequently checked and treated than in the general population. Whether the outcome of COVID-19 patients on previous vitamin D might have been influenced by vitamin D status itself or by the presence of an important number of patients with IMIDs on corticosteroids and/or immunosuppressant agents is difficult to determine due to the size of the sample.

COVID-19 hospitalized patients had lower serum corrected calcium levels than the control population. In this regard, Di Filippo et al. (34) conducted a hospital-based retrospective study on 531 COVID-19 patients in Italy. Hypocalcemia, defined as serum ionized calcium level <1.18 mmol/L, was observed in 82% of patients, mainly elderly males. They also found that hypocalcemia was an independent predictor for hospitalization. However, and despite lower calcium and vitamin D levels in our COVID-19 patients, serum parathyroid hormone was higher in controls. This could be related to a lower glomerular filtration rate in the control population, since there is no reason to suspect relative hypoparathyroidism.

Finally, it is worth mentioning that the SARS-CoV-2 pandemic represents a challenging scenario in the management of osteoporosis and fragility fractures. Thus, COVID-19 hospitalized patients are mainly frail, older individuals with comorbidities, who are in many cases exposed to systemic corticosteroids as part of the treatment of the disease and may require prolonged immobilization periods for a complete recovery. Besides, as we observed, they have a high percentage of vitamin D deficiency that may also contribute to a loss of muscle strength and to an increase in the risk of falls. All these factors put these individuals at an increased risk for fragility fractures (35, 36). Under these circumstances, prevention strategies should be implemented. According to our results, vitamin D treatment should be recommended in COVID-19 patients with serum 25OHD deficiency, since this approach might have beneficial effects in both the musculoskeletal and the immune system (36).

Our study has several limitations. First of all, those inherent to an observational study that does not permit one to establish whether vitamin D is simply a biomarker of exposure or a biomarker of effect on the disease. Other vitamin D–related parameters such as the free fraction of 25OHD, 1,25 dihydroxyvitamin D, and vitamin D binding protein were not measured. The number of COVID-19 patients who were on oral vitamin D supplements is too small and on different dosages to draw solid conclusions about its role in the clinical outcomes of the disease, although we think that it represents a unique opportunity to preliminary explore the differences between both groups of COVID-19 patients. Furthermore, the study has been conducted in a single Spanish tertiary care hospital, and data may not be generalized to other settings, ethnicities, or countries, especially those with specific policies for vitamin D supplementation or food fortification. The methods to assess serum 25OHD levels in cases and controls were different, although, as stated, we have found a very good correlation between both techniques. Finally, no dietary assessment was carried out, and therefore information on dietary habits is lacking.

In summary, serum 25OHD levels of hospitalized COVID-19 patients are lower than sex-matched population-based controls of similar age. Men with this viral disease represent the group with lower serum vitamin D levels than women. Serum vitamin D levels below 20 ng/mL were detected in 82% of COVID-19 patients, indicating that they represent a population with a higher risk for vitamin D deficiency. In our COVID-19 patients, 25OHD was inversely associated with some inflammatory parameters, such as ferritin and D-dimer. We did not found any relationship between vitamin D concentrations or vitamin deficiency and the severity of the disease, including mortality, although further studies including a large sample size should be done to determine the real impact of vitamin D deficiency on the severity of COVID-19. Probably the best approach should be to identify and treat vitamin D deficiency, especially in high-risk individuals such as elderly people, patients with comorbidities, and nursing home residents, to maintain serum 25OHD levels above 20 ng/mL, and probably with a target between 30 ng/mL and 50 ng/mL. Whether the treatment of vitamin D deficiency will play some role in the prevention of the viral disease or improve the prognosis of patients with COVID-19 remains to be elucidated in large randomized controlled trials, which will be certainly necessary to precisely define the role of vitamin D supplementation in futures waves of SARS-CoV-2 infection.

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RESEARCH ARTICLE

SARS-CoV-2 positivity rates associated with circulating 25-hydroxyvitamin D levels

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Abstract

Until treatment and vaccine for coronavirus disease-2019 (COVID-19) becomes widely available, other methods of reducing infection rates should be explored. This study used a retrospective, observational analysis of deidentified tests performed at a national clinical laboratory to determine if circulating 25-hydroxyvitamin D (25(OH)D) levels are associated with severe acute respiratory disease coronavirus 2 (SARS-CoV-2) positivity rates. Over 190,000 patients from all 50 states with SARS-CoV-2 results performed mid-March through mid-June, 2020 and matching 25(OH)D results from the preceding 12 months were included. Residential zip code data was required to match with US Census data and perform analyses of race/ethnicity proportions and latitude. A total of 191,779 patients were included (median age, 54 years [interquartile range 40.4–64.7]; 68% female. The SARS-CoV-2 positivity rate was 9.3% (95% C.I. 9.2–9.5%) and the mean seasonally adjusted 25(OH)D was 31.7 (SD 11.7). The SARS-CoV-2 positivity rate was higher in the 39,190 patients with "deficient" 25(OH)D values (<20 ng/mL) (12.5%, 95% C.I. 12.2–12.8%) than in the 27,870 patients with "adequate" values (30–34 ng/mL) (8.1%, 95% C.I. 7.8–8.4%) and the 12,321 patients with values ≥55 ng/mL (5.9%, 95% C.I. 5.5–6.4%). The association between 25(OH)D levels and SARS-CoV-2 positivity was best fitted by the weighted second-order polynomial regression, which indicated strong correlation in the total population ($R^2 = 0.96$) and in analyses stratified by all studied demographic factors. The association between lower SARS-CoV-2 positivity rates and higher circulating 25(OH)D levels remained significant in a multivariable logistic model adjusting for all included demographic factors (adjusted odds ratio 0.984 per ng/mL increment, 95% C.I. 0.983–0.986; p<0.001). SARS-CoV-2 positivity is strongly and inversely associated with circulating 25(OH)D levels, a relationship that persists across latitudes, races/ethnicities, both sexes, and age ranges. Our findings provide impetus to explore the role of vitamin D supplementation in reducing the risk for SARS-CoV-2 infection and COVID-19 disease.

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Competing interests: HWK, JKN, MHK, and CB are employees of Quest Diagnostics. HWK, MHK and CB own stock in Quest Diagnostics. MFH is a consultant to Quest Diagnostics and was on the speakers' bureau for Abbott Inc. and Hyatt Pharmaceutical Industries Company PLC.

Introduction

Studies suggest an association between vitamin D deficiency and risk of viral upper respiratory tract infections and mortality from coronavirus disease-2019 (COVID-19) [1, 2]. This relationship is anticipated, given that vitamin D has numerous actions affecting the innate and adaptive immune systems. Respiratory monocytes/macrophages and epithelial cells constitutively express the vitamin D receptor. Acting through this receptor, vitamin D may be important in protection against respiratory infections [3]. In addition, an important action of vitamin D is suppressing excessive cytokine release that can present as a "cytokine storm," a common cause of COVID-19-related morbidity and mortality [4]. The role of vitamin D supplementation in reducing the risk of infection by severe acute respiratory disease coronavirus-2 (SARS-CoV-2) has not been studied. Better understanding of the relation between vitamin D status and SARS-CoV-2 NAAT positivity rates is appropriate before evaluating this potential intervention.

Previous studies examined latitude-related differences in COVID-19 outcomes related to vitamin D [1, 5]. However, to our knowledge, only two studies investigated the direct relationship between vitamin D status and SARS-CoV-2 positivity, and these came to opposite conclusions [6, 7]. Both were based on small numbers of paired SARS-CoV-2 and 25(OH)D results, and neither involved US patients. In this study, we evaluated the association of circulating 25-hydroxyvitamin D [25(OH)D] levels, a measure of vitamin D status, with positivity for SARS-CoV-2 as assessed with nucleic acid amplification testing (NAAT).

Methods

Study population

In this retrospective, observational analysis of deidentified test results from a clinical laboratory, a Quest Diagnostics-wide unique patient identifier was used to match all results of SARS-CoV-2 testing performed March 9 through June 19, 2020, with 25(OH)D results from the preceding 12 months. Analysis was limited to one SARS-CoV-2 result per patient; patients were considered to have a positive SARS-CoV-2 result if any test result indicated positivity. When multiple 25(OH)D results were available, the most recent was selected. We excluded specimens with inconclusive results (one out of two SARS-CoV-2 targets detected) or missing residential zip code data, which are needed to assign race/ethnicity proportions and latitude.

Laboratory methods

All SARS-CoV-2 RNA NAATs were performed by Quest Diagnostics using one of four United States Food and Drug Administration (FDA) Emergency Use Authorized tests (Quest Diagnostics SARS-CoV-2 RNA [COVID-19], Qualitative NAAT; Hologic Panther Fusion SARS--CoV-2 assay; Roche Diagnostics cobas® SARS-CoV-2 test; or Hologic Aptima SARS-CoV-2 assay). We combined results from all four tests due to their very similar sensitivity and specificity [8–11]. Total 25(OH)D was measured using a chemiluminescent immunoassay (DiaSorin LIAISON® XL 25-hydroxyvitamin D, total) or a laboratory-developed test based on liquid chromatograph/tandem mass spectrometry. The laboratory categorizes 25(OH)D results <20 ng/mL as deficient, 20–29 ng/mL as suboptimal, and ≥ 30 ng/mL as optimal. The laboratory assays are standardized and performed identically throughout Quest Diagnostics.

Estimates by zip code

To analyze race/ethnicity, patient data were linked to estimated race/ethnicity proportions reported by zip code in the 2018 5-year American Community Survey (ACS) [12]. Zip codes

with estimated proportions of black non-Hispanic population over 50% are referred to as "predominately black non-Hispanic." The same pattern was followed for "predominantly Hispanic" and "predominantly white non-Hispanic" zip codes. Latitude for each zip code, acquired from SAS reference data, was stratified into three groups: >40 degrees ("northern"); 32–40 degrees ("central"); or <32 degrees ("southern").

Vitamin D seasonality adjustment

We adjusted for vitamin D seasonality with a model based on a previous $25(OH)D_3$ study, utilizing Quest Diagnostics results that fit the present study data well [13].

Statistical analyses

Comparisons of proportions were analyzed using the chi-square test. Comparisons of means were analyzed using the t-test. Concentrations of circulating 25(OH)D are reported in ng/mL. Values <20 ng/mL or ≥60 ng/mL were assigned a value of 19 ng/mL or 60 ng/mL, respectively. Age was stratified into two groups: <60 years and ≥60 years for convenience. The correlation between 25(OH)D values and SARS-CoV-2 positivity were fitted the best by the weighted second-order polynomial regression. For regressions of predominately black non-Hispanic and Hispanic zip codes, 25(OH)D values were grouped into bins with two values from 20–29 (20–21, 22–23, etc.), and bins with 5 values thereafter (30–34, 35–39, etc.), because of the relatively low number of patients with 25(OH)D values ≥30. For all other polynomial regressions, 25(OH)D values were grouped into bins with two values. Multivariable logistic regression was performed using a stepwise entry criterion of $p<0.05$, after excluding patients with missing values for any included factor. Analyses were performed using SAS Studio 3.6 on SAS 9.4 (SAS Institute) and R, version 3.6.1 (R Project for Statistical Computing). HIPAA clearly defines research use of data as analyzed for this and numerous other studies based on the Quest Diagnostics Data Informatics Warehouse (45 CFR 164.501, 164.508, 164.512(i) (See also 45 CFR 164.514(e), 164.528, 164.532) Quest Diagnostics takes the additional step of having its process reviewed annual by the Western Institutional Review Board (Puyallup, Washington) who has determined the process is "deemed exempt."

Results

Our potential cohort included 218,372 patients. After excluding patients with missing residential zip code data (n = 26,387) or inconclusive SARS-CoV-2 NAAT results (n = 206), results from 191,779 (87.8%) patients remained for analysis. This cohort comprised patients from all 50 states and the District of Columbia. The median age and sex distribution of included and excluded patients were nearly identical: age, 54.0 years, IQR 40.4–64.7, vs. 53.7 years, IQR 39.7–64.5, and female, 68% vs. 67%, respectively. SARS-CoV-2 positivity was lower among included (9.3%, 95% C.I. 9.2–9.5%) than excluded (10.1%, 95% C.I. 9.7–10.4%) patients ($p<0.001$). 98.8% of included patients had 25(OH)D levels assessed with immunoassay testing methodology.

There was an association between lower SARS-CoV-2 positivity rates and higher circulating 25(OH)D levels (unadjusted odds ratio 0.979 per 1 ng/mL increment, 95% C.I. 0.977–0.980). Regression analysis indicated strong correlation (R-squared = 0.96) between 25(OH)D levels and SARS-CoV-2 positivity in the total population (Fig 1) and in northern, central, and southern latitudes (Fig 2A). The decrease in positivity rate associated with 25(OH)D levels appeared to plateau as values approached 55 ng/mL; SARS-CoV-2 positivity rates were similar between the 4,016 patients with values 55–59 ng/mL (6.0%, 95% C.I. 5.2–6.7%) and the 8,305 patients with higher values (5.9%, 95% C.I. 5.4–6.4%). The SARS-CoV-2 positivity rate was lower in

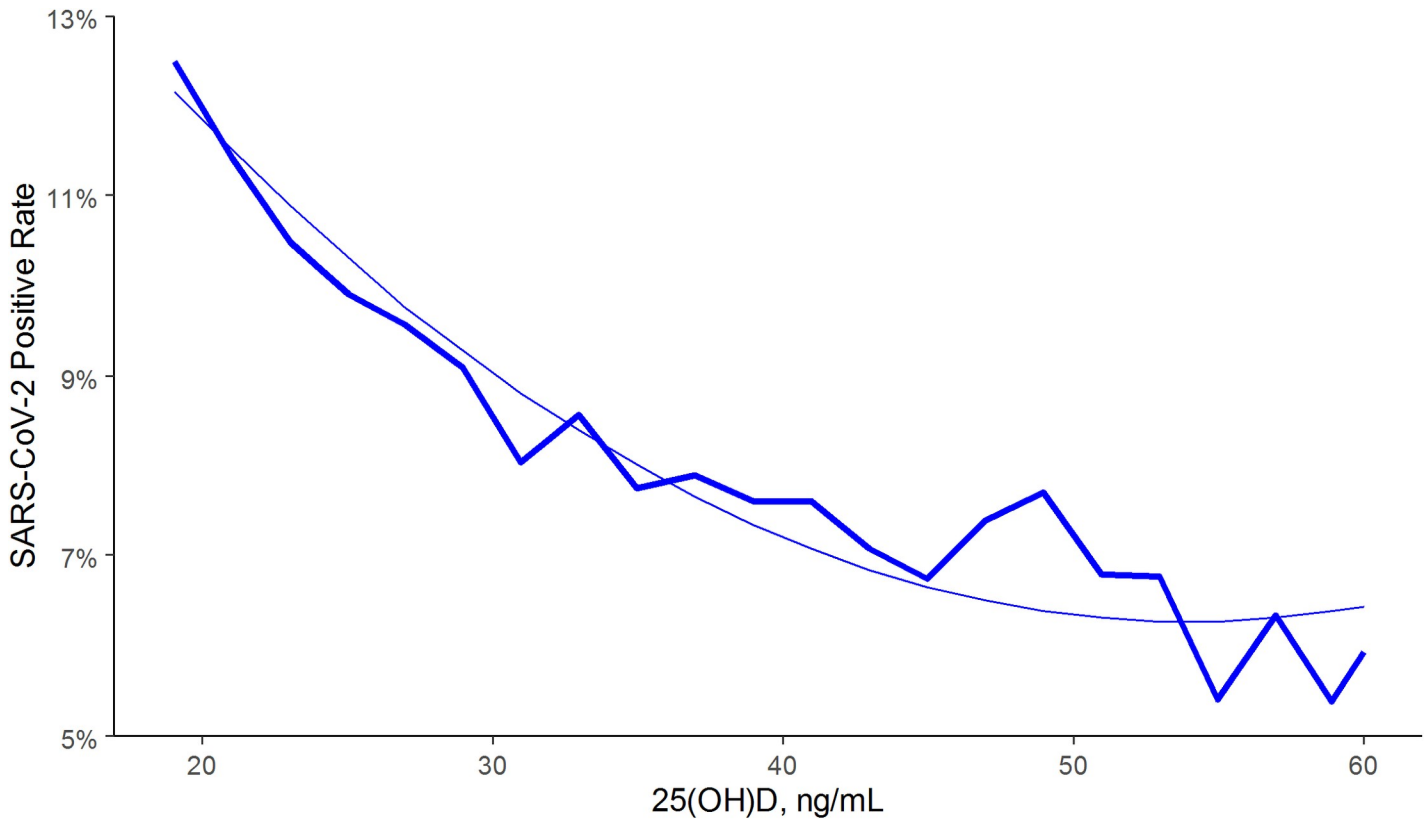

Fig 1. SARS-CoV-2 NAAT positivity rates and circulating 25(OH)D levels in the total population. Smooth line represents the weighted second order polynomial regression fit to the data associating circulating 25(OH)D levels (x) and SARS-CoV-2 positivity rates (y) where: $y = 0.2029–0.0052^{*}x + 4.8e\text{-}05^{*}x^{2}$; $R^2 = 0.96$. SI conversion factor: 1 ng/mL = 0.400641 nmol/L.

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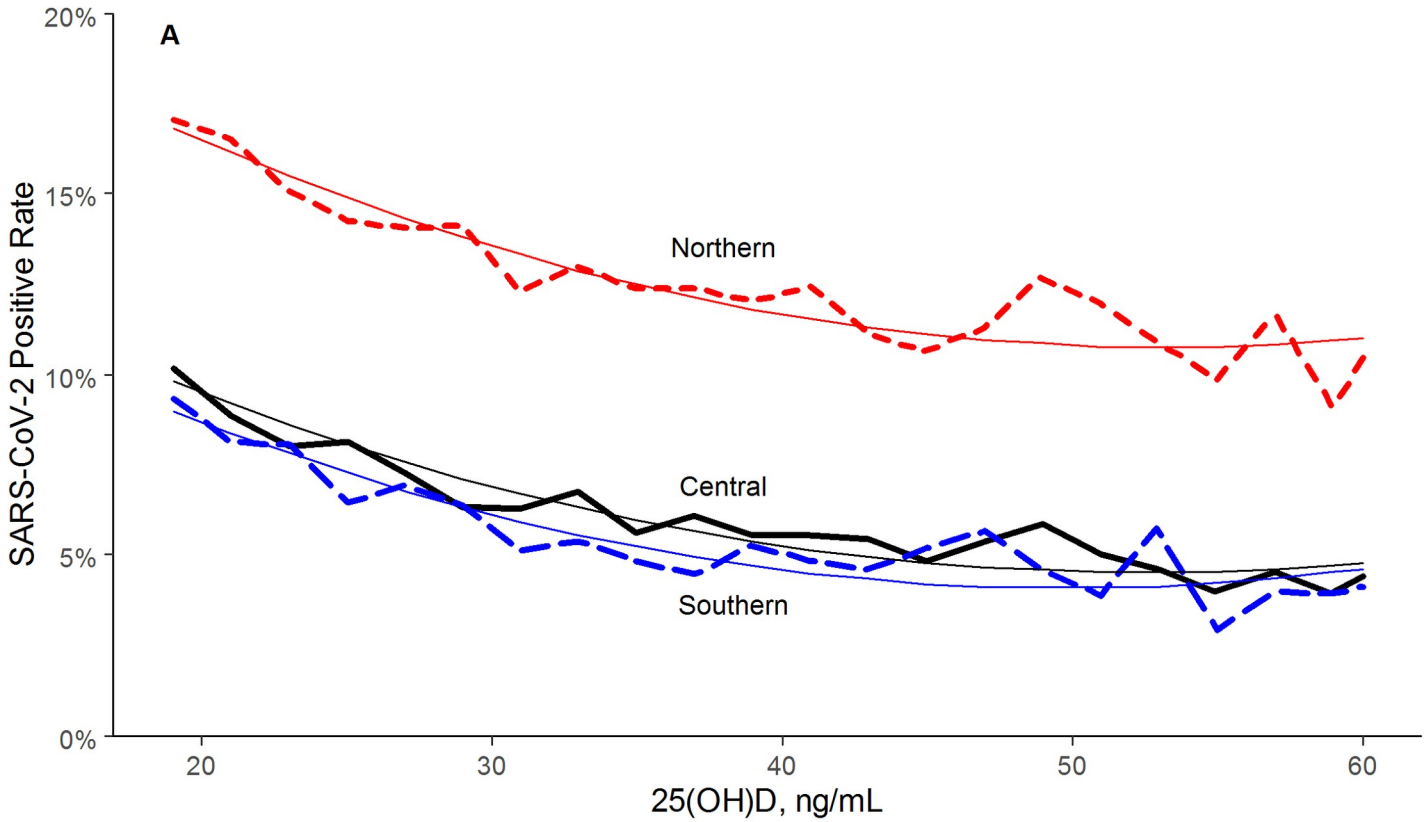
the 27,870 patients with "adequate" 25(OH)D values (30–34 ng/mL) (8.1%, 95% C.I. 7.8–8.4%) than in the 39,190 patients with "deficiency" (<20 ng/mL) (12.5%, 95% C.I. 12.2–12.8%) (difference 35%; p<0.001). Similarly, the SARS-CoV-2 positivity rate was lower in the 12,321 patients with 25(OH)D values ≥55 ng/mL (5.9%, 95% C.I. 5.5–6.4%) than in patients with adequate values (difference 27%; p<0.001).

SARS-CoV-2 positivity rates were higher in the 9,529 patients from predominately black non-Hispanic zip codes (15.7%, 95% C.I. 15.0–16.4%) and the 26,242 patients from predominately Hispanic zip codes (12.8%, 95% C.I. 12.4–13.2%) than in the 112,281 patients from predominately white non-Hispanic zip codes (7.2%, 95% C.I. 7.1–7.4%; p<0.001 for both comparisons). Mean (±SD) 25(OH)D levels were also higher in patients from predominately white non-Hispanic zip codes (33.0±11.9 ng/mL; 1 ng/mL = 0.400641 nmol/L) than in patients from predominately black non-Hispanic (29.1±11.0 ng/mL; p<0.001) or Hispanic (28.8±10.7 ng/mL; p<0.001) zip codes. Regression analysis indicated strong correlation between 25(OH)D levels and SARS-CoV-2 positivity in each of these groups (Fig 2B).

Compared to the 67,667 patients age ≥60 years, the 120,362 younger patients had significantly higher SARS-CoV-2 positivity (10.2%, 95% C.I. 10.0–10.3%, vs. 7.7%, 95% C.I. 7.5–7.9%; p<0.001) and lower mean 25(OH)D levels (29.4±10.8 ng/mL vs. 35.4±12.1 ng/mL; p<0.001). Compared to the 130,473 female patients, the 61,305 male patients had higher SARS-CoV-2 positivity (10.7% 95% C.I. 10.5–11.0% vs. 8.7%, 95% C.I. 8.5%-8.8%; p<0.001) and lower mean 25(OH)D levels (31.3±11.4 ng/mL vs. 31.9±11.8 ng/mL; p<0.001). Regression


A
20%
15%
10%
5%
0%
SARS-CoV-2 Positive Rate
Northern
Central
Southern
20
30
40
50
60
25(OH)D, ng/mL

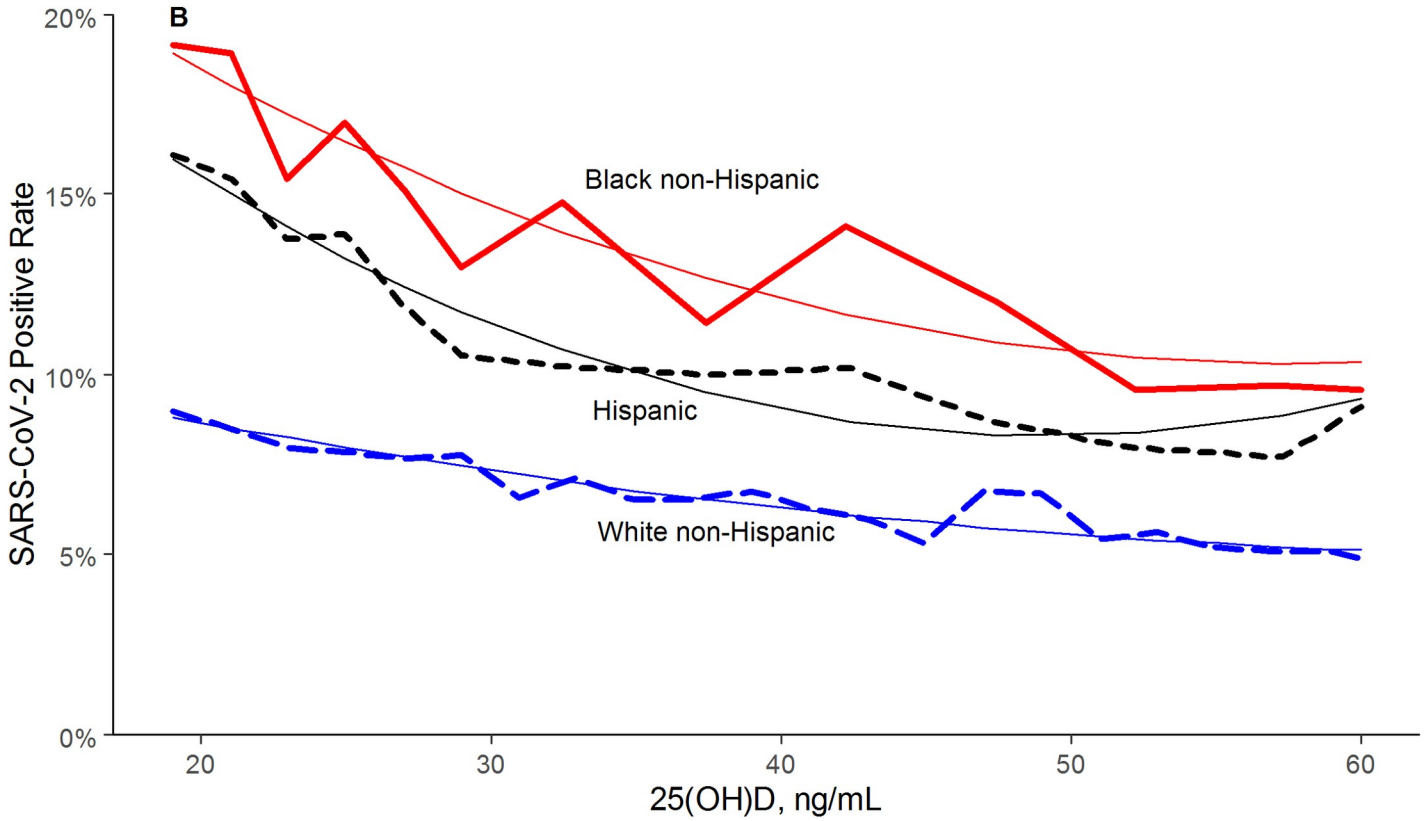

B
20%
15%
10%
5%
0%
SARS-CoV-2 Positive Rate
Black non-Hispanic
Hispanic
White non-Hispanic
20
30
40
50
60
25(OH)D, ng/mL

Fig 2. SARS-CoV-2 NAAT Positivity Rates and Circulating 25(OH)D Levels, (A) by Latitude Region and (B) Predominately Black non-Hispanic, Hispanic, and White non-Hispanic Zip Codes. Smooth lines represent the weighted second order polynomial regression fit to the data associating circulating 25(OH)D levels (x) and SARS-CoV-2 positivity rates (y) where: Northern: $y = 0.2544–0.0055^{*}x + 5.2e\text{-}05^{*}x^2$; $R^2 = 0.94$. Central: $y = 0.1745–0.0049^{*}x + 4.7e\text{-}05^{*}x^2$; $R^2 = 0.94$. Southern: $y = 0.1693–0.0052^{*}x + 5.2e\text{-}05^{*}x^2$; $R^2 = 0.90$. Black non-Hispanic: $y = 0.2948–0.0067^{*}x + 5.8e\text{-}05^{*}x^2$; $R^2 = 0.87$. Hispanic: $y = 0.2873–0.0083^{*}x + 8.5e\text{-}05^{*}x^2$; $R^2 = 0.95$. White non-Hispanic: $y = 0.1219–0.0021^{*}x + 1.5e\text{-}05^{*}x^2$; $R^2 = 0.92$. SI conversion factor: 1 ng/mL = 0.400641 nmol/L.

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analysis indicated strong correlation between 25(OH)D level and SARS-CoV-2 positivity in all these groups (Fig 3A and 3B).

The association between lower SARS-CoV-2 positivity rates and higher circulating 25(OH)D levels per ng/mL remained significant in a multivariable logistic model (adjusted odds ratio 0.984, 95% C.I. 0.983–0.986; $p<0.001$). Other significant factors in both the adjusted and unadjusted models were male sex, northern and central latitudes, predominately black non-Hispanic zip codes, and predominately Hispanic zip codes (Table 1).

Discussion

These results demonstrate an inverse relationship between circulating 25(OH)D levels and SARS-CoV-2 positivity. For the entire population those who had a circulating level of 25(OH)D <20 ng/mL had a 54% higher positivity rate compared to those who had a blood level of 30–34 ng/mL. The risk of SARS-CoV-2 positivity continued to decline until the serum levels reached 55 ng/mL. This finding is not surprising, given the established inverse relationship between risk of respiratory viral pathogens, including influenza, and 25(OH)D levels [14–16]. Vitamin D supplementation may reduce acute respiratory infections, especially in people with vitamin D deficiency [17]. A previous study found that each 4 ng/mL increase in circulating 25(OH)D levels was associated with a 7% decreased risk of seasonal infection, a decrement of approximately 1.75% per ng/mL [18]. This is remarkably similar to the 1.6% lower risk of SARS-CoV-2 positivity per ng/mL found in our adjusted multivariable model.

Patients with the lowest circulating levels of 25(OH)D had approximately 5–7% higher absolute SARS-CoV-2 positivity across northern, central, and southern latitudes. Indeed, Covid-19 diagnoses and particularly mortality exhibit a decreasing worldwide North-South latitude gradient [19]. The inverse relationship between SARS-CoV-2 positivity and 25(OH)D levels was most striking in predominately black non-Hispanic zip codes, followed by predominately Hispanic zip codes. Although 25(OH)D levels appeared to play a role for all race/ethnicities, patients from predominately black non-Hispanic zip codes had higher SASR-CoV-2 positivity than those from predominately white non-Hispanic zip codes at every 25(OH)D level. Other potential reasons including chronic diseases, occupations, housing, and lifetime risk exposures for the increased impact of COVID-19 on African Americans and Latinos have been published previously [20–22].

Northern and central latitudes, predominately Hispanic zip codes, predominately black non-Hispanic zip codes, age <60 years, and male sex were independently associated with both 25(OH)D levels and SARS-CoV-2 positivity. Yet, both in stratified analyses and in a model that controlled for all of these factors, the relationship between SARS-CoV-2 positivity and circulating 25(OH)D levels remained.

Limitations of this retrospective study include that testing for SARS-CoV-2 was based on selection factors, including presence and gravity of symptoms and exposure to infected individuals. High-risk groups, such as healthcare workers and first responders, are also more likely to be tested. Another limitation is that race/ethnicity estimates were based on aggregate U.S. Census proportions by zip code. There may be many other potentially confounding factors that were neither identified nor controlled for in this study. As expected, the multivariable model displayed poor overall fit and correlation statistics, given SARS-CoV-2 can infect

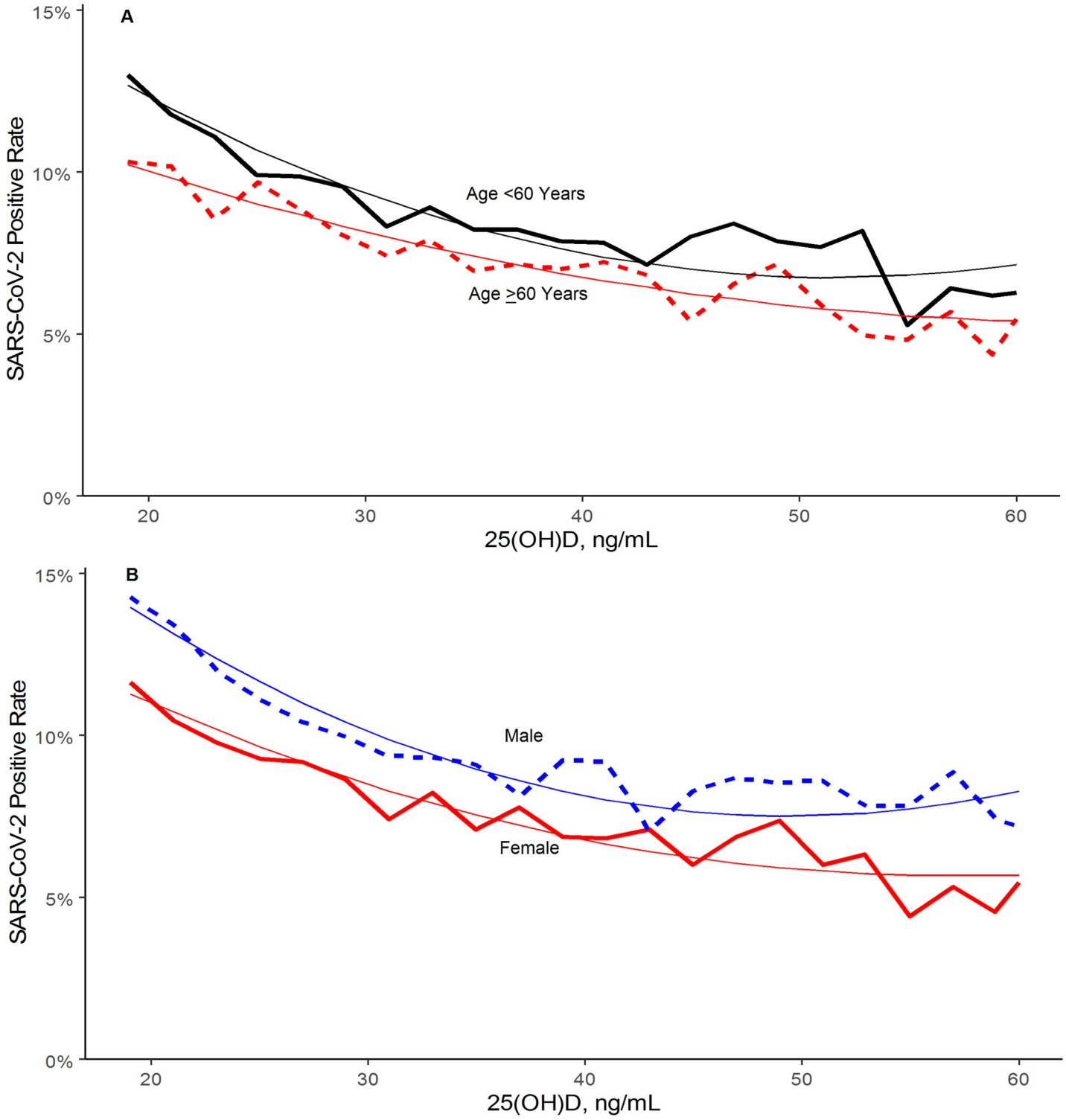

Fig 3. SARS-CoV-2 NAAT Positivity Rates and Circulating 25(OH)D Levels by (A) Age Group and (B) Sex. Smooth lines represent the weighted second order polynomial regression fit to the data associating circulating 25(OH)D levels (x) and SARS-CoV-2 positivity rates (y) where: Age <60: $y = 0.2161–0.0058^{*}x + 5.6e\text{-}05^{*}x^2$; $R^2 = 0.94$. Age ≥60: $y = 0.1515–0.0030^{*}x + 2.4e\text{-}05^{*}x^2$; $R^2 = 0.91$. Female: $y = 0.1837–0.0045^{*}x + 3.9e\text{-}05^{*}x^2$; $R^2 = 0.94$. Male: $y = 0.2445–0.0068^{*}x + 6.9e\text{-}05^{*}x^2$; $R^2 = 0.94$. SI conversion factor: 1 ng/mL = 0.400641 nmol/L.

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Table 1. Associations with SARS-CoV-2 positivity.

	Unadjusted Odds Ratio (95% C.I.)	Adjusted Odds Ratio (95% C.I.)
25(OH)D (per ng/mL increment)	0.979 (0.977–0.980)	0.984 (0.983–0.986)
Male	1.26 (1.22–1.31)	1.24 (1.20–1.28)
Female	reference	reference
Age ≥60 years	0.74 (0.71–0.76)	0.84 (0.81–0.87)
Age <60 years	reference	reference
Latitudes		
Northern (>40 degrees)	2.43 (2.32–2.54)	2.66 (2.54–2.79)
Central (32–40 degrees)	1.17 (1.12–1.23)	1.22 (1.16–1.28)
Southern (<32 degrees)	reference	reference
Race/Ethnicity zip codes		
Predominately black non-Hispanic	2.04 (1.93–2.17)	2.03 (1.91–2.15)
Predominately Hispanic	1.61 (1.54–1.67)	1.95 (1.87–2.04)
All other zip codes	reference	reference

Adjusted model H-L Fit: $p = 0.003$; $R^2 = 0.024$. SI conversion factor: 1 ng/mL = 0.400641 nmol/L. Adjusted model included 188,028 patients with no missing values (98% of included patients).

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anyone. The intent of the model was to determine whether circulating 25(OH)D levels remained significantly associated with SARS-CoV-2 positivity after adjustment for other identified factors.

The major strength of this study is the direct assessment of circulating 25(OH)D levels in a large cohort; this approach can more clearly elucidate the relationship of circulating 25(OH)D levels to SARS-CoV-2 positivity than is possible when using latitude as a surrogate for vitamin D status.

In conclusion, SARS-CoV-2 NAAT positivity is strongly and inversely associated with circulating 25(OH)D levels, a relationship that persists across latitudes, races/ethnicities, sexes, and age ranges. Our findings provide further rationale to explore the role of vitamin D supplementation in reducing the risk for SARS-CoV-2 infection and COVID-19 disease. If controlled trials find this relationship to be causative, the implications are vast and would present a cheap, readily-available method for helping prevent infection, especially for those with vitamin D deficiency. This could be of increased importance for the African American and Latinx community, who are disproportionately affected by both COVID-19 and vitamin D deficiency. In the interim, the authors recommend responsible vitamin D supplementation based on personal needs, risk factors, and advice from personal physicians in accordance with existing Endocrine Society Guidelines [23].

Author Contributions

Conceptualization: Harvey W. Kaufman, Justin K. Niles, Martin H. Kroll, Caixia Bi, Michael F. Holick.

Data curation: Harvey W. Kaufman, Justin K. Niles, Martin H. Kroll, Caixia Bi.

Formal analysis: Harvey W. Kaufman, Justin K. Niles, Martin H. Kroll, Caixia Bi, Michael F. Holick.

Investigation: Justin K. Niles, Martin H. Kroll, Caixia Bi, Michael F. Holick.

Methodology: Harvey W. Kaufman, Justin K. Niles, Martin H. Kroll, Caixia Bi, Michael F. Holick.

Project administration: Harvey W. Kaufman, Michael F. Holick.

Software: Harvey W. Kaufman, Justin K. Niles, Martin H. Kroll, Caixia Bi.

Supervision: Michael F. Holick.

Validation: Michael F. Holick.

Visualization: Harvey W. Kaufman, Justin K. Niles, Martin H. Kroll, Caixia Bi, Michael F. Holick.

Writing – original draft: Harvey W. Kaufman, Justin K. Niles, Martin H. Kroll, Caixia Bi, Michael F. Holick.

Writing – review & editing: Harvey W. Kaufman, Justin K. Niles, Martin H. Kroll, Caixia Bi, Michael F. Holick.

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PLOS ONE

RESEARCH ARTICLE

Vitamin D sufficiency, a serum 25-hydroxyvitamin D at least 30 ng/mL reduced risk for adverse clinical outcomes in patients with COVID-19 infection

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Data Availability Statement: The data is part of the inpatient database of Sina Hospital COVID-19 Registry (SHCo-19R) and was used under license for the current study. The datasets used and analyzed during the current study will be available from the Research Development Center of Sina Hospital (Dr. Hale Ashraf; sina.research.development.center@gmail.com) on reasonable request. Based on the ethics board of Tehran University of Medical Sciences, access to data

Abstract

Background

To investigate the association between serum 25-hydroxyvitamin D levels and its effect on adverse clinical outcomes, and parameters of immune function and mortality due to a SARS-CoV-2 infection.

Study design

The hospital data of 235 patients infected with COVID-19 were analyzed.

Results

Based on CDC criteria, among our study patients, 74% had severe COVID-19 infection and 32.8% were vitamin D sufficient. After adjusting for confounding factors, there was a significant association between vitamin D sufficiency and reduction in clinical severity, inpatient mortality serum levels of C-reactive protein (CRP) and an increase in lymphocyte percentage. Only 9.7% of patients older than 40 years who were vitamin D sufficient succumbed to the infection compared to 20% who had a circulating level of 25(OH)D< 30 ng/ml. The significant reduction in serum CRP, an inflammatory marker, along with increased lymphocytes percentage suggest that vitamin D sufficiency also may help modulate the immune response possibly by reducing risk for cytokine storm in response to this viral infection.

should be permitted after considered in the COVID-19 research committee.

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Conclusion

Therefore, it is recommended that improving vitamin D status in the general population and in particular hospitalized patients has a potential benefit in reducing the severity of morbidities and mortality associated with acquiring COVID-19.

Background

Coronavirus disease (COVID-19) is a respiratory and systemic disorder caused by "severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)" with a range of severity from mild respiratory symptoms to severe lung injury, multi-organ failure, and death [1]. On March 11, the COVID-19 outbreak was characterized as a pandemic by the WHO [2]. It is now affecting 212 countries and territories around the world with approximately 5,041,620 confirmed Coronavirus cases, of whom 327,062 deaths as of May 20, 2020.

The virus spread was rapid in Iran and by March 20, 2020, all 31 provinces were infected. The total number of confirmed cases by May 20, 2020, was 126,949, with 7,183 deaths; 86 deaths per 1M population (https://www.worldometers.info/coronavirus/Iran).

The rapid spread of COVID-19 has identified as a public health emergency of international concern. The complete clinical picture of COVID-19 is not fully known but is associated with significant respiratory symptoms and in some cases induces the acute respiratory distress syndrome (ARDS) with multiple organ failure especially in elder patients with history of being treated for chronic disorders [3].

Recently, some clinical trials have been conducted such as convalescent plasma, clustered regularly interspaced short palindromic repeats (CRISPR), mesenchymal stem cell (MSC), remdesivir as an antiviral therapy and dexamethasone as an anti-inflammatory medication for people suffering from COVID-19. The safety and efficiency of these antiviral strategies are not yet proven to be efficacious and are under consideration [4–7].

Among confirm therapies by Food and Drug Administration (FDA), remdesivir and dexamethasone are the only treatments that have been shown a possibility to reduce death and improve the primary outcomes in patients with COVID-19 [6, 7].

Of note, there is a great need not only to develop a vaccine to prevent the infection but also there is a need for more therapeutics to treat the infection for all COVID-19 patients and other interventions to help reduce risk of infection and its serious health consequences.

The virus infects type II pneumocytes and enterocytes as the primary target cells [8]. Spike proteins of the virus facilitates viral entry into the target cells through binding with the angiotensin converting enzyme 2 (ACE-2) on the surface of the cells [9]. ACE-2, a regulator of the renin-angiotensin system is distributed in many tissues in the body including lung, kidney, gastrointestinal (GI) tract, and cardiovascular system [10] that could explain multi-organ failure in susceptible patients.

It has been suggested that vitamin D has a protective effect against COVID-19. Vitamin D has been shown to have immunomodulatory activity. Vitamin D [1,25-dihydroxyvitamin D; 1,25(OH)2D], interacting with its receptor (VDR) in immune cells, modulates the innate and acquired immune systems in response to invasion of bacterial and viral pathogens. [11]. It also acts as a modulator of renin-angiotensin pathway and down-regulates ACE-2 [12]. Therefore, vitamin D might help in treatment of COVID-19 by preventing the cytokine storm and subsequent ARDS which is commonly the cause of mortality [13].

Iran is sunny country but the prevalence of vitamin D deficiency is high especially in elder people [14, 15] who present with more severe clinical manifestations after exposure to SARS-CoV-2. It is our hypothesis that vitamin D sufficiency would reduce risk for clinical severity and adverse clinical outcomes including mortality associated with a COVID-19 infection.

Material and methods

Study design and participants

This is a cross-sectional analysis of a COVID-19 database in Sina hospital, Tehran, Iran.

Data were collected until May 1, 2020.

The current study was approved by the ethics review board at Tehran University of Medical Sciences (IR.TUMS.VCR.REC.1399.338).

Data source

Hospital medical records were analyzed from inpatient database of Sina Hospital COVID-19 Registry (SHCo-19R) [16]. SHCo-19R is an ongoing, prospective, hospital-based registry of patients diagnosed with COVID-19 presenting to emergency department of Sina Hospital, affiliated to Tehran University of Medical Sciences.

Patients and data collection. Diagnosis was made by infectious disease specialists based on WHO interim guidance and the recommendations by the Iranian National Committee of COVID-19 [16]. The patients were 18 years of age and older with acute respiratory tract infection symptoms (e.g. fever, cough, dyspnea) with no other etiology that fully explained the clinical presentation. The diagnosis was supported by chest computed tomography (CT) scan findings compatible with COVID-19 or a definitive diagnosis of COVID-19 with real-time polymerase chain reaction (RT-PCR).

CDC criteria were used for the disease severity and prognosis; which includes mild-moderate (mild respiratory symptoms and fever, on an average of 5–6 days after infection), severe disease (dyspnea, respiratory frequency $\geq$30/minute, blood oxygen saturation $\leq$ 93%, and/or lung infiltrates $>$50% of the lung field within 24–48 hours) and critical (respiratory failure, septic shock, and/or multiple organ dysfunction/failure). Patients with at least two complications, including acute respiratory distress syndrome (ARDS), acute cardiac injury (ACI), acute kidney injury (AKI) or acute liver injury consider as multiple organ damage. Hypoxia defines as an arterial blood oxygen saturation levels below 90%. Severe and critical categories were defined "severe" in data analysis.

Study measurements

Data were included following information: demographic information (age, sex, body mass index (BMI)), smoking habit, medical history, principal clinical symptoms and their onset time, RT-PCR results, radiological findings, laboratory findings, comorbidities, and disease progression.

Laboratory examination at the time of admission to the hospital or soon thereafter included a complete blood count, blood biochemistries (total 25-hydroxyvitamin D [25(OH)D], calcium (Ca), Phosphorus (P), magnesium (Mg), sodium (Na), potassium (K), alanine transaminase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), C-reactive protein (CRP), procalcitonin (PCT), troponin I and erythrocyte sedimentation rate (ESR), and also arterial blood gas (PO2, PCO2, HCO3, pH).

Total serum 25(OH)D was measured by electrochemiluminescence (Abbott Architect), with the limit of quantitative value at 2.2ng/ml at 20% coefficient variation (CV).

Overall, a cutoff point of 30 ng/mL was used for the definition of vitamin D sufficiency based on the Endocrine Society's Practice Guidelines on Vitamin D that defined vitamin D deficiency and insufficiency as a circulating level of 25(OH)D of <20 ng/mL and 20–29 ng/mL respectively [17].

Diagnostic results of chest computed tomographic (CT) scans was provided by two radiologists independently, and then were cross-checked. For the patients with inconsistent diagnostic results or who were suspected of having COVID-19, the final diagnosis was made after the deliberation of the two radiologists.

Only first laboratory findings and CT scan results after hospitalization were used in the present study.

Statistical analysis. Data were analyzed by SPSS statistical software (version 20). 25(OH)D, CPK and LDH levels, did not have a normal distribution, a log transformation was applied to correct their normality distribution. Continue variables were presented as mean (standard deviation [SD]) for with normally distributed or median (interquartile range [IQR]) for non-normally distributed data and Student's t-test and Mann-Whitney U test were used to compare normal and non-normal distributed variables. The categorical variables were presented as percentage and chi square test was applied to examine the percentage differences of the sign and symptom, requiring mechanical ventilation, shock, multiple organ failure and requires intensive care and hospital mortality rates in patients with and without vitamin D deficiency/insufficiency.

A back-ward logistic regression model was used to determine the independent association of vitamin D sufficiency with severity of the disease. P values <0.05 were considered significant.

Results

A total of 611 patients with COVID-19 were registered in database of Sina Hospital COVID-19 until May 1, 2020. Among them, 235 patients were analyzed in this cross-sectional study who had laboratory documentation of a 25(OH)D level at the time of hospitalization. The mean age was 58.7 years ± 15.2 SD (range: 20–90 years) and 37.4% of patients were 65 years or older. All patients had CT scan report but 31.06% of patients had RT-PCR results. Among all patients, 66% had at least a history of a chronic disorder; 36.6% diabetes, 44.4% hypertension, 1.3% immunological disorders, 1.3% COPD, 22.1% heart disorders, 0.9% malignancy, 5.5% lung disorders, 4.3% asthma, 3% rheumatology disorders. Also, 0.4% of patients had cirrhosis and 0.9% of patients were HIV positive. The baseline characteristics of the 235 patients are presented in Tables 1 and 2.

COVID clinical features and vitamin D sufficiency

A cutoff point equal or higher than 30 ng/mL of 25(OH)D was used for the definition of vitamin D sufficiency. In total, 67.2% of the patients had a 25(OH)D level of less than 30ng/mL.

To assess the role of vitamin D status in relation to the disease clinical features, all data were classified into two subgroups based on 25(OH) D levels that were less than or 30 ng/mL.

Vitamin D sufficiency was associated with a statistically significant lower risk of unconsciousness and hypoxia, defined by an arterial blood oxygen saturation levels below 90%. The serum CRP and lymphocyte percentage in the blood were significantly lower and higher respectively in patients who were vitamin D sufficient (Tables 3 and 4). There were no

Table 1. Demographic characteristics and clinical outcomes of the study population.

Demographic characteristic	N = 235
Age (years)†	58.72±15.22
Sex (Men)‡	61.3% (144)
BMI (kg/m^2)†	27.41±4.55
Current smoker ‡	38.6% (66/171)
Systolic Blood Pressure† (mmHg)	126.28±21.55
Diastolic Blood Pressure† (mmHg)	76.95±12.68
Clinical outcomes	
Duration of hospitalization (days)†	5.96±3.57
ICU admission ‡	18.7% (44)
O2 saturation (%)†	90.60±6.37
Hypoxia: O2 saturation less than 90%‡	32.5% (76)
Intubation ‡	10.2% (24)
Bilateral lung involvement in chest CT ‡	26.3%(62)
Unconsciousness	6% (14)
Chest pain ‡	10.2%(24)
Dyspnea ‡	57.4%(135)
Multi organ dysfunction ‡	16.2%(38)
Acute hypoxia respiratory failure ‡	15.3% (36)
Shock ‡	9.4% (22)
Severity (Mild-moderate) ‡	27.2% (64)
Severity (Severe-critical) ‡	72.8%(172)
A history of chronic disorders ‡	66% (155)

Numerical variables were expressed as the mean ± SD or median (IQR). Body mass index (BMI), computerized tomography (CT), diastolic blood pressure (DBP), intensive care unit (ICU), systolic blood pressure (SBP)

† mean± SD,

††median (IQR),

‡ % (N),

*N = available data for each variable.

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significant differences in hospitalization duration and ICU admissions between patients with and without vitamin D sufficiency (Table 3).

An evaluation of mortality in the patient population revealed that no one under the age of 40 years died as a result of being infected with COVID 19. However, 16.3% of patients 40 years and older succumbed to the infection. Of the 206 patients who were 40 years and older, 20% had a blood level of 25(OH)D<30 ng/mL whereas only 9.7% who perished had a blood level of (25OH)D of at least 30 ng/mL($p = 0.04$). Furthermore only 6.3% of the patients over 40 years of age died with a blood level of 25(OH)D of 40 ng/mL or higher (Fig 1).

Severity of COVID-19 and vitamin D sufficiency

Based on CDC criteria, among our study patients, 74% of those had severe COVID-19 infection. The data analyses revealed that the severe disease infection was less prevalent in patients with vitamin D sufficiency (63.6% vs. 77.2% $p = 0.02$).

In backward logistic regression model after adjusting for age, sex, BMI, smoking and history of a chronic medical disorder, there were significant independent associations between vitamin D sufficiency ($p = 0.01$) and lower BMI ($p = 0.02$) with decreased disease severity.

Table 2. Biochemical and laboratory analysis of the study population.

Biochemical and laboratory analysis	N = 235
R.B.C. (Mil C/ml)†	4.56±0.75
W.B.C. (*1000C/ml) ††	6.50 (4.40)
Neutrophil (%)†	73.69±11.65
Lymphocyte (%)†	20.01±10.29
ANC (*1000C/ml) ††	4.76 (3.84)
ALC (*1000C/ml) ††	1.19 (0.65)
Hb (gr/dl) †	13.26±2.13
HCT(g/dl) †	37.86±5.92
PLT (*1000 C/ml) †	211.14±88.72
BS (mg/dl)†	139.08±72.46
Urea (mg/dl) ††	31.00(26.00)
BUN (mg/dl††	20.09 (42.06)
Cr (mg/dl) ††	1.08 (0.47)
Na (mEq/L)†	135.57±5.58
K(mEq/L)†	4.32±0.53
Ca (mg/dl)†	8.62±0.73
P (mg/dl)†	3.54±1.01
Mg (mg/dl)†	2.26±0.46
Ln-25OHD (ng/ml)†	3.03±0.69
ESR-1 hr (mm/hr)†	53.41±30.48
CRP (mg/l)†	74.97±51.01
Ln.CPK (U/lit)†	5.04±0.92
Ln.LDH (U/lit)†	6.35±0.41
PCT (ng/ml) ††	0.36 (0.84)
Troponin I (ng/ml)††	5.4 (10.91)
ALT (U/L)††	36 (24.75)
AST (U/L)††	48.00 (28.75)
ALP (U/L)†	199.61±135.03
Arterial blood gas analysis	
PO2 (mmHg)††	29 (16.75)
pH	7.42±0.07
PCO2 (mmHg)	38.03±7.55
HCO3 (mmol/L)†	25.4 (5.80)

Numerical variables were expressed as the mean ± SD or median (IQR).

Categorical variables were presented as percentages. 25(OH)D, CPK and LDH levels, did not have a normal distribution, a log transformation (Ln) was applied to correct their normality distribution.

Absolute neutrophil count (ANC), absolute lymphocyte count (ALC), hemoglobin (Hb), hematocrit (HCT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood sugar (BS), blood urea nitrogen (BUN), calcium (Ca), creatinine (Cr), creatine phosphokinase (CPK), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), bicarbonate (HCO3), potassium (K), lactate dehydrogenase (LDH), magnesium (Mg), sodium (Na), platelets count (PLT), phosphorus (P), procalcitonin (PCT), partial pressure of oxygen (PO2), partial pressure of carbon dioxide (PCO2).

† mean± SD,

††median (IQR),

‡ % (N),

*N = available data for each variable.

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Table 3. The COVID-19 clinical outcomes based on vitamin D status.

Clinical outcomes	25OHD ≥30	25OHD < 30	P-value
	N = 77	N = 158	
Hospitalization (day)†	5 (5)	5 (5)	0.28
Duration from illness onset to first admission (day) †	7 (7)	7 (7)	0.30
Chest pain ‡	14.3% (11)	8.2% (13)	0.17
Dyspnea ‡	51.9% (40)	60.1% (95)	0.26
ICU admission ‡	14.3% (11)	20.9% (33)	0.33
Acute respiratory distress syndrome ‡	11.7% (9)	17.1% (27)	0.33
Intubation ‡	7.8% (6)	11.4% (18)	0.49
Multi-organ damage ‡	13% (10)	17.7% (28)	0.45
Acute kidney injury ‡	13% (10)	15.2% (24)	0.69
Bilateral lung involvement‡	31.7 (19)	33.3% (43)	0.86
Shock‡	6.5 (5)	10.8% (17)	0.34
Unconsciousness	1.3%(1)	8.2%(13)	0.03
Hypoxia† b	19.4% (15)	39.2%(62)	0.004
Quantitative C-reactive protein (CRP)>40mg/L ‡	61(47)	77.2(122)	0.01
blood lymphocyte percentage<20% ‡	45.5(35)	60.1(95)	0.03
Severity † c	63.6% (49)	77.2%(122)	0.02

Numerical variables were expressed as median (IQR). Categorical variables were presented as percentages.

Hospitalization range: 1–23 days in patients with vitamin D deficiency/insufficiency and 1–19 days in patients with vitamin D sufficiency.

Duration form illness onset to first admission: 0–30 days in patients with vitamin D deficiency/insufficiency and 0–21 days in patients with vitamin D sufficiency.

† median (IQR),

‡ % (N),

[a] only in patients older than 40 years,

[b] defined as an arterial blood oxygen saturation levels below 90%,

[c] Severe-critical.

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Discussion

To assess the association between vitamin D sufficiency and severity of the disease, all patients were categorized based on cut point of 30 ng/mL for 25(OH)D as recommended by the Endocrine Society.[14] Our data revealed that a 25(OH)D levels of at least 30 ng/mL was associated with a significant decrease in the severity of clinical outcomes related to a COVID-19 infection.

Table 4. Relative risk of COVID-19 clinical outcomes associated with patients who had a 25(OH)D<30 ng/mL.

Clinical outcomes	Relative Risk	95% CI (lower, upper)	P-value
Severity ‡	1.59	1.05, 2.41	**0.02**
Unconsciousness	1.07	1.02, 1.13	**0.03**
Hypoxia† b	1.32	1.11, 1.57	**0.004**
C-reactive protein (CRP)>40mg/L	1.7	1.13,2.56	**0.01**
lymphocyte percentage<20%	1.36	1.03, 1.80	**0.03**

Values in bold indicate statistical significance (P<0.05).

† Only in patients older than 40 years.

‡ Severe-critical.

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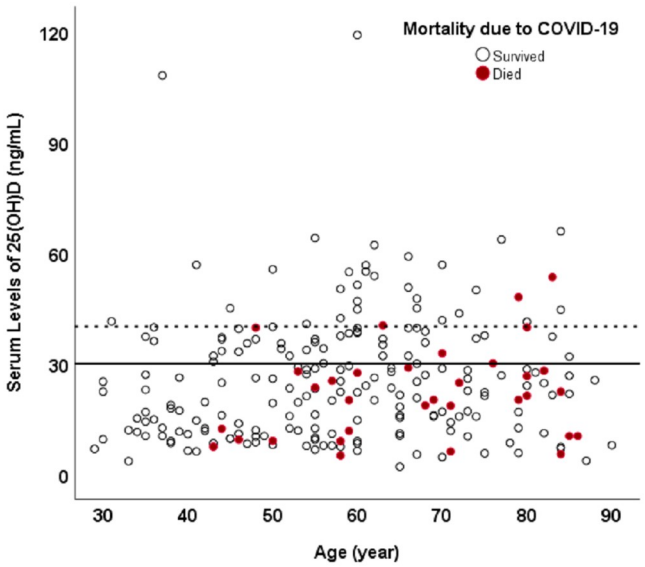

Fig 1. The association between vitamin D status and inpatient mortality because of COVID-19. A scatter plot relating mortality in patients with a serum 25(OH)D level. The red dots represent the inpatients who perished and the black dots represented the patients who have survived. The solid black line separates the patients with vitamin D deficiency/insufficiency (below the solid line) from the vitamin D sufficient patients (above the solid line). The number of red dots (inpatient mortality) above the solid line is significantly less compared to the dots below the line. Also, the trend of reducing inpatient mortality is continued for higher levels of serum 25(OH)D. The dotted line represents a serum level of 25(OH)D of 40 ng/mL. The mortality (red dots) is very rare in patients with serum 25(OH)D of at least 40ng/mL (above the dotted line). An evaluation of mortality in the patient population revealed that no one under the age of 40 years died as a result of being infected with COVID 19. However 16.3% of patients 40 years and older succumbed to the infection. Of the 206 patients who were 40 years and older, 20% had a blood level of 25(OH)D<30 ng/mL whereas only 9.7% who perished had a blood level of 25OH)D of at least 30 ng/mL($p = 0.04$). Furthermore only 6.3% of the patients over 40 years of age died with a blood level of 25(OH)D of 40 ng/mL or higher.

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Based on different guidelines, the threshold for serum 25(OH)D has been set at 20–30 ng/mL for bone health [17–20]. With respect to vitamin D's non-skeletal effects, including the immune system, it has been suggested that a higher blood level of 25(OH)D of at least 30 ng/mL is required [21–23].

In our study only 32.8% of patients with documented COVID-19 infection were vitamin D sufficient. It is notable that the COVID-19 outbreak began during the winter. In 1981, a "seasonal stimulus" hypothesis had been suggested to explain epidemics of influenza A around the winter solstice [24]. The biology, physiology, and epidemiology of vitamin D point to vitamin D as a likely candidate for the "seasonal stimulus" since the blood levels of 25(OH)Dare lowest at the end of the winter [25].

In a nationwide 1958 British birth cohort, Berry et al. [26] reported the association between vitamin D status and seasonal infections. The authors evaluated 25(OH)D, lung function, forced vital capacity, and respiratory infections of 6789 participants from the age of 45 years. The authors reported "The prevalence of respiratory infections had a strong seasonal pattern in the opposite direction to the pattern for 25(OH)D concentrations". Also they presented a linear association between vitamin D status and seasonal infections and lung function. Each 10 nmol/L (4 ng/mL) increase in serum 25(OH)D level was associated with a 7% lower risk of infection.

Recently several researchers have mentioned the impact of vitamin D on prevention of COVID-19 or using vitamin D as an intervention strategy in patients affected by SARS-CoV-2. The suggestion is largely based on the impact of vitamin D status on influenza infectious

disease. A meta-analysis of randomize control trials shows that improving the vitamin D status in children and adults has been associated with reduced risk of upper or lower respiratory tract infections [27].

The possible role of vitamin D in infectious diseases like COVID-19 is explained by its regulatory role on acquired immunity and innate immunity [11]. There is a complex interaction between vitamin D, infection and the immune system. To help regulate innate immunity, 1,25 (OH)2D is produced in macrophages in response to the stimulation of toll-like receptors by the binding of an infectious agent. 1,25(OH)D binds to the VDR in the macrophage resulting in an increase in the production of antimicrobial peptides (AMPs) such as defensin and cathelicidin that have antiviral effects [28]. In acquired immunity pathways, 1,25(OH)2D has more modulating effect. 1,25(OH)2D inhibits activation of B-cells [29] and immunoglobulin synthesis [29]. This hormone also promotes Treg cells, which are responsible for anti-infectious action by inducing IL-10 production. This leads to suppression of Th1, and Th17 cells and IFNγ, IL-17, IL-6, IL-23 and IL-2 production and makes Th2 cells predominant. Th2 cells limits inflammatory processes by inhibiting Th1 cell-mediated cytokines and tumour necrosis factor α (TNFα) [11, 30, 31]. Of note, the active form of vitamin D regulates invariant NK T cells (iNKT) that are regulatory cells to link innate and adaptive immunity systems [30]. Our results are very consistent with the immunomodulatory effect of vitamin D. Our results indicated that the lymphocyte percentage in patients with vitamin D deficiency/insufficiency were lower than patients with vitamin D sufficiency. A recent study suggested that lymphocyte percentage can be used as a reliable indicator to classify the moderate, severe, and critical ill types independent of any other auxiliary indicators [32].

Indeed, the anti-inflammatory role of 1,25(OH)2D could explain the protective role of vitamin D against immune hyper reaction and cytokine storm in a subgroup of patients with severe COVID-19. This is also consistent with the recent observation that C-reactive protein (CRP), a surrogate for vitamin D status, was associated with severity of COVID-19 [33]. They concluded that higher CRP levels associated with vitamin D deficiency were related to an increased risk for severe COVID-19 [33]. Their finding is consistent with our results. Our findings indicated that CRP levels in patients with higher levels of serum 25(OH)D was lower than patients with a serum level of 25(OH)D< 30 ng/mL (Tables 3 and 4). Also, the severity of COVID-19 infection in patients with vitamin D sufficiency was lower than other patients with higher levels of 25(OH)D. This finding can be explained by the anti-inflammatory effect of vitamin D on reducing the inflammatory markers like CRP that was observed in our study. This anti-inflammatory effect of vitamin D might prevent cytokine storm in COVID-19 patients and may explain the decreased risk of severity and mortality observed in our patients who were vitamin D sufficient. Recent study showed that in the early stage of COVID-19 CRP levels were positively correlated with lung lesions and could reflect disease severity [34]. Also, CRP levels in severe COVID-19 patients increased significantly at the initial stage, before CT findings [35]. Importantly consistent with our study, serum levels of CRP, which was associated with disease development, predicted early severe COVID-19 [35].

In a preliminary estimate of underlying health conditions among patients with COVID-19, in the United States, 37.6% patients had one or more underlying health condition or risk factor [36]. The percentage of COVID-19 patients with at least one underlying health condition or risk factor was higher among those requiring intensive care unit (ICU) admission (358 out of 457, 78%) [36].

To assess the independent role of vitamin D sufficiency on severity of COVID-19, the logistic regression model was used to adjust age, sex, smoking and at least one underlying health condition as well as obesity. Our finding showed that patients with a 25(OH)D of less than 30 ng/mL had a RR = 1.59 associated with COVID-19 severity.

In a recent study, the mean level of 25(OH)D for 20 European countries was related to morbidity and mortality caused by COVID-19 [37]. Negative correlations between mean levels of 25(OH)D (average 56 mmol/L, SD 10.61; 22.4 ng/mL, SD 4.2) in each country and the number of COVID-19 cases/1 M (mean 295.95, SD 298.7, and mortality/1 M (mean 5.96, SD 15.13) were observed. Their results are consistent with our findings that show that the patient's risk of mortality was lower in patients who were vitamin D sufficient (Fig 1).

Some limitations in our study are worth noting. Firstly, we included patients who had recorded 25(OH)D levels. Some confounding factors, such as smoking, and social economic status were not recorded for all patients and could have a plausible impact on the COVID-19 severity. Also, the RT-PCR test was not performed on all patients with clinical signs of COVID-19. Secondly, the design of our study is cross-sectional. So, we cannot explain the cause and effect relationship of vitamin D sufficiency and the reduced risk of severity from a COVID-19 infection. Designing large-scale studies and randomized clinical trials (RCTs) needs to evaluate the interaction between them.

Conclusion

The present study revealed an independent association between vitamin D sufficiency [25 (OH)D $\geq$ 30 ng/mL] and decreased risk of adverse clinical outcomes from COVID-19. The severity of clinical outcomes from COVID-19 and mortality were reduced in patients who were vitamin D sufficient. Clinical features were also significantly different in patients who were vitamin D sufficient. They had a lower risk of becoming unconscious and becoming hypoxic. Patients who were vitamin D sufficient had significantly lower blood levels of the inflammatory marker CRP and had a higher total blood lymphocyte count suggesting that vitamin D sufficiency had improved the immune function in these patients and raising the inflammatory markers. This beneficial effect on the immune system may also reduce the risk of acquiring this insidious potentially life-threatening viral infection. It is recommended that further studies including RCTs are need be designed to evaluate the role of vitamin D status on risk of developing COVID-19 infection and mitigating complications and mortality in those infected with the virus. It remains debatable as to what the optimum serum level of 25(OH)D should be for maximizing its effect on the immune system. We did observe that 6.3% of the patients who had a blood level of 25(OH)D of at least 40 ng/mL succumbed to the infection compared to 9.7% and 20% who died and had a circulating blood level above and below 30 ng/mL respectively. Thus, a blood level of at least 40 ng/mL may be optimal for vitamin D's immunomodulatory effect. Therefore, based on the available literature and results from this study it is reasonable to recommend vitamin D supplementation, along the guidelines recommended by the Endocrine Society to achieve a blood level of 25(OH)D of at least 30/mL, to children and adults to potentially reduce risk of acquiring the infection and for all COVID-19 patients especially those being admitted into the hospital.

Supporting information

S1 Video.
(MP4)

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