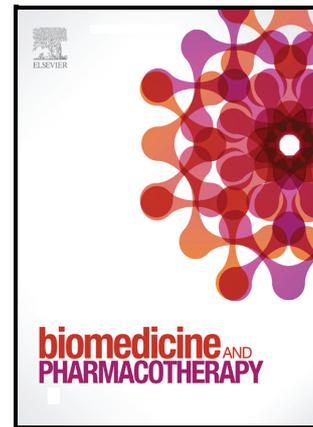


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Immunomodulatory effect of vitamin D supplementation in patients with COVID-19

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## **Changes in the immune response against SARS-CoV-2 in individuals with severe COVID-19 treated with high dose of vitamin D**

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## **Abstract**

Main cause of severe illness and death in COVID-19 patients appears to be an excessive but ineffectual inflammatory immune response that may cause severe acute respiratory distress syndrome (ARDS). Vitamin D may favour an anti-inflammatory environment

and improve cytotoxic response against some infectious diseases. A multicenter, single-blind, prospective, randomized clinical trial was approved in patients with COVID-19 pneumonia and levels of 25-hydroxyvitamin D (25(OH)D) of 14.8 ng/mL (SD: 6.18) to test antiviral efficacy, tolerance and safety of 10,000 IU/day of cholecalciferol (vitamin D<sub>3</sub>) for 14 days, in comparison with 2,000 IU/day. After supplementation, mean serum 25(OH)D levels increased to 19 ng/mL on average in 2,000 IU/day *versus* 29 ng/mL in 10,000 IU/day group ( $p < 0.0001$ ). Although levels of inflammatory cytokines were not modified by treatment with 10,000 IU/day, there was an increase of anti-inflammatory cytokine IL-10 and higher levels of CD4<sup>+</sup> T cells, with predominance of T central memory subpopulation. Cytotoxic response against pseudotyped SARS-CoV-2 infected cells was increased more than 4-fold in patients who received 10,000 IU/day. Moreover, levels of IFN $\gamma$  were significantly higher in this group. Beneficial effect of supplementation with 10,000 IU/day was also observed in participants who developed ARDS and stayed at the hospital for 8.0 days, whereas those who received 2,000 IU/day stayed for 29.2 days ( $p=0.0381$ ). Administration of high doses of vitamin D<sub>3</sub> as adjuvant of the standard care treatment during hospitalization for COVID-19 may improve the inflammatory environment and cytotoxic response against pseudotyped SARS-CoV-2 infected cells, shortening the hospital stay and, possibly, improving the prognosis.

## Keywords

COVID-19; SARS-CoV-2; Vitamin D supplementation; Immune cytotoxic response

## Introduction

In December 2019, several viral pneumonia cases of unknown origin were detected in Wuhan City (Hubei Province, China), associated with an outbreak in a seafood market. Sequencing of the pathogen genome identified a new member of *Coronaviridae* family which was named severe acute respiratory syndrome coronavirus

2 (SARS-CoV-2). This virus rapidly spread all over the world and the Coronavirus Disease 2019 (COVID-19) was declared a global public health threat by the World Health Organization on March 11<sup>th</sup>, 2020<sup>1</sup>.

COVID-19 may cause different clinical manifestations, from asymptomatic disease to severe acute respiratory distress syndrome (ARDS), and ultimately death<sup>2</sup>. The host receptor for SARS-CoV-2 entry into intestinal and alveolar cells is angiotensin converting enzyme 2 (ACE2), and the subsequent dysregulation of the renin-angiotensin system (RAS) may lead to a massive cytokine activation, known as cytokine storm. This is a life-threatening syndrome characterized by high-level activation of immune cells and excessive production of a large number of inflammatory cytokines and chemical mediators<sup>3,4</sup>, ultimately resulting in prospective fatal ARDS. Individuals with severe presentations of COVID-19 show high levels of proinflammatory cytokines such as interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF $\alpha$ ) that may interfere with the cytotoxic response of Natural Killer (NK) and CD8<sup>+</sup> T cells by reducing the production of granzymes and perforins<sup>5,6</sup>, as well as lymphopenia, mainly of CD4<sup>+</sup> T lymphocytes and NK cells that also show an exhausted phenotype<sup>7,8</sup>. Moreover, individuals with severe COVID-19 present an impaired antiviral immune response as a consequence of reduced levels of cytokines with antiviral activity such as interferons (IFN)<sup>9</sup>.

For a long time, there was no clinically proven specific treatment for COVID-19<sup>10</sup>. Supportive treatment, including oxygen and conservative fluid therapy, remains the most important strategy for patient management<sup>2,11</sup>. Drugs designed for other viral infections (e.g. remdesivir), used in rheumatology (e.g. baricitinib) and humanized monoclonal IL-6R antibodies (e.g. tocilizumab) were some of the options that were assayed, but none proved to have a decisive beneficial effect<sup>12,13</sup>, except for unspecific treatments to control the excessive inflammatory immune responses such as

corticosteroids<sup>14</sup>. Recently, FDA authorized the oral administration of molnupiravir for the treatment of mild-to-moderate COVID-19 in adults, as well as nirmatrelvir and ritonavir for children and adults, but only in case of emergency for those individuals with high risk to progress to severe disease<sup>15,16</sup>.

It is still undetermined why some patients show an asymptomatic disease whereas other individuals require invasive mechanical ventilation and admission to the Intensive Care Unit (ICU). The development of these severe and critical forms of COVID-19 have been associated with some predisposing factors such as obesity, hypertension and diabetes<sup>17</sup>. Other factor that has also been related to an increased risk of dying from COVID-19 is vitamin D deficiency. In fact, several reports have described an association between serum levels of 25-hydroxyvitamin D [25(OH)D] below 20 ng/mL (50nmol/L), and concomitant diseases, including systemic infection<sup>18–21</sup>. Vitamin D exerts an immunomodulatory role by promoting the development of an anti-inflammatory environment. Besides, it has been described that vitamin D stimulates ACE2 that may bind to SARS-CoV-2, preventing it from attaching to ACE2 receptors and infecting the target cells<sup>22–24</sup>. Moreover, some studies described that vitamin D insufficiency during COVID-19 may compromise the respiratory immune response, thereby increasing the risk of severity and mortality<sup>25</sup>, and that there is a correlation between the vitamin D levels and the severity of the disease<sup>26,27</sup>. However, there is still no clear evidence that vitamin D supplementation can prevent the severity and/or mortality of COVID-19.

In this study, we evaluated whether vitamin D supplementation to avoid its insufficiency during COVID-19 may influence positively the disease progression by reducing the levels of pro-inflammatory cytokines and increasing the cytotoxic antiviral response. Therefore, we analyzed the changes in the immune response of individuals

with COVID-19 who were recruited for a randomized clinical trial to receive a moderate dose of 2,000 International Units (IU)/daily of cholecalciferol or a higher dose of 10,000 IU/daily for 14 days, in combination with the standard drug therapeutic regimen. The efficacy of both treatments at reducing the duration and severity of COVID-19 was analyzed.

## **Materials and methods**

### **Study design and participants**

This study was a multicenter, single blind, prospective, randomized clinical trial to evaluate the effect of cholecalciferol (vitamin D) supplementation in combination with the standard drug regimen therapeutic in patients hospitalized with pneumonia due to COVID-19. For ethical reasons, it was not felt possible to treat one arm with placebo because a potentially effective treatment would be denied to the patients. This study was sponsored by the Hospital Universitario Severo Ochoa (Madrid, Spain) and was conducted in other four Tertiary Care Hospitals in Spain: Hospital Universitario Infanta Leonor (Madrid), Hospital Universitario Fundación Alcorcón (Madrid), Hospital Universitario Príncipe de Asturias (Madrid), and Hospital Universitario de Cabueñes (Asturias) between June 2020 and March 2021.

The inclusion criteria for the participants in the study were being adults (>18 years old) hospitalized for at least seven days from the onset of COVID-19 symptoms, which is when usually began the inflammatory phase, with a diagnosis of pneumonia due to COVID-19 based on clinical-radiological criteria and a laboratory confirmed SARS-CoV-2 infection diagnosis, oxygen saturation <94% and 25(OH)D serum levels <30 ng/mL. Participants were excluded if they were lactating, pregnant women, were participating in other clinical trials with drugs with potential antiviral action for

COVID-19, were on treatment with digoxin, had evidence of Multiple Organ Dysfunction Syndrome (MODS), were requiring mechanical ventilation at the time of inclusion, had hypersensitivity to cholecalciferol or the excipient refined olive oil, had hypercalcemia or hypercalciuria, were diagnosed with hereditary fructose intolerance, sarcoidosis or hyperparathyroidism, had glucose-galactose malabsorption or sucrose insufficiency or had chronic kidney disease (stage 4; estimated glomerular filtration rate (eGFR) $<30$ ). Participants who were expected to be transferred to another Hospital Care Centre in the following 96 hours were also excluded. A sample size of 41 evaluable participants in each group was estimated to have 90% power, with a 0.05 two-sided significance level, to detect a difference of 15 ng/mL in 25(OH)D serum levels between groups before and after the cholecalciferol supplementation. The randomization was done by blocks of 5 using the block randomization-sampling module with the user command “rndseq block” included on the STATA 14.2 software (StataCorp LLC, College Station, TX). Eligible patients were assigned randomly in a 1:1 ratio to receive either a supplementation of a dose of 10,000 IU of cholecalciferol or a dose of 2,000 IU once daily for fourteen days. In both groups, participants were given the oral solution Thorens 25,000 IU/2.5 mL (Grupo Italfarmaco, Madrid, Spain). Throughout the study, all patients received any concomitant medication deemed necessary to provide adequate standard care, according to local recommendations.

Peripheral blood samples and clinical data were collected at baseline (day 0), day 7 and day 14. Patients were required to fulfil the treatment and follow up visits even if the hospital discharge occurred before day 14. Patient data were registered at the Research Electronic Data Capture tool (REDCap) online platform (developed by Vanderbilt University, Nashville, TN), which included demographics, respiratory status, radiological score, biochemical parameters, coexisting comorbidities and treatments.

Serious adverse events, the onset of the symptoms, length of stay at the hospital (LOS), ARDS, ICU admission and mortality were also collected. The primary endpoint was the increase of 25(OH)D serum level  $\geq 30$  ng/ml after 14 days of supplementation (end of supplementation), without any related adverse events. The secondary endpoints were the LOS and, in a subgroup of patients, the analysis of changes in the plasma inflammatory profile and the cytotoxic immune response from baseline to end of the supplementation.

### **Ethical statement**

The protocol (ID 01052020) for this study was developed under the Declaration of Helsinki and previously reviewed and approved by the Spanish Agency for Medicines and Health Products (AEMPS) (EudraCT Number 2020-002312-43), as well as by the Ethics Committee for Research involving Medicines (CEIm) of the Hospital Universitario Severo Ochoa (Madrid). All patients recruited for the study gave their written informed consent to participate in the study. The Spanish and European Data Protection Laws in force guarantee the confidentiality of the participants.

### **Cells**

Whole blood was collected in EDTA Vacutainer tubes (Becton Dickinson, Madrid, Spain) and then treated by Ficoll-Hypaque (Pharmacia Corporation, North Peapack, NJ) density gradient centrifugation to isolate peripheral blood mononuclear cells (PBMCs) and plasma that were cryopreserved until the analysis. PBMCs were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 2mM L-glutamine, 100 $\mu$ g/ml streptomycin, 100UI/ml penicillin (Lonza, Basel, Switzerland). Cells viability after thawing was assessed by flow cytometry. K562 (Human Caucasian chronic myelogenous leukaemia) cell line (ECACC 89121407) was kindly provided by Dr Cristina Eguizabal (Basque Centre Transfusions and Human Tissue, Álava, Spain). These cells were also cultured in RPMI 1640 medium supplemented with 10% FCS,

2mM L-glutamine, 100µg/ml streptomycin, 100 IU/ml penicillin. Vero E6 (African green monkey kidney) cell line (ECACC 85020206) was kindly provided by Dr. Antonio Alcami (CBM Severo Ochoa, Madrid) and was cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine and 100 IU/ml penicillin and streptomycin (Lonza).

### **Vitamin D detection**

The quantification of the level of 25(OH)D (ng/ml) was performed from serum samples with the automated immunoassays Liaison 25(OH) Vitamin D Total assay DiaSorin Liaison XL (DiaSorin, Italy) in the participating hospitals, according to the manufacturer's instructions

### **Luminex assay**

The detection and quantification of cytokines in plasma samples of the participants was performed using a customized Human Magnetic Luminex Assay kit (R&D Systems) panel for IL1 $\beta$ , IL2R, IL6, IL8, IL10, sCD14, TNF $\alpha$ , MIP-1 $\alpha$ , MIP-1 $\beta$ , GM-CSF, IFN $\alpha$ , IFN $\beta$ , and IFN $\gamma$  according to the supplier's guidelines and recommendations. The analysis was performed on a Bio-Plex 200 System (Bio-Rad).

### **Antibodies and flow cytometry**

For staining of cell surface markers, the following conjugated antibodies were used: CD3-APC (BD Biosciences; San Jose, CA), CD4-PercP (BD), CD8-APC-H7 (BD), CD16-PercP (BD), CD56-FITC (BD), CD107a-PE-Cy7 (BD), TCR $\gamma\delta$ -PE (BioLegend, London) and CD158f/KIR2DL5-BV421 (R&D Systems) for analysis of CD4+ conventional T-cells (CD4+ T), CD8+ conventional T-cells (CD8+ T), NK, and NKT cell populations. CD4-PercP, CD25-PE-Cy5 and CD127-FITC (R&D Systems) for CD4+ natural regulatory T-cells (Treg) and CCR7-FITC (Biosciences) and CD45RA-PE-Cy7 (Biosciences) for CD4+, CD8+ T cell memory subpopulations that were

determined as follows: naïve (CD45RA+CCR7+), central memory (TCM) (CD45RA-CCR7+), effector memory (TEM) (CD45RA-CCR7-) and terminally differentiated effector memory (TEMRA) (CD45RA+CCR7-) cells. Samples were acquired on BD LSRFortessa X-20 flow cytometer (BD Biosciences, San Jose, CA) and analyzed using Flow Jo software v10.0.7 (Tree Star Inc., Ashland, OR, USA).

### **Direct NK cell-mediated cytotoxicity assay**

The analysis of the NK-mediated direct cell-mediated cytotoxicity (DCC) of PBMCs of the participants was performed using the NK-sensitive target K562 cell line as previously described<sup>9</sup>. K562 cells are lymphoblasts isolated from an individual with chronic myeloid leukemia that are used as specific targets of NK cells due to they do not express HLA Class I. Briefly, K562 cells were stained with PKH26 Red Fluorescence Cell Linker kit (Sigma Aldrich-Merck) and co-cultured 1:1 for 1 hour with PBMCs isolated from the patients. Cells were then collected and Annexin V conjugated with FITC (Thermofisher) was used to measure early apoptosis by flow cytometry. Data were acquired on BD LSRFortessa X-20 flow cytometer (BD Biosciences) and analyzed using Flow Jo software v10.0.7 (Tree Star Inc.).

### **Direct cell-mediated cytotoxicity assay against pseudotyped SARS-CoV-2-infected cells**

In order to evaluate the specific antiviral DCC against SARS-CoV-2-infected cells of PBMCs from the participants in the study, the single-cycle pseudotyped virus pNL4-3 $\Delta$ env\_SARS-CoV-2-S $\Delta$ 19(G614)\_Ren was used to infect a monolayer of sensitive Vero E6 cells. This virus encodes SARS-CoV-2 spike glycoprotein, within the HIV-1 genome, as well as the Renilla luciferase gene generated as previously described<sup>28,29</sup>. Briefly, Vero E6 cells were infected with pNL4-3 $\Delta$ env\_SARS-CoV-2-S $\Delta$ 19(G614)\_Ren (100 ng p24 Gag/well) for 48 hours. Cells were then washed and co-

cultured for 1 hour with PBMC from the participants at a ratio of 1:2. PBMCs were collected and the cytotoxic cell populations were analyzed by flow cytometry. Vero E6 monolayer was dissociated from the plate with trypsin-EDTA solution (Sigma Aldrich-Merck, Darmstadt, Germany), and the caspase-3 activity was quantified by luminescence using Caspase-Glo 3/7 Analysis system (Promega) as a measurement of induction of apoptosis by the cytotoxic cells.

### **Statistical Analysis**

Categorical data were expressed as percentages, while the median was used to describe the central tendency of the non-normally distributed numerical data. Group comparisons were done using Mann–Whitney U-test or ordinary one-way ANOVA and Tukey’s multiple comparisons test for numerical data and Chi-square ( $\chi^2$ ) or Fisher’s exact test for categorical data. All statistical analyses and graphics were performed using the GraphPad Prism software version 8.4.3 (GraphPad Software Inc., San Diego, California). P-values lower than 0.05 (two-tailed) were considered to be statistically significant.

## **Results**

### **Demographics and clinical characteristics of the participants**

A total of 86 patients were recruited for this study. Eighty-five participants fulfilled the inclusion criteria and agreed to participate in the study, and one patient was excluded from the study due to not being able to meet the study’s schedule. After randomization, 41 patients received the supplementation of 10,000 IU/day (high dose) and 44 patients received 2,000 IU/day (moderate dose) of vitamin D. All patients in both groups completed the entire study. A summary of patient demographics and clinical data of the groups are described in Table 1. The median age of the participants was 65.0 years

(interquartile range [IQR]: 53.0-74.0), and it took a median of seven days (IQR: 6-10) from the onset of the symptoms of COVID-19 to the hospitalization. Most of them (70.6%) were men and, according to the body mass index (BMI) classification, 54.1% of the patients were obese. Additionally, the main coexisting conditions were hypertension (48.2%), dyslipidemia (36.5%), and diabetes (22.3%). Most of the participants (85.9%) showed bilateral pneumonia at x-rays. The most common symptoms of COVID-19 were malaise (94.2%), fever (84.7%), cough (75.3%), and dyspnea (65.9%) (Supplemental Table 1). In agreement with adequate randomization, we did not observe differences at baseline in demographic characteristics, vital signs, blood biochemistry data, distribution of ordinal scale scores, or treatments by group, although the levels of haemoglobin and bilirubin were significantly higher ( $p=0.006$  and  $p=0.010$ , respectively) in the group of participants who received 10,000 IU/day (Supplemental Table 1).

#### **Maintenance of vitamin D serum levels**

At the start of the study, mean serum 25(OH)D levels were 14 ng/ml (SD:6) in the group of patients who received 2,000 IU/day and 15 ng/ml (SD: 6) in the group who received 10,000 IU/day. After seven days of supplementation, the increase in serum vitamin D levels was 1.31-fold ( $p=0.0001$ ) in the 10,000 IU/day group in comparison with the 2,000 IU/day group, and after 14 days the levels were increased 1.53-fold ( $p<0.0001$ ) in the 10,000 IU/day group, in comparison with the 2,000 IU group/day. Within the 2,000IU/day group, serum vitamin D levels were increased 1.24- ( $p=0.0061$ ) and 1.33- ( $p=0.0006$ ) fold at days 7 and 14, respectively, whereas within the 10,000 IU/day group, serum vitamin D levels increased 1.51-fold ( $p<0.0001$ ) and 1.91-fold ( $p<0.0001$ ) after 7 and 14 days of supplementation, respectively (Figure 1). Ten (11.76%) participants of the study developed ARDS, six (13.64%) were assigned to the

2,000 IU/day group (5 of 6 individuals of this group were admitted to the ICU) and four (9.76%) to the 10,000 IU/day group (2 of 4 individuals of this group were admitted to the ICU) but no significant differences were observed in the serum vitamin D levels of these participants between groups after seven and fourteen days of treatment. The quantification values of serum 25(OH)D of the participants measured at each time point of the study are shown in Supplemental Table 2. After the supplementation, 9.09% (4/44) of the participants who received the moderate dose achieved serum 25(OH)D levels  $\geq 30$ ng/mL, while 39.02% (16/41) of the participants who received the high dose achieved the target 25(OH)D level of 30ng/mL ( $p=0.0027$ ).

#### **Differences in the length of hospital stay between groups**

The participants who received 10,000 IU/day spent an average of 6.44 days in the hospital, while in the group of participants who received 2,000 IU/day the average LOS was 9.36 days, but there was no significant difference between both groups (Figure 2A). The analysis of the LOS among the participants who developed ARDS showed that those participants (9.76%) who received the highest dose of vitamin D stayed at the hospital an average of 8.0 days (SD: 5.099), whereas those patients (13.6%) who received the moderate dose, stayed for an average of 29.2 days (SD: 18.76) ( $p=0.0381$ ) (Figure 2B).

#### **Safety and mortality**

During the study, 13 adverse effects were reported in the participants, of which 7 adverse effects occurred in the 2,000 IU/day group and 8 adverse effects occurred in the 10,000 IU/day group. However, none of these adverse events was directly linked to the treatment with cholecalciferol. Nine participants (20.45%) experienced adverse events in the moderate dose group and 8 (19.51%) in the high dose group, with no statistically significant differences between both groups. One (2.27%) participant in the 2,000

IU/day group and one (2.44%) participant in the 10,000 IU/day group died as a result of COVID-19 complications during the study.

### **Changes in the cytokine profile during treatment with vitamin D**

A subgroup of 30 patients of each supplementation group was randomly selected to analyze the profile of cytokines in plasma. The levels of pro-inflammatory cytokines TNF $\alpha$  and IL-6 were not significantly different between both groups of participants neither at 7 or 14 days after treatment, whereas the levels of IL-1 $\beta$  in the high dose group were increased 1.45- ( $p < 0.0001$ ) and 1.44- ( $p < 0.0001$ ) fold after 7 and 14 days, respectively (Figure 3A). The comparison between groups showed that the levels of the chemokine CCL4/MIP-1 $\beta$  were increased 1.29-fold ( $p = 0.0002$ ) after 7 days of treatment, and 1.27-fold ( $p = 0.0039$ ) after 14 days in the 10,000 IU/day group (Figure 3B). Similarly, the levels of chemokine CCL3/MIP-1 $\alpha$  were increased 1.12-fold ( $p = 0.0021$ ) after 14 days in the 10,000 IU/day group. There were no significant differences between groups for IL8-CXCL8 levels and for cytokines related to cellular activation such as GM-CSF, sCD25/IL-2R $\alpha$  and sCD14 (Figure 3C). In the case of sCD14, the levels detected were higher than 20,000 pg/mL in some of the patients from both groups (normal values in the absence of infection  $< 200$  pg/ml).

On the other hand, the level of the anti-inflammatory cytokine IL-10 was increased 1.48-fold ( $p = 0.0286$ ) after 7 days in the 10,000 IU/day group in comparison with the 2,000 IU/day group (Figure 4A). Regarding the antiviral cytokines, the level of IFN $\gamma$  was significantly increased 1.54- ( $p = 0.0020$ ) and 1.4- ( $p = 0.0272$ ) fold at 7 and 14 days, respectively, in the 10,000 IU/day group (Figure 4B). We did not find significant differences in the levels of IFN type I  $\alpha$  and  $\beta$  between groups.

### **Effect of the dose of vitamin D on cytotoxic cell populations**

We randomly selected 8 participants from each group to evaluate the direct cytotoxic activity of PBMCs. When K562 cells were used as target, there was an increase of 1.5-fold in the cytotoxic activity of PBMCs from participants who received 10,000 IU/day after 14 days of treatment, in comparison with the 2,000 IU/day group, but this was a non-significant trend (Figure 5A). However, when pseudotyped SARS-CoV-2-infected Vero E6 cells were used as target, there was a significant increase of 4.27-fold ( $p=0.0205$ ) in the activity of caspase-3 in these cells after co-culture with PBMCs isolated from patients supplemented for 14 days with 10,000 IU/day of vitamin D, in comparison with PBMCs from the 2,000 IU/day group (Figure 5B). The analysis of NK cells in these PBMCs revealed that although the levels of CD3-CD56<sup>+</sup> cells were similar between both groups (Figure 5C, left graph), the expression of the degranulation marker CD107a was reduced 1.2-fold ( $p=0.0313$ ) after 14 days of treatment in the group of 2,000 IU/day of vitamin D, whereas in the group treated with 10,000 IU/day the expression of CD107a was increased 1.2-fold after 7 days of treatment ( $p=0.0078$ ) (Figure 5C, right graph). No significant differences were found in total levels of NKT cells or in the expression of CD107a between both groups of treatment (Figure 5D). Interestingly, the expression of the NK cells inhibitory marker CD158f/KIR2DL5 was increased 2.2-fold ( $p=0.0078$ ) in the PBMCs of participants from the group treated with 2,000 IU/day after 14 days of treatment, whereas it was less increased in the PBMCs of participants treated with 10,000 IU/day (1.7-fold;  $p=0.0078$ ) (Figure 5E).

There were no significant differences between both groups in the total count of CD8<sup>+</sup> T cells, the distribution of memory subpopulations, or the levels of CD3<sup>+</sup>CD8<sup>±</sup>TCR $\delta\gamma$ <sup>+</sup> T cells (Supplemental Figure 1).

### **Distribution of CD4+ T cell subpopulations**

CD4+ T cell levels significantly increased 1.31-fold ( $p=0.0464$ ) after 7 days of supplementation with 10,000 IU/day of vitamin D (Figure 6A). In this group, TCM CD4+ T cells were increased 1.42-fold ( $p=0.0053$ ) whereas effector CD4+ T cells such as TEMRA were reduced 2.5-fold ( $p=0.0051$ ) after 7 days of treatment (Figure 6B). No significant differences were found between both groups in the levels of Tregs (Figure 6C).

### **Discussion**

COVID-19 mortality has shown a decreasing North-South gradient that may be explained by the high prevalence of older people in Northern European populations, who are predisposed to a higher probability of cardio-pulmonary and metabolic comorbidities<sup>30</sup>. However, countries such as Italy or Spain have also shown high mortality rates, suggesting that other risk factors might also contribute to COVID-19 infection severity<sup>31</sup>. Due to the immunomodulatory effect of vitamin D, its deficiency may contribute to airway and gastrointestinal infectious diseases, and it has been suggested as an additional risk factor associated with COVID-19 severity and mortality<sup>25,27</sup>, although there is a great controversy about the effect of vitamin D supplementation during COVID-19<sup>32-35</sup>. Though Spain is a country with adequate sunlight, some studies describe that vitamin D intake is lower than the recommended levels of 10  $\mu\text{g}/\text{day}$ <sup>36</sup>, resulting in a high proportion of the Spanish population with deficient levels of vitamin D<sup>37</sup>. In our study, the majority (81.7%) of participants who were hospitalized due to severe COVID-19 showed 25(OH)D serum levels in a deficient range ( $<20 \text{ ng/mL}$ )<sup>38</sup>. The daily supplementation of 10,000 IU cholecalciferol achieved the increase of 25(OH)D serum to levels close to sufficiency, whereas in those patients who received

the 2,000 IU/day dose only a slight increase was observed after 14 days. These results are in line with other randomized clinical trials on vitamin D supplementation in hospitalized COVID-19 patients in which one single dose of 200,000 IU or 5,000 IU/day for 14 days achieved 25(OH)D serum levels of sufficiency ( $\geq 30$  ng/mL)<sup>39,40</sup>. However, 90.90% of the participants who received the moderate dose and 53.66% of the participants who received the high dose did not reach the primary endpoint of 30 ng/mL 25(OH)D in serum. The reduction of 25(OH)D levels during COVID-19 are expected due to this prohormone is an acute phase reactant and it is usually reduced during acute inflammatory procedures<sup>41,42</sup>. Moreover, although there is controversy about the possible role of vitamin D supplementation to improve the health status during COVID-19, the deficiency of vitamin D at hospital admission due to COVID-19 has been appointed as a potential prognostic marker of disease severity in these patients, as it would indicate the progression to the inflammatory form of the disease<sup>43,44</sup>. Therefore, this inflammatory process that is developed during COVID-19 could be responsible for the impairment in achieving the primary endpoint of  $>30$ ng/ml in most participants, even with the higher dose of 10,000 IU/day.

The severe and critical presentations of COVID-19 are characterized by an enhanced inflammatory response<sup>3,6</sup>. Consequently, the administration of corticosteroids has been one of the most effective treatments since the beginning of the pandemic<sup>45</sup>. Although with a much more modest effect, vitamin D has an immunomodulatory role that may favour an anti-inflammatory environment<sup>46</sup>, mostly based on the inhibition of the production of interleukins responsible for Th1 and Th17 polarization, which is characterized by the release of pro-inflammatory cytokines such as IL-6 and TNF $\alpha$ <sup>47</sup>. In our pilot study, we did not find significantly increased levels of pro-inflammatory cytokines in the plasma of those individuals who developed ARDS, despite both events

are usually related during COVID-19<sup>48</sup>. This could be due to the levels of cytokines are frequently higher in the lungs than in plasma and do not often correlate<sup>49</sup>. Therefore, the daily supplementation with a high dose of cholecalciferol did not reduce significantly the levels of inflammatory markers in plasma that had been already released, not even in individuals with ARDS, in accordance with previous studies<sup>50</sup>. However, the participants who received the supplementation of 10,000 IU/day of cholecalciferol showed a significant increase in plasma of the anti-inflammatory cytokine IL-10, which may contribute to reduce the signs and symptoms of the disease related to the inflammatory environment. This result corroborated other studies that also described a significant increase in IL-10 levels after the administration of vitamin D<sup>51,52</sup>. Therefore, the supplementation with vitamin D could at least partially contribute to reducing the inflammatory environment characteristic of COVID-19, which may also be related with the shortening of the length of the hospital stay observed in those participants supplemented with 10,000 IU/day of vitamin D who developed ARDS during the study. Although the number of participants who developed ARDS was quite reduced in our cohort, the improvement in the clinical recovery and the shortening of the stay at the hospital has also been described for individuals who received 50,000 IU/day of cholecalciferol for five days<sup>53</sup>.

On the other hand, COVID-19 is characterized by causing CD4+ T cell lymphopenia, which may be involved in the progression to more severe forms of the disease<sup>54</sup>. Although vitamin D may affect T-cell proliferation by interfering with the production of IL-2<sup>55</sup>, in our study, the CD4 count was significantly higher in patients treated with 10,000 IU/day for 7 days, which has also been described in patients with inflammatory diseases who receive vitamin D supplementation<sup>56</sup>. Moreover, those participants from our cohort who received 10,000 IU/day showed significantly

enhanced levels of long-lived CD4<sup>+</sup> TCM cell subpopulation and reduced levels of CD4<sup>+</sup> TEMRA cells, which are short-lived cells that die upon activation<sup>57</sup> during protective immunity against pathogens<sup>58</sup>. Therefore, the reduced levels of CD4<sup>+</sup> TEMRA cell subpopulation observed in those participants who received 10,000 IU/day may be a consequence of an increased antiviral activity. In fact, we also observed a significant increase in the production of IFN $\gamma$  in the participants who received 10,000 IU/day after 7 days that was maintained after 14 days of treatment, which corroborated other studies that reported a significant increase of IFN $\gamma$  levels after the administration of vitamin D<sup>51,52</sup>. Therefore, supplementation with vitamin D could also be beneficial to induce a higher antiviral effect. Although vitamin D supplementation did not modify the proliferative capacity of cell populations with cytotoxic activity such as NK or CD8<sup>+</sup> T cells, we observed a significant increase in the cytotoxic activity on SARS-CoV-2-infected cells exerted by PBMCs from those participants who received 10,000 IU/day for 14 days. This antiviral activity seemed to rely mostly on NK cell-mediated cytotoxic response, due to the degranulation capacity of these cells increased after 14 days of receiving 10,000 IU/day. Interestingly, an impairment in the expression of the degranulation marker CD107a has been described in NK cells from patients with the most severe forms of COVID-19<sup>59</sup>, which appeared to be counteracted by treatment with vitamin D. We cannot rule out that vitamin D was also promoting a similar effect on CD8<sup>+</sup> T cells because there are precedents that deficiency in vitamin D receptor gene (Vdr<sup>-/-</sup>) may cause an impairment in the cytotoxic response against viral infections, reducing the diversity of effector CD8<sup>+</sup> T cell repertoire and the survival of antigen-specific cytotoxic clones after viral clearance<sup>60</sup>.

In conclusion, the efficacy of vitamin D supplementation in patients with COVID-19 is still controversial<sup>32-35</sup>, likely due to variations in the clinical

presentations of the disease and the design of the different studies. In our pilot clinical trial, the supplementation of 10,000 IU/day for 14 days in patients with severe outcomes of COVID-19 was safe and well-tolerated and it appeared to have a beneficial clinical effect, despite the serum levels of vitamin D did not reach the primary clinical endpoint in all individuals. However, the participants showed improved biochemical and haematological parameters in plasma, such as vitamin D levels close to sufficiency and enhanced CD4 count, as well as increased levels of the anti-inflammatory cytokine IL-10, which may contribute to decreasing the inflammatory environment characteristic of the severest forms of COVID-19. Besides, our results are consistent with previous observations that vitamin D may enhance the lytic activity of NK cells<sup>61-63</sup>, although this is the first description that this vitamin D-mediated lytic activity may be directed against cells expressing the spike protein of SARS-CoV-2 on the surface after infection with pseudotyped virus. We cannot rule out that early administration of vitamin D, just after being hospitalized due to severe COVID-19, would have been more beneficial than waiting for the inflammatory phase to begin. However, due to the controversy, more studies are needed to establish a solid association between vitamin D and clinical recovery of COVID-19.

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### **Conflicts of interest**

The authors declare no conflict of interest.

### **Author Contributions**

MCer and MCo conceptualized the project. MT, GC and MCo wrote the manuscript. MT, GC and LV performed the study of cytotoxicity. MT, GC and SRM performed the analysis of cell populations by flow cytometry with technical assistance from EM.

MRLH and GC performed the analysis of cytokines in plasma. FR and EM processed and stored all blood samples. DLW, MNM, PRM, MLTM and MCer identified, selected, and recruited the patients, and also collected the blood samples. MT, MCer and MRLH collected and analyzed the clinical data. All co-authors read and approved the final version of the manuscript.

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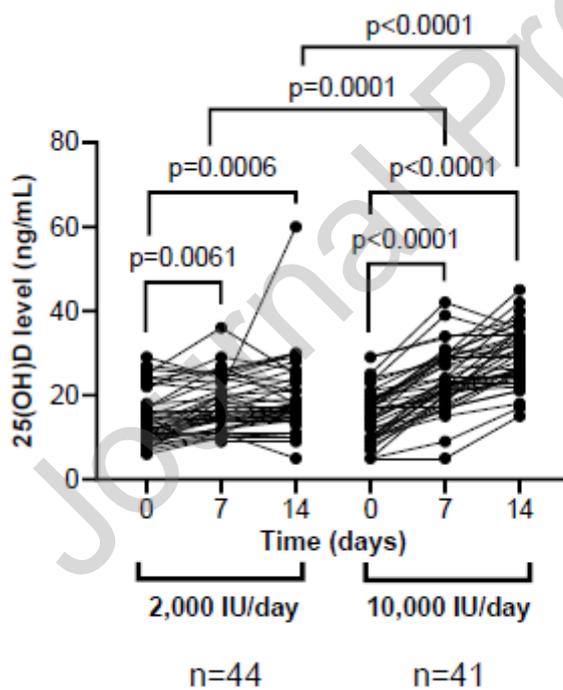
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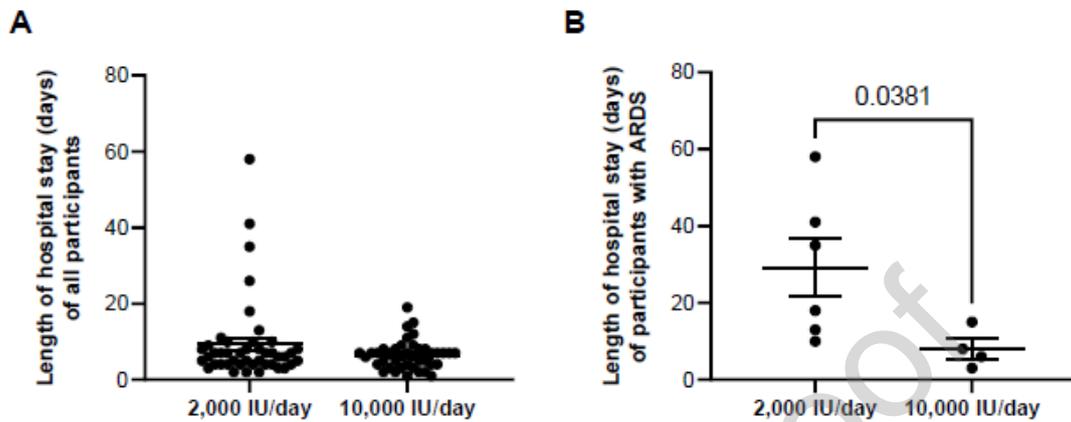
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### Figure legends



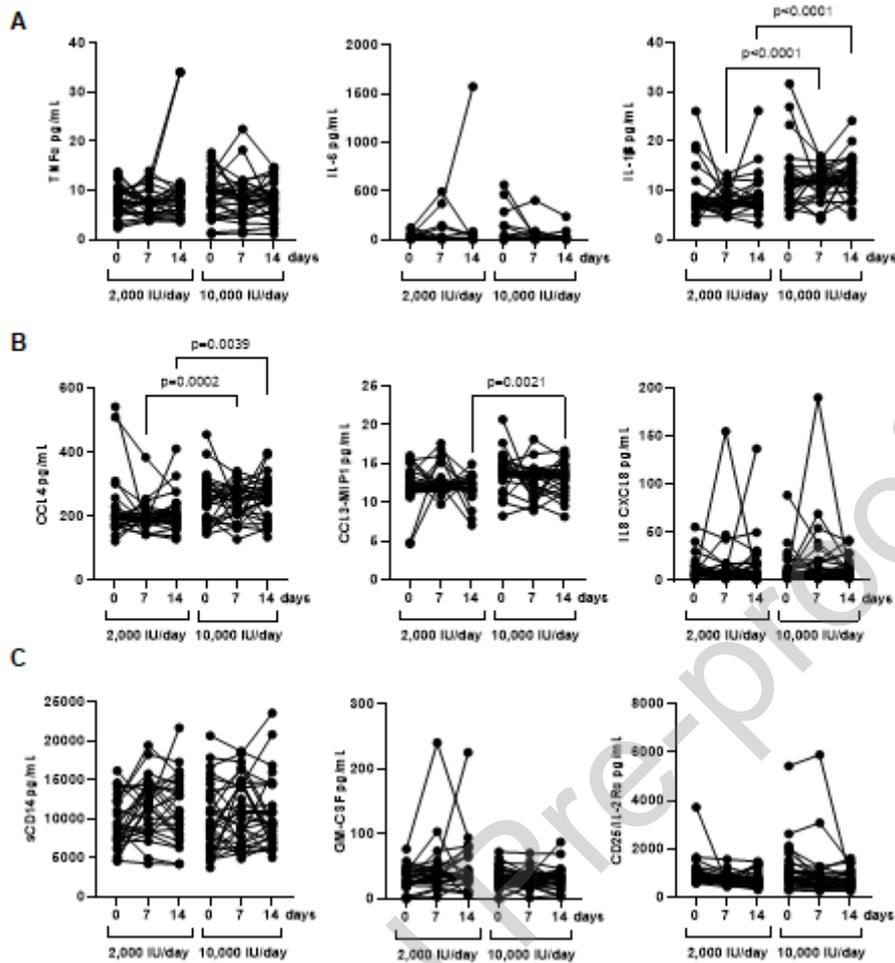
**Figure 1. Quantification of 25(OH)D plasma levels.** The levels of 25(OH)D (ng/ml) in serum samples of hospitalized patients with COVID-19 who received 2,000 IU/day and 10,000IU/day of cholecalciferol was determined at baseline and after 7 and 14 days

of treatment. Each dot corresponds to one sample. Statistical significance was calculated using Mann–Whitney U-test.

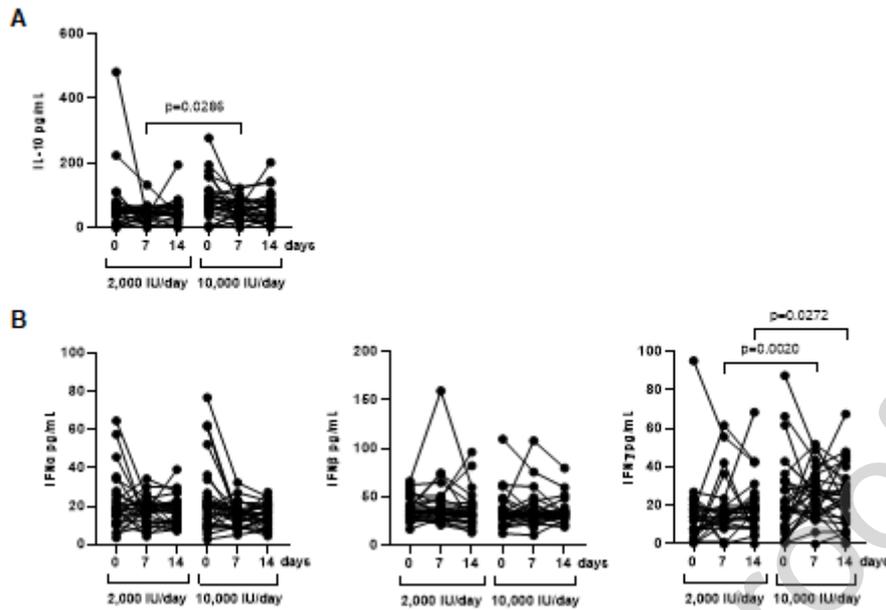


**Figure 2. Length of hospital stay in patients treated with 2,000 or 10,000 IU/day.**

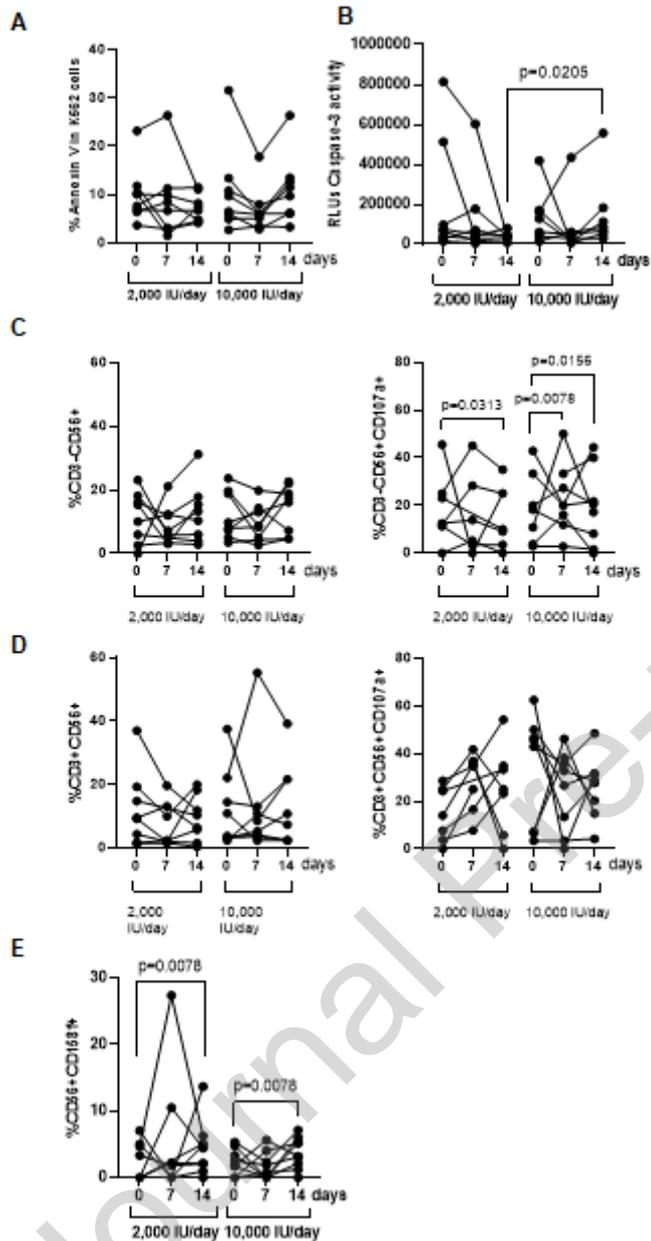
(A) Total length of hospital stay (days) in hospitalized patients with COVID-19 who received 2,000 IU/day and 10,000IU/day of cholecalciferol and (B) length of hospital stay (days) according to ARDS diagnosis. Each dot corresponds to one sample and lines represent mean  $\pm$  standard error of the mean (SEM). Statistical significance was calculated using Mann–Whitney U-test.



**Figure 3. Pro-inflammatory and activating cytokine profile in plasma samples of hospitalized patients with COVID-19 who received each dose of vitamin D.** (A) Levels (pg/ml) of pro-inflammatory cytokines TNF $\alpha$ , IL-6, and IL-1 $\beta$  were quantified in plasma of patients hospitalized with COVID-19 who received 2,000 IU/day and 10,000IU/day of cholecalciferol at baseline, 7 and 14 days of treatment. Levels (pg/ml) of chemokines CCL4, CCL3-MIP1, and IL8 CXCL8 (B), as well as cytokines related to cell activation sCD14, GM-CSF, and IL25/I2R- $\alpha$  (C) were also quantified in plasma of the same patients. Each dot corresponds to one sample. Statistical significance was calculated using Mann–Whitney U-test.

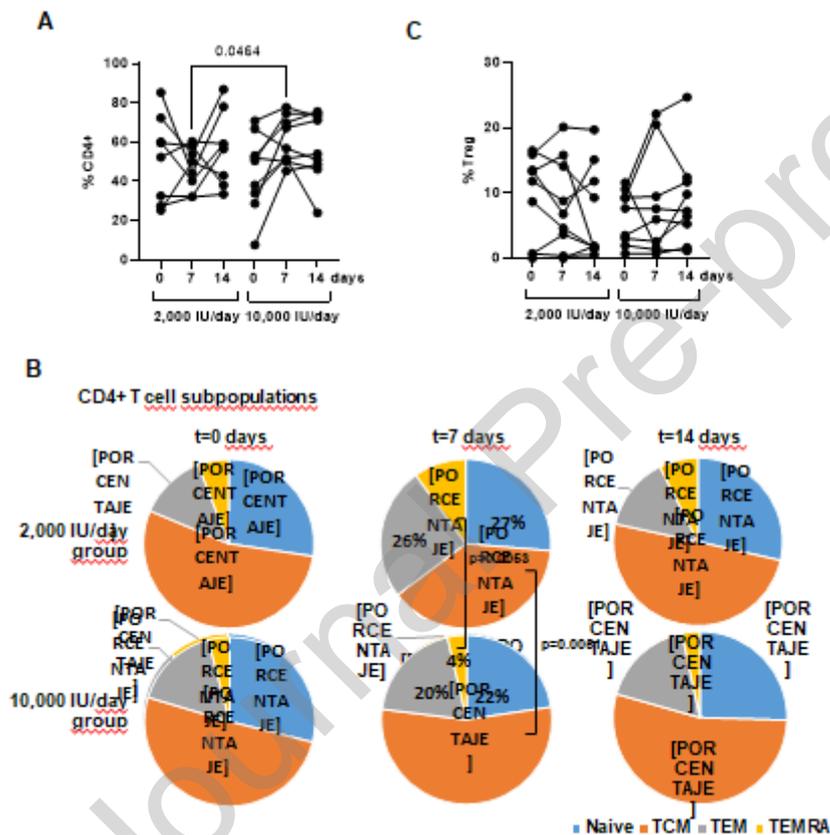


**Figure 4. Anti-inflammatory and antiviral cytokine profile in plasma samples of hospitalized patients with COVID-19 who received each dose of vitamin D.** Levels (pg/ml) of the anti-inflammatory cytokine IL-10 (A) and antiviral cytokines IFN $\alpha$ , IFN $\beta$  and IFN $\gamma$  (B) were quantified in plasma of patients hospitalized with COVID-19 who received 2,000 IU/day and 10,000IU/day of cholecalciferol at baseline, 7 and 14 days of treatment. Each dot corresponds to one sample. Statistical significance was calculated using Mann–Whitney U-test.



**Figure 5. Evaluation of the cytotoxic response in PBMCs of hospitalized patients with COVID-19 who received each dose of vitamin D.** (A) Cytotoxicity of PBMCs from patients with COVID-19 who received 2,000 IU/day and 10,000IU/day of cholecalciferol at baseline, 7 and 14 days was analyzed by quantifying early apoptosis in K562 cells as unspecific target after co-culture (1:1) for 1 hour. (B) Direct antiviral cytotoxicity against SARS-CoV-2 infected cells was evaluated in the same PBMCs by quantifying caspase-3 activity in a monolayer of Vero E6 cells infected with

pseudotyped SARS-CoV-2 virus G614 that were co-cultured with PBMCs (1:1) for 1 hour. Both NK (C) and NKT (D) cell populations were analyzed by evaluating the expression of the activation marker CD56 and the degranulation marker CD107a. (D) The expression of the inhibitory marker CD158f/KIR2DL5 was evaluated in the same cells. Each dot corresponds to one sample. Statistical significance was calculated using Mann–Whitney U-test.



**Figure 6. Analysis of CD4+ T cell subsets in PBMCs of hospitalized patients with COVID-19 who received each dose of vitamin D.** Percentage of total CD4+ T cells (A), the distribution of CD4 subpopulations (B), and Tregs (C) was determined in PBMCs from hospitalized patients with COVID-19 who received 2,000 IU/day or 10,000IU/day of cholecalciferol at baseline, 7 and 14 days. Each dot corresponds to one sample. Statistical significance was calculated using Mann–Whitney U-test.

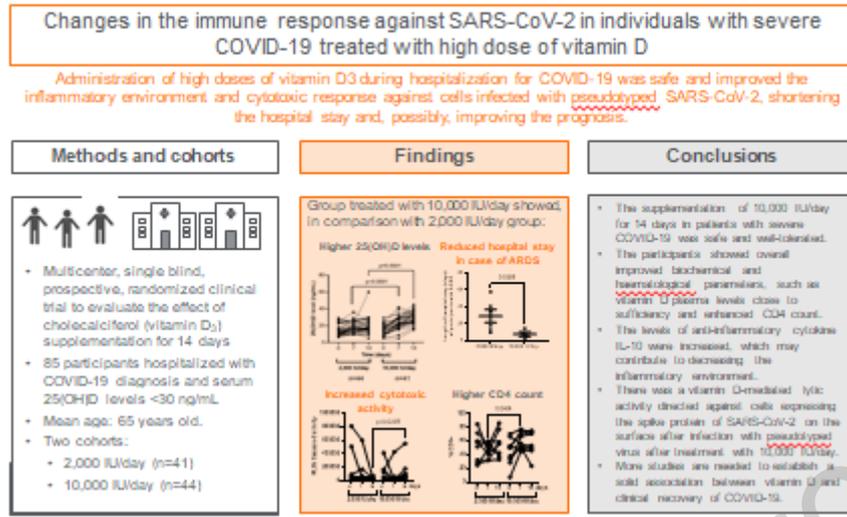
**Table 1.** Main demographics and clinical data of the participants in this study at baseline (day 0), before

Characteristics	All (n=85)	Supplementation group		p-value
		2,000 IU/day (n=44)	10,000 IU/day (n=41)	
Age at diagnosis, median (IQR)— yr.	65.0 (53.0-74.0)	65.3 (44.0-72.3)	67.0 (58.0-75.0)	0.0665
Time from onset of symptoms to hospitalization, median (IQR) — days	7 (6-10)	7 (6-9)	7 (6-10)	0.5440
<b>Gender</b>				
Male— no. (%)	60 (70.6)	30 (68.2)	30 (73.2)	0.7901
Female— no. (%)	25 (29.4)	14 (31.2)	11 (26.8)	
<b>Classification by BMI</b>				
Normal Weight (18-5-24.99) — no. (%)	7 (8.2)	4 (9.1)	3 (7.3)	0.7747
Overweight (25.0-25.9) — no. (%)	32 (37.6)	15 (34.1)	17 (41.5)	
Obesity (>30) — no. (%)	46 (54.1)	25 (56.8)	21 (51.2)	
<b>Smoker</b>				
No — no. (%)	57 (67.1)	30 (68.2)	27 (65.8)	0.9260
Former Smoker— no. (%)	25 (29.4)	13 (29.5)	12 (29.3)	
Yes— no. (%)	3 (3.5)	1 (2.3)	2 (4.9)	
<b>Alcohol consumption</b>				
No— no. (%)	72 (84.7)	38 (86.4)	34 (82.9)	0.8973
Former drinker— no. (%)	4 (4.7)	2 (4.5)	2 (4.9)	
Yes— no. (%)	9 (10.6)	4 (9.1)	5 (12.2)	
<b>Coexisting conditions</b>				
Hypertension— no. (%)	41 (48.2)	18 (40.1)	23 (56.1)	0.2368
Dyslipidemia— no. (%)	31 (36.5)	12 (27.3)	19 (46.3)	0.1097
Diabetes— no. (%)	19 (22.3)	8 (18.2)	11 (26.8)	0.4866
<b>Pneumonia at Rx</b>				
Unilateral — no. (%)	12 (14.1)	7 (15.1)	5 (12.2)	0.8574
Bilateral— no. (%)	73 (85.9)	37 (84.1)	36 (87.8)	
<b>Blood biochemistry data</b>				
Leucocytes, mean (SD)— mil/ $\mu$ L	8.1 (4.3)	7.3 (3.8)	8.9 (4.7)	0.0815
Neutrophils, mean (SD)— mil/ $\mu$ L	6.3 (4.0)	5.7 (3.6)	7.0 (4.4)	0.1614
Lymphocytes, mean (SD)— mil/ $\mu$ L	1.2 (0.9)	1.1 (0.5)	1.4 (1.2)	0.0646
<b>Treatments— no. (%)</b>				
Dexamethasone	56 (65.9)	29 (65.9)	27 (65.8)	1
Methylprednisolone	21 (24.7)	9 (20.45)	12 (29.3)	0.4903
Tocilizumab	21 (24.7)	9 (20.45)	12 (29.3)	0.4903
LMWH	73 (85.9)	36 (81.8)	37 (90.2)	0.4219
Ceftriaxone	50 (58.9)	24 (54.5)	26 (59.1)	0.5421
Azithromycin	36 (42.35)	20 (45.5)	16 (39)	0.7040

BMI, body mass index; IQR, interquartile range; LMWH, Low-molecular-weight heparin; no., number; SD, standard deviation; yr., year.

the administration of 2,000 IU/day or 10,000 IU/day of cholecalciferol.

## Graphical abstract



### **Author Contributions**

MCer and MCo conceptualized the project.

MT, GC and MCo wrote the manuscript. MT, GC and LV performed the study of cytotoxicity.

MT, GC and SRM performed the analysis of cell populations by flow cytometry with technical assistance from EM.

MRLH and GC performed the analysis of cytokines in plasma.

FR and EM processed and stored all blood samples.

DLW, MNM, PRM, MLTM and MCer identified, selected, and recruited the patients, and also collected the blood samples.

MT, MCer and MRLH collected and analyzed the clinical data.

All co-authors read and approved the final version of the manuscript.

**Declaration of Interest**

The authors declare no conflict of interest.

Journal Pre-proof

### Highlights

- Treatment with 10,000 IU/day of cholecalciferol was safe during severe COVID-19.
- Anti-inflammatory cytokine IL-10 was significantly increased in 10,000 IU/day group.
- Individuals who received 10,000 IU/day of cholecalciferol showed increased CD4 count.
- Individuals with ARDS in 10,000 IU/day group stayed at the hospital less time.
- The 10,000 IU/day group showed increased antiviral cytotoxic activity.

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