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Association of Vitamin D with Severity and Outcome of COVID-19: Clinical and Experimental Evidence

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Keywords
Vitamin D · Tumor necrosis factor · Interleukin-6 · Interferon gamma · Acute respiratory distress syndrome · COVID-19

Abstract
Introduction: The role of vitamin in COVID-19 remains controversial. We investigated the association between endogenous vitamin D and the severity of COVID-19 as well as the mechanisms of action of vitamin D supplementation. ***Methods:*** 25(OH)D3 in serum was associated with disease severity and outcome in 190 COVID-19 patients. In a COVID-19 animal model using intravenous injection of plasma from patients with COVID-19 acute respiratory distress syndrome into C57/BL6 mice, mice were treated with 0.25 μg human 1,25(OH)D3 or vehicle. Mice were sacrificed on day 4. Cytokines and myeloperoxidase (MPO) in tissues were measured. Changes in gene expression after vitamin D supplementation were measured. ***Results:*** Vitamin D deficiency and insufficiency were associated with increased severity and unfavorable outcome after 28 days. Vitamin D levels were negatively associated with biomarkers of COVID-19 severity. Vitamin D supplementation after challenge of mice with COVID-19 plasma led to reduced levels of TNFα, IL-6, IFNγ, and MPO in the lung, as well as down-regulation of pro-inflammatory pathways. ***Conclusion:*** Normal levels of endogenous vitamin D are associated with reduced severity and risk of unfavorable outcome in COVID-19, possibly through attenuation of tissue-specific hyperinflammation.

Introduction

The novel viral infection caused by SARS-CoV-2, named COVID-19, was declared a pandemic by the WHO on March 11, 2020 (https://www.who.int/emergencies/diseases/novel-coronavirus-2019). Based on several clinical indicators, such as oxygen saturation, respiratory rate, and signs of severe respiratory distress, WHO classifies COVID-19 into non-severe, severe, and critical cases [1]. There is an ongoing effort to elucidate the mechanisms that drive the progression in COVID-19 cases

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from lower respiratory tract infections to acute respiratory distress syndrome (ARDS). It has been demonstrated that SARS-CoV-2 stimulates pro-inflammatory responses, including NLRP3 inflammasome activation in monocytes and macrophages, a macrophage-activation like syndrome, and formation of cytokine-overproducing monocytes [2, 3]. Therefore, research has focused on possible treatments to reduce this hyper-inflammatory state.

Vitamin D, in its calcitriol form (1,25(OH)2D3), interacts with nuclear vitamin D receptors in several immune cells, modulating both the innate and the adaptive arms of the immune system [4]. Vitamin D has been demonstrated to attenuate the Th1 and Th7 responses and enhance the Th2 response, suggesting an anti-inflammatory effect [5]. Moreover, insufficient vitamin D levels have been correlated with a higher risk of respiratory tract infections [6, 7].

However, the role of vitamin D in COVID-19 remains controversial. Whereas some studies link low vitamin D levels with increased risk of infection, disease severity, and hospitalization, studies in larger populations have failed to relate vitamin D to COVID-19 outcome [8, 9].

In this study, we investigated the role of vitamin D in progression of COVID-19 as well as the underlying mechanism of action. We used a two-step approach. In the first step, we measured and found lower concentrations of vitamin D in the plasma of patients with critical COVID-19 compared to patients with non-severe COVID-19. Vitamin D deficiency was associated with severity, risk of development of ARDS, and death in COVID-19. In the second step, we studied in a COVID-19-like animal model the effect of vitamin D supplementation on COVID-19-related hyperinflammatory response.

Patients and Methods

Clinical Study

Plasma samples were analyzed in 190 patients participating in the SAVE trial (suPAR-guided Anakinra treatment for validation of the risk and early management of severe respiratory failure by COVID-19; EudraCT number 2020-001466-11; National Ethics Committee of Greece approval 38/20; National Organization for Medicines of Greece approval ISO 28/20; ClinicalTrials.gov registration NCT04357366) and the ESCAPE trial (EudraCT number 2020-001039-29; Clinicaltrials.gov NCT04339712; approval 30/20 by the National Ethics Committee of Greece; approval IS 021-20 by the National Organization for Medicines of Greece). Adult patients of both genders were enrolled after written informed consent provided by themselves or legal representative. Blood was sampled within the first 24 h of hospital admission. In some patients, blood sampling was repeated after 4 days for evaluation of changes of vitamin D levels.

All patients had positive molecular testing of SARS-CoV-2 from respiratory secretions with lower respiratory tract consolidation defined by the presence of diffuse infiltrates by chest X-ray or computed tomography of the chest. Exclusion criteria were (a) HIV-1 infection and (b) neutropenia defined as less than 1,000 neutrophils/mm^3.

The following clinical variables were recorded: (i) demographics; (ii) severity scores, namely Acute Physiology and Chronic Health Evaluation (APACHE) II score, Charlson's Comorbidity Index (CCI); Sequential Organ Failure Assessment (SOFA) score; and Pneumonia Severity Index (iii) absolute blood cell counts, biochemistry, and blood gases; (iv) and progression into ARDS during the entire hospital stay. ARDS was defined as a ratio of partial oxygen pressure to fraction of inspired oxygen (PaO_2/FiO_2) below 150 mm Hg necessitating mechanical ventilation. All patients were categorized according to the WHO severity classification scale for COVID-19 into non-severe, severe, and critical [1].

Five milliliters of whole blood were collected into a sterile tube containing ethylenediaminetetraacetic acid (EDTA) and centrifuged. 25(OH)D was measured by HPLC. Vitamin D deficiency was defined, according to international cut-off limits, as 25(OH)D levels below 20 ng/mL, and vitamin D insufficiency as 25(OH)D levels of 21–29 ng/mL [10]. Procalcitonin (PCT) was measured by a time-resolved amplified cryptate emission technology assay according to the manufacturer's instructions (Kryptor, Brahms, Hennigsdorf, Germany). The lower detection limit was 0.06 ng/mL. C-reactive protein (CRP) was measured in duplicate by a nephelometric assay (Behring, Berlin, Germany). The lowest limit of detection was 0.2 mg/dL.

Animal Model of COVID-19 Pathogenic Inflammation

Animal experiments were conducted in the Unit of Animals for Medical and Scientific Purposes of the University General Hospital "Attikon" (Athens, Greece). All experiments were licensed from the Greek Veterinary Directorate under the protocol numbers 471955/06.07.2020 and 846137/07.07.2023. We studied a total of 74 male and female C57Bl6 mice (7–8 weeks old). Mice were allowed to acclimate for 7 days before the experiments. They were housed in typical mouse cages, up to 5 mice per cage on 12-h dark/light cycle, and allowed free access to standard dry rodent diet and water. Analgesia was achieved with paracetamol suppositories to avoid interactions with the immune system.

One published model of COVID-19-like inflammatory disease was studied [11] which is utilizing the intravenous (i.v.) injection of 100 μL of plasma from patients with ARDS due to SARS-CoV-2 infection for 3 consecutive days via mouse tail vein; control mice were treated with 100 μL of plasma from healthy volunteers for 3 consecutive days. On each of these days, some mice of both groups were treated intraorally (i.o.) with 0.25 μg human 1,25(OH)D3 dissolved in 0.9% N/S, as suggested elsewhere [12]. D3 was provided as a ready-made water-soluble solution in drops, where each drop provides 200 IU (5 μg 100% nutritional reference value) of vitamin D3 (D3fix, Ioulia, and Irini Tseti Pharmaceutical Laboratories SA, d.t. "Intermed SA"). On the fourth day, mice were sacrificed by subcutaneous (sc) injection of 300 mg/kg ketamine, followed by cervical dislocation. Under sterile conditions, a midline abdominal incision

was performed. Then, segments of the right lung were excised and collected into sterile tubes with 1 mL NaCl 0.9%. The samples were weighed and homogenized.

In separate experiments, mice treated with plasma of COVID-19 plasma for 3 days were challenged on the fourth day ip with 2×10^7 cfu/mL *Acinetobacter baumannii*. In these experiments, mice were randomized to treatment with and without 1,25(OH)D3 before bacterial challenge in parallel to COVID-19 plasma, at the dose regimen described above. Survival was recorded every 12 h for 7 days.

Concentrations of tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), and IL-6 were measured in duplicate in supernatants from tissue samples by an enzyme immunosorbent assay (ThermoFisher Scientific, Massachusets, USA) according to the manufacturer's instructions. The lowest detections limits were as follows: for TNFα 19 pg/mL; for IFNγ 16 pg/mL and for IL-6 10 pg/mL.

Myeloperoxidase (MPO) activity in all collected tissues was determined. Tissue segments were homogenized with T-PER® (ThermoFisher Scientific) and centrifuged at 10,000 rpm at 4°C. Then the homogenates were incubated in wells of a 96-well plate at 37°C with 4.2 mM tetramethylbenzidine (Serva, Heidelberg, Germany), 2.5 mM citrate, 5 mM NaH_2PO_4, and 1.18 mM H_2O_2, pH 5.0, at a final volume of 150 μL. After 5 min, the reaction was terminated by adding 50 mL 0.18 M H_2SO_4. Absorbance was read at 450 nm against blank wells. Results were adjusted for tissue sample protein content on Bradford assay (Sigma-Aldrich), and they were expressed as MPO units/mg protein/g. Creatinine was measured via the Jaffe method, with the lowest detection limit 0.1 mg/dL.

RNA was isolated from mouse lung segments stored in RNAlater (Qiagen, Hilden, Germany), following the RNeasy mini kit protocol (QIAGEN) according to the manufacturer's recommendations. RNA concentration was measured spectrophotometrically using the absorbance ratio of 260/280 nm. Gel electrophoresis was used to check the purity and integrity of the total RNA. RNA-seq libraries were prepared with the NebNext Ultra II directional RNA library prep kit for Illumina. Library size distribution and quality control were performed using the Agilent Bioanalyzer DNA1000 chip, and quantitation was done with the qubit HS spectrophotometric method. Between 13 and 63 million 100-bp single-end reads were generated per sample, using the Illumina NovaSeq 6000 sequencer. RNA-seq experiments were carried out at the Greek Genome Center (GGC) of the Biomedical Research Foundation of the Academy of Athens (BRFAA).

Bioinformatics analyses were performed using the Galaxy Suite [13]. The quality of sequencing reads was assessed using the FastQC algorithm (Galaxy Version 0.72+galaxy1). To evaluate RNA integrity at the transcript level, transcript integrity number (Galaxy Version 2.6.4.1) from RseQC package was calculated [14]. Calculation of the distribution of sequencing reads over genomic features was performed using the Read Distribution option (Galaxy Version 2.6.4.1) from RseQC package [14]. Fastq files were aligned to the mouse genome version mm9 with the use of HISAT2 (Galaxy Version 2.1.0+galaxy7) [15] using the Reverse Strand parameter. Sequencing reads falling into genes were counted with HT-seq count algorithm (Galaxy Version 0.9.1) [16] using the Union mode and Reverse Stranded options. Finally, differentially expressed genes were identified with the DESeq2 package (Galaxy Version 2.11.40.6+galaxy1) [17] using a fold change cutoff of 1.5 and p adj. value <0.05. Coverage files in bigwig format were constructed using the bamCoverage option (Galaxy Version 3.3.2.0.0) from deepTools using RPKM normalization [18]. Gene ontology and pathway analysis were performed for differentially expressed genes using the EnrichR software [19]. Heatmaps were constructed using the Morpheus software (https://software.broadinstitute.org/morpheus/). Volcano plot was constructed using the Volcano plot option from Galaxy (Galaxy Version 0.0.3). The IGV genome browser was used to visualize RNA-seq signals around certain genes [20]. RNA-seq raw and processed files have been deposited at GEO under accession number GSE222471. The data can be accessed using the edyjoiaqzhcjjar token.

Statistics

Categorical data were presented as frequencies and quantitative variables as mean ± SE. Comparisons between groups were done using the Fisher's exact test for categorical data. Comparison for quantitative data was performed using the Mann-Whitney U test for two group comparisons and the one-way ANOVA with the Bonferroni correction for multiple group comparisons. Correlations were performed using Spearman's rank of order. Any p value below 0.05 was considered statistically significant. 28-day survival was compared between groups by the log-rank test. Other demographic variables associated with unfavorable outcome were transformed into dichotomous variables after ROC curve analysis. For each parameter, the coordinate point with the maximum value of the Youden index was used as a cut-off. Univariate and multivariate Cox regression analysis with odds ratios (ORs) and confidence intervals (CIs) was used to investigate if vitamin D deficiency is an independent variable for unfavorable outcome of COVID-19. Any p value below 0.05 was considered statistically significant.

Results

190 patients with COVID-19 were analyzed. Their demographics according to 28-day survival are shown in Table 1. 58, 79, and 53 patients were categorized according to the WHO classification of severity [1] into moderate, severe, and critical COVID-19, respectively. Serum 25(OH)D3 levels were significantly lower among critical cases compared to moderate and severe cases (Fig. 1a). Vitamin D deficiency and vitamin D insufficiency were more common among patients in critical condition compared to patients in moderate or severe condition (Fig. 1b, c). In 29 patients, blood sampling was repeated on day 4 after admission. Increase of day 1 values was found in survivors compared to non-survivors (online suppl. Fig. S1; for all online suppl. material, see https://doi.org/10.1159/000535302). None of the 190 patients was treated with any supplement of vitamin D.

After 28 days, 17 (28.3%) patients with vitamin D deficiency and 17 (13.1%) patients with 25(OH)D3 levels above 20 ng/mL survived (Fig. 1d). In respect to vitamin

Table 1. Baseline clinical and laboratory characteristics of patients according to 28-day survival from pneumonia by the SARS-CoV-2 coronavirus

	Survivors (n = 156)	Non-survivors (n = 34)	p value
Age, years, mean±SD	56.61±14.03	70.53±10.14	<0.0001[b]
Male sex, *n*, (%)	98 (62.8)	23 (67.6)	0.696[a]
Female sex, *n*, (%)	58 (37.2)	11 (32.4)	0.696[a]
CCI, mean±SD	2.06±2.01	3.59±2.09	<0.0001[b]
APACHE II score, mean±SD	6.42±7.36	13.16±12.50	<0.0001[b]
SOFA, mean±SD	1.91±1.67	4.06±2.11	<0.0001[b]
PSI, mean±SD	62.98±26.92	96.03±32.27	<0.0001[b]
Main comorbidities, *n* (%)			
Type 2 diabetes mellitus	32 (20.5)	11 (32.4)	0.173[a]
Congestive heart failure	3 (1.9)	3 (8.8)	0.072[a]
Coronary heart disease	13 (8.3)	8 (23.5)	0.029[a]
Chronic renal disease	3 (1.9)	3 (8.8)	0.072[a]
Stroke	3 (1.9)	3 (8.8)	0.072[a]
COPD	4 (2.6)	5 (14.7)	0.010[a]
Solid tumor	9 (5.8)	2 (5.9)	0.619[a]
Laboratory values, mean±SD			
25(OH)D_3, ng/mL	28.26±14.00	23.92±13.50	0.022[b]
White blood cells, mm^3	6,628.93±3,003.82	8,993.55±7,604.56	0.131[b]
Absolute neutrophil counts, mm^3	4,902.39±2,939.24	7,565.85±7,379.16	0.025[b]
Absolute lymphocyte counts, mm^3	1,191.79±935.85	862.93±378.73	0.003[b]
Platelets, $\times 10^3$ mm^3	228.8±96.7	205.9±91.0	0.120[b]
CRP, mg/L	58.83±93.67	118.58±81.81	<0.0001[b]
Fibrinogen, mg/dL	502.42±143.21	465.84±201.45	0.381[b]
Ferritin, ng/mL	684.44±898.64	1,307.94±1,991.36	0.213[b]
PCT, ng/mL	1.26±5.29	1.43±2.03	0.016[b]
D-dimers, mg/dL	0.88±0.88	1.36±1.05	0.021[b]
AST, U/L	44.44±27.87	140.74±493.15	0.106[b]
ALT, U/L	42.22±30.86	62.32±142.59	0.590[b]
Creatinine, mg/dL	0.95±0.29	1.25±0.69	0.019[b]
Total bilirubin, mg/dL	0.76±0.83	0.66±0.36	0.845[b]
Lactate, mmol/L	1.47±1.67	2.04±1.62	0.001[b]
pO2/FiO2	313.38±106.28	193.93±110.04	<0.0001[b]

APACHE, acute physiology and chronic health evaluation; ALT, alanine aminotransferase; ARDS, acute respiratory distress syndrome; AST, aspratate aminotransferase; CCI, Charlson's comorbidity index; COPD, chronic obstructive pulmonary disease; SD, standard deviation; SOFA, sequential organ fialure assessment; PSI, pneumonia severity index. [a]Comparison by Fischer exact test. [b]Comparison by the Mann-Whitney U test.

D insufficiency, 29 (23.4%) patients with vitamin D insufficiency and 5 (7.6%) patients with 25(OH)D3 levels above 30 ng/mL survived after 28 days (Fig. 1e).

Following ROC curve analysis and univariate analysis of the documented parameters, it was found that the following baseline values were associated with unfavorable outcome: age >61 years, APACHE II score >7, CCI >2, SOFA score on day 1 > 3, and PSI score >81. Additionally, history of coronary heart disease and COPD were associated with death due to COVID-19 (Table 1). All above variables entered Cox regression analysis. Analysis showed that vitamin D deficiency was an independent factor associated with unfavorable outcome (Table 2).

Serum 25(OH)D3 levels on day 1 were positively associated with the absolute lymphocyte count but not with the absolute neutrophil count of the studied patients (Fig. 2a, b). The neutrophil-to-lymphocyte ratio was inversely associated to vitamin D levels (Fig. 2c). A negative correlation was also found between serum 25(OH)D3 levels and d-dimers, ferritin, CRP, and PCT on day 1 (Fig. 2d–g). Lastly, 25(OH)D3 in serum was positively associated with the PaO_2/FiO_2 ratio of the patients on day 1 (Fig. 2h).

In order to investigate the effect of vitamin D on the progression of COVID-19 to ARDS, we studied a COVID-19- like murine model [11]. Mice were challenged

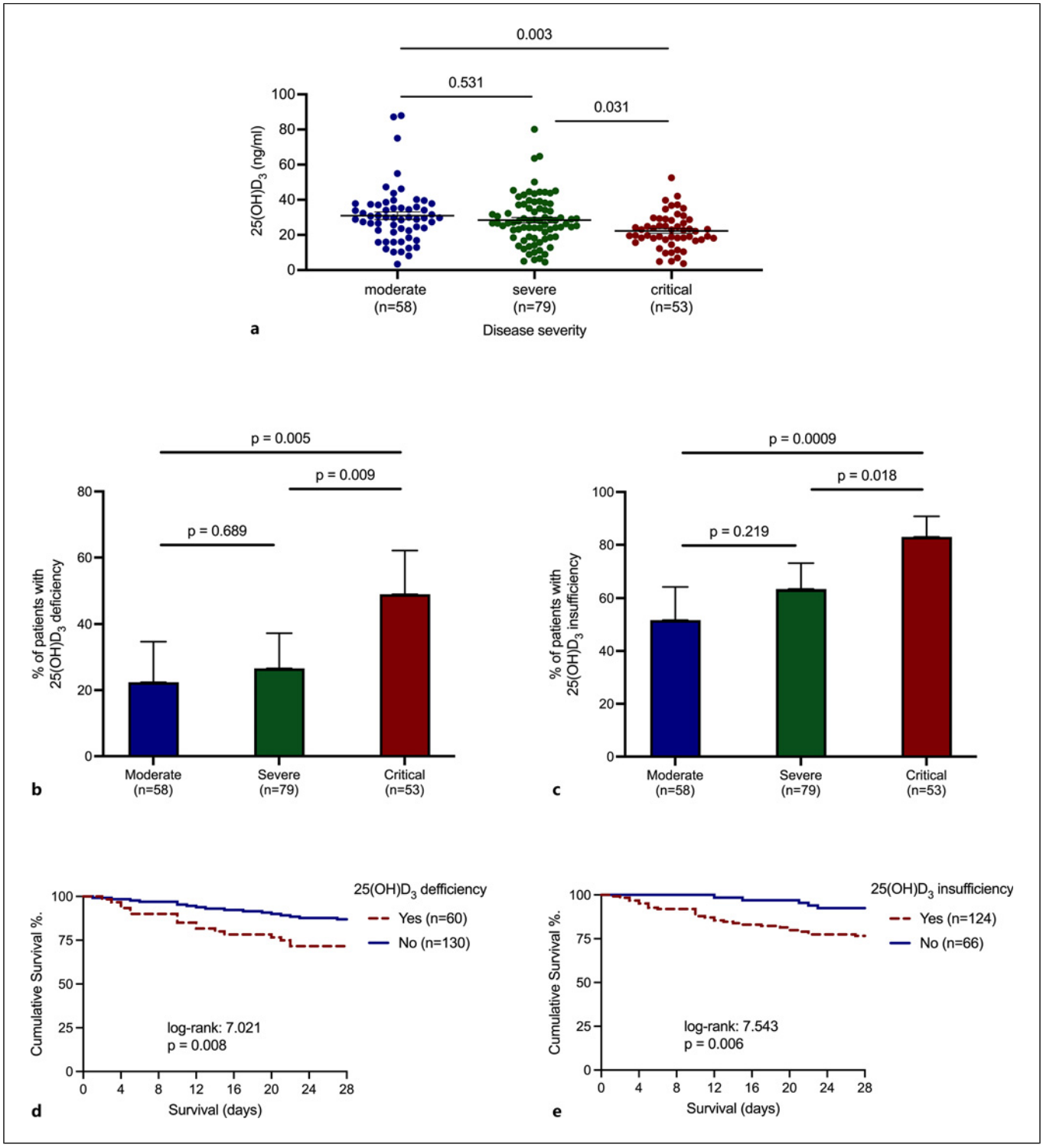

Fig. 1. Association of vitamin D levels and COVID-19 severity and outcome (**a**) Concentrations of 25(OH)D3 were measured in the plasma of 190 COVID-19 patients. Concentrations are presented according to the WHO severity scale for COVID-19. Comparison by the Mann-Whitney U test; the relevant *p* values are given. **b**, **c** Prevalence of 25(OH)D3 deficiency (**b**) and insufficiency (**c**) among COVID-19 patients according to the WHO severity scale for COVID-19. The rp values are given. **d**, **e** Kaplan-Meier analysis for survival of COVID-19, among patients with and without $25(OH)D_3$ deficiency (**d**) or insufficiency (**e**). Results of the log-rank test and the *p* value are given.

Table 2. Univariate and step-wise Cox regression analysis of parameters associated with unfavorable outcome of pneumonia by the SARS-CoV-2 coronavirus

	Survivors (n = 156)	Non-survivors (n = 34)	Univariate analysis		Step-wise cox regression analysis	
			HR (95% CIs)	p value	OR (95% CIs)	p value
Age >61 years, n (%)[a]	54 (34.6)	23 (67.6)	3.95 (1.79–8.71)	0.0005		
APACHE II 1 > 7, n (%)[a]	48 (31.6)	26 (83.9)	11.27 (4.08–31.13)	<0.0001		
CCI >2, n (%)[a]	55 (36.2)	23 (67.6)	3.69 (1.67–8.13)	0.001		
SOFA >3, n (%)[a]	18 (11.8)	17 (53.1)	8.44 (3.60–19.76)	<0.0001		
PSI >81, n (%)[a]	26 (18.1)	25 (78.1)	16.21 (6.33–41.48)	<0.0001	10.03 (4.28–23.50)	0.0001
25(OH)D_3 deficiency, n (%)[#]	43 (27.6)	17 (50.0)	2.62 (1.23–5.62)	0.014	2.10 (1.03–4.31)	0.042
Coronary heart disease, n (%)	13 (8.3)	8 (23.5)	3.39 (1.28–8.97)	0.029	2.61 (1.11–6.14)	0.028
COPD, n (%)	4 (2.6)	5 (14.7)	6.55 (1.66–25.87)	0.010		

APACHE, acute physiology and chronic health evaluation; CCI, Charlson's Comorbidity Index; CI, confidence intervals; COPD, chronic obstructive pulmonary disorder; HR, hazard ratio; SOFA, sequential organ failure assessment; PSI, pneumonia severity index; [#]25$(OH)_2D_3$ deficiency defined as 25$(OH)_2D_3$ below 20 ng/mL. [a]Cut-off point of each variable was determined based on the coordinate point with the maximum value of the Youden index.

with plasma either from healthy volunteers (HV) or from patients with ARDS of COVID-19. Following challenge with plasma by COVID-19 patients, TNFα, IL-6, IFNγ, and MPO in the lung (Fig. 3a–d) increased. This phenomenon was attenuated with 1,25(OH)D3 supplementation. These findings demonstrated an anti-inflammatory action of vitamin D in the lung of mice after challenging with COVID-19 patient plasma. Serum creatinine was decreased with 1,25(OH)D3 supplementation (online suppl. Fig. S2).

Sequenced samples were subjected to rigorous quality control metrics (supplementary table). Differentially gene expression analysis using DeSeq2 (log2foldchange >1.5, p adj <0.05) identified 200 upregulated and 221 downregulated genes following vitamin D supplementation. Differentially expressed genes and highlighted examples are presented using a Volcano plot (Fig. 4a). In order to identify the biological processes in which differentially expressed genes partake, we performed Gene Ontology analysis (Fig. 4d) which identified processes related to inflammation for downregulated genes such as regulation of natural killer cell migration and cytokine-mediated signaling pathway. No statistically robust biological processes were identified for upregulated genes. Characteristic examples of downregulated genes include members of the CCL chemokine family (*Ccl4*, *Ccl5*, *Ccl6*, *Ccl17*, and *Ccl22*), other chemokines such as *Cxcl9* and Interferon Stimulated genes (ISGs) such as *Ifit1*, *Ifit2*, *Mx1*, *Mx2*, *Oas1b*, *Oas3*, *Usp18*, and *Irf9*. Characteristic IGV browser snapshots depicting RNA-seq signal around the *Ccl5* and *Cxcl9* loci and their downregulated expression levels following vitamin D supplementation are presented in Figure 4b. All downregulated genes are presented in a heatmap form in Figure 4c, while all modulated genes are presented in online supplementary Tables 1–4.

The studied COVID-19-like animal model is not lethal but favors lethality of secondary infection by *A. baumannii* [11]. Oral supplementation with 1,25(OH)D3 could not prevent mortality from this secondary infection (online suppl. Fig. S3).

Discussion

This study provides evidence which supports some anti-inflammatory effect of vitamin D in COVID-19. Normal levels of vitamin D provide independent protection against severe respiratory failure and lethal outcome by SARS-CoV-2 pneumonia, whereas levels were increased over time among survivors. The anti-inflammatory properties of vitamin D are partly shown in a COVID-19-like animal model. Although oral supplementation with vitamin D protected mice from renal dysfunction, it did not protect them from death after secondary infection by *A. baumannii*.

Multiple pathways have been investigated in the pathogenesis of COVID-19. Immune dysregulation is a core characteristic of progression into ARDS [3]. One strategy of management is the early recognition of patients at risk for ARDS and the start of biologicals to attenuate the exaggerated immune response. One such

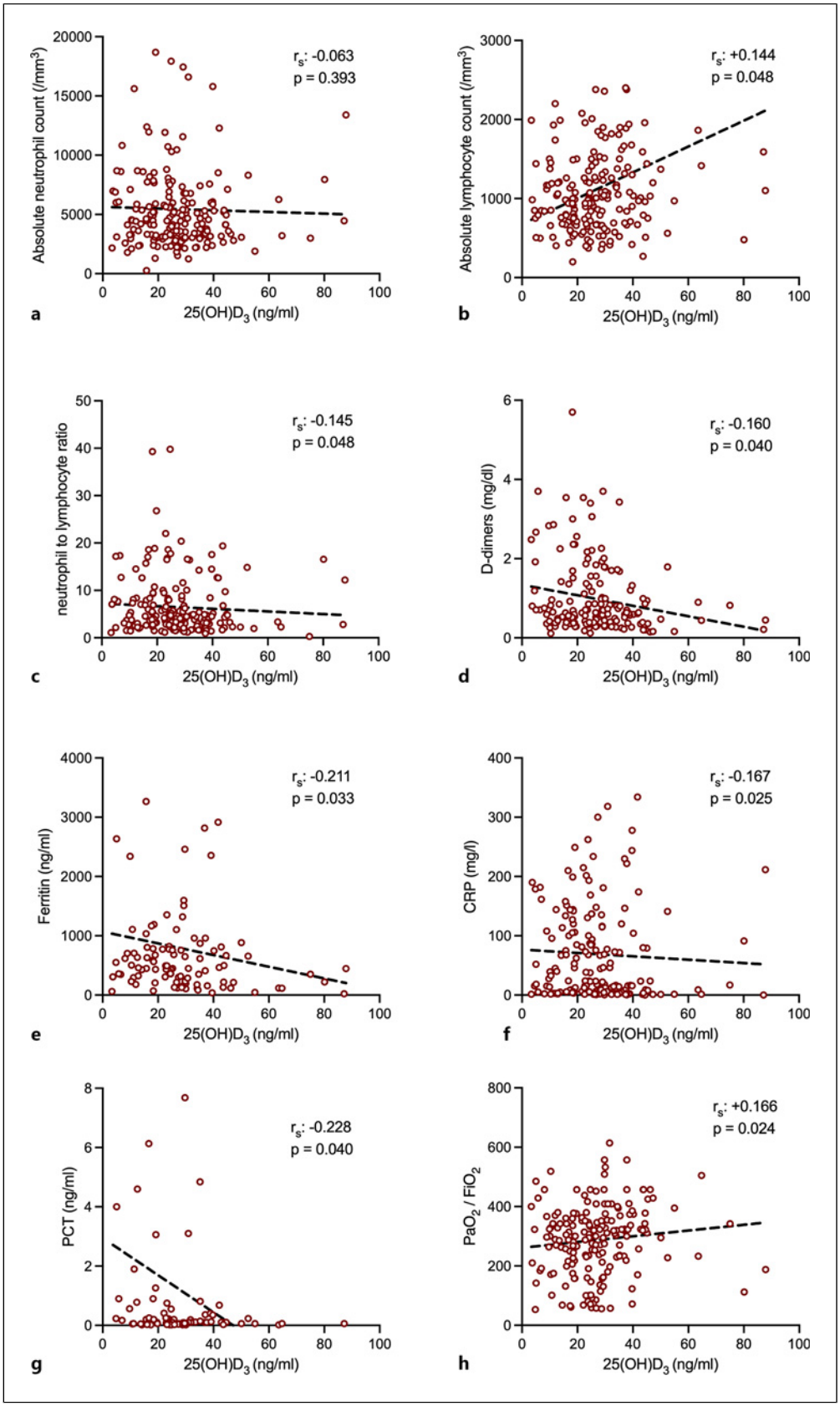

Fig. 2. Association of vitamin D to other markers predictors of outcome of pneumonia by the SARS- CoV-2 coronavirus. Correlation of absolute neutrophil counts (**a**); absolute lymphocyte counts (**b**); neutrophil to lymphocyte ratio (**c**); d-dimers (**d**); ferritin (**e**); C-reactive protein (CRP) (**f**); procalcitonin (PCT) (**g**), and PaO_2/FiO_2 ratio (**h**) in plasma of patients with COVID-19 with concentrations of 25(OH) D_3 on day of hospital admission. Spearman rank correlation coefficients (r_s), interpolation lines and *p* values are provided.

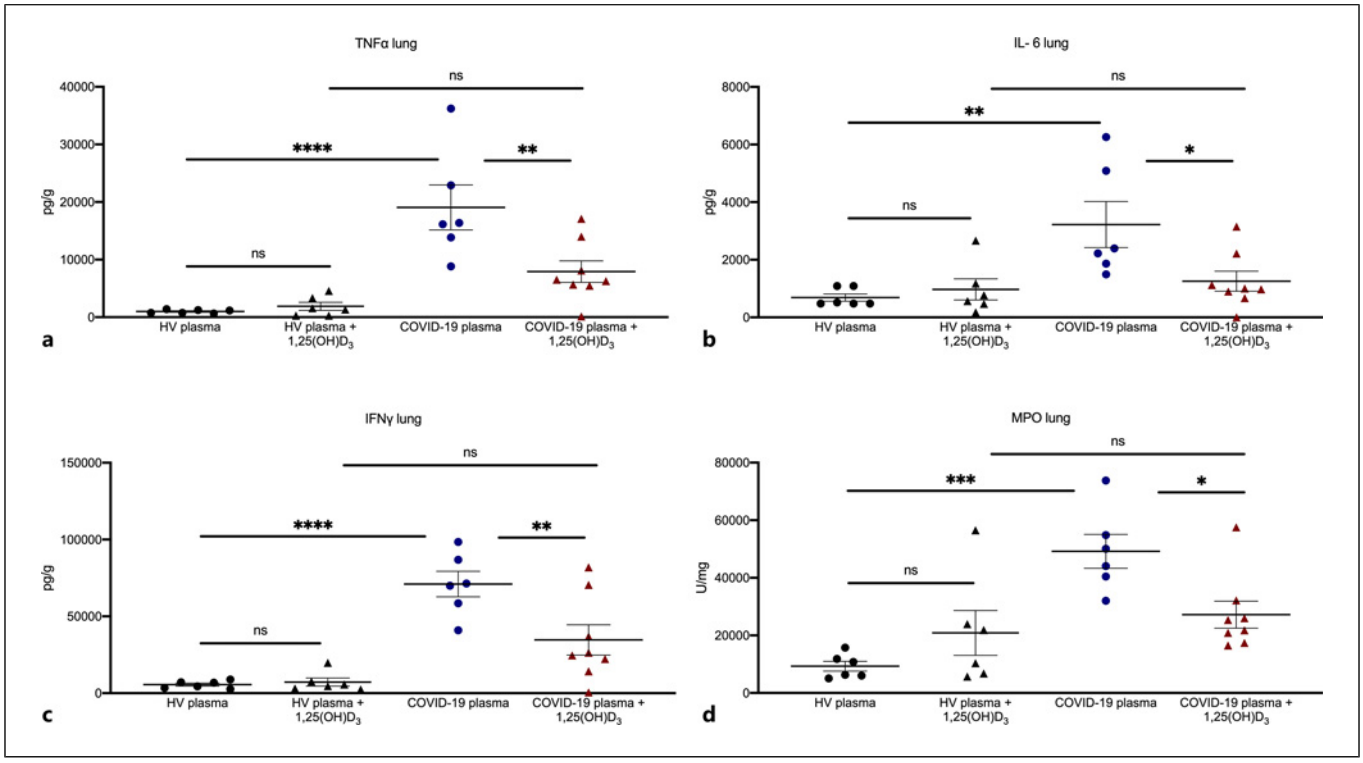

Fig. 3. Vitamin D supplementation attenuates the compartmentalized hyperinflammation in a COVID-like murine model. In this COVID-like infection model, C57Bl6 mice were challenged intravenously (i.v.) with plasma of healthy volunteers (HV) or patients with ARDS due to COVID-19 for three consecutive days. In separate experiments, on each day of plasma challenge, mice were treated with $25(OH)D_3$. Tumor necrosis factor alpha (TNFα) (**a**), interleukin (IL)-6 (**b**), interferon gamma (IFNγ) (**c**), and myeloperoxidase (MPO) (**d**) activity was determined in the lung. Comparison by the Mann-Whitney U test; ns non-significant; $*p < 0.05$; $**p < 0.01$.

strategy is already registered by the European Medicines Agency and the Food and Drug Administration (FDA) of the USA. The results of the SAVE-MORE pivotal double-blind randomized trial showed that early start of anakinra decreases by 0.36 the odds for unfavorable outcome compared to placebo treatment [21]. The early recognition of risk is done by measurement of the biomarker suPAR (soluble urokinase plasminogen activator receptor) upon hospital admission; levels 6 ng/mL or more suggest the need for anakinra treatment to prevent progression into ARDS [22]. The biomarker suPAR is not yet available for commercial use in the USA. The FDA has recently provided emergency use authorization of anakinra using a separate clinical score to early recognize the risk of progression into ARDS. The score contains eight variables, and patients who meet at least three of the variables should receive treatment [23].

Studies with miRNA analysis have implicated changes in vitamin D synthesis and function as part of the dysregulation of COVID-19 and suggest early oral supplementation to prevent these changes. It is suggested that vitamin D helps maintaining the physical barrier and regulates both innate and adaptive immune dysregulation [24]. In our animal model, vitamin D was administered early, i.e., in parallel to animal challenge. The prevention of lung inflammation, as shown by the reduction of TNFa, IL6, IFNγ, and MPO in murine lung specimens, was associated with the down-regulation of several pro-inflammatory processes including natural killer cell chemotaxis, lymphocytes migration, and cytokine-mediated signalling pathways.

Our clinical data reveal positive association between vitamin D levels and lymphocyte counts as well as negative correlation between vitamin D levels and inflammatory markers like D-dimers, ferritin, CRP, and PCT. In one randomized prospective trial, patients with COVID-19 and hypovitaminosis D were treated with vitamin D. Restoration of vitamin D levels between 80 and 100 ng/mL was accompanied by significant decrease of ferritin, IL-6, and CRP and increases in the neutrophil-to-lymphocyte ratio [25]. In one study of 474 patients risk

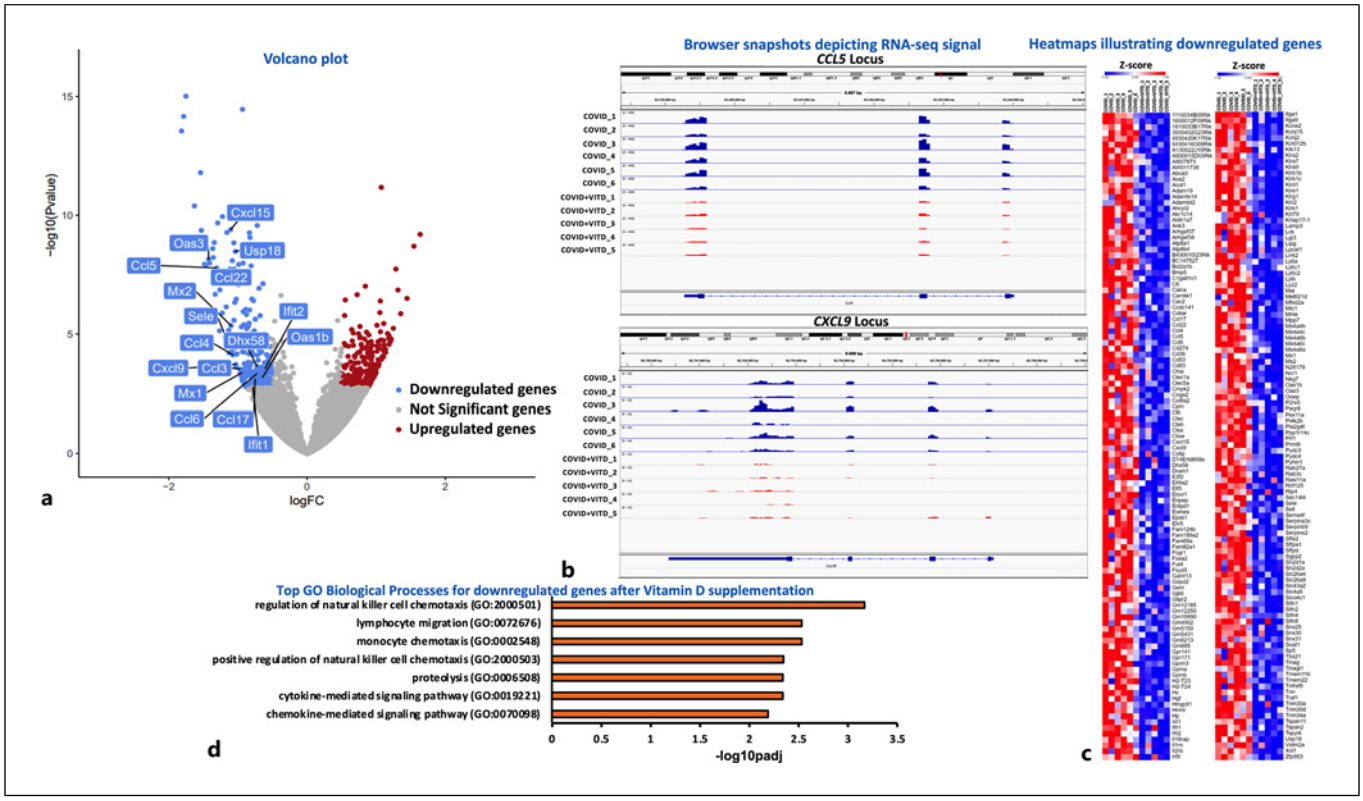

Fig. 4. Vitamin D supplementation downregulates the expression of core proinflammatory genes in a COVID-like murine model. In a COVID-like infection model, C57Bl6 mice were challenged intravenously (i.v.) with plasma of healthy volunteers (HV) or patients with ARDS due to COVID-19 for three consecutive days. In separate experiments, on each day of plasma challenge, mice were treated with 25(OH)D_3. **a** Volcano plot highlighting differentially expressed genes following vitamin D supplementation. **b** IGV browser snapshots depicting RNA-seq signal around the *Ccl5* (up) and *Cxcl9* locus (down). Both genes are downregulated following vitamin D supplementation. **c** Heatmaps illustrating all downregulated genes following vitamin D supplementation. **d** Top statistically significant GO Biological Processes for downregulated gene following vitamin D supplementation.

of death by COVID-19 was greater among patients with vitamin D insufficiency [26]. The results of the recent REsCue trial revealed that treatment with capsules of vitamin D for 2 weeks affected earlier resolution of symptoms only among the subgroup of patients who restored vitamin D circulating levels to normal [27]. In a recent clinical trial, where 930 COVID-19 patients were randomized to receive calcifediol or placebo, mortality was more than three times greater and requirement of ICU treatment was almost four times greater in the control group [28].

However, it must be pointed out that various meta-analyses have failed to provide conclusive evidence if vitamin D supplementation provides protection against infection or improves outcomes in COVID-19 [29–31]. This led to an inconclusive recommendation in the 2022 NIH guidelines (https://www.covid19treatmentguidelines.nih.gov/) [32]. However, a most recent meta-analysis and trial sequential analysis of five RCTs reported that vitamin D administration resulted in decreased risk for death and ICU admission (standardized mean difference (95% CI): 0.49 (0.34–0.72) and 0.28 (0.20–0.39), respectively) [33].

The existing data indicate possible clinical associations between defective vitamin D levels and severity of COVID-19. Therefore, we strongly believe that additional experimental and clinical data are needed for a better understanding of the role of vitamin D in COVID-19, as well as to highlight the possible mechanisms of action.

Statement of Ethics

The SAVE trial (EudraCT number 2020-001466-11; ClinicalTrials.gov registration NCT04357366) was approved by the National Ethics Committee of Greece (approval 38/20) and by the National Organization for Medicines of Greece

(approval ISO 28/20). The ESCAPE trial (EudraCT number 2020-001039-29; Clinicaltrials.gov NCT04339712) was approved by the National Ethics Committee of Greece (approval 30/20) and by the National Organization for Medicines of Greece (approval IS 021-20). Non mechanically ventilated patients were enrolled after written informed consent provided by themselves. Patients under mechanical ventilation were enrolled after written informed consent provided by their legal representative. Animal experiments were conducted in the Unit of Animals for Medical and Scientific Purposes of the University General Hospital "Attikon" (Athens, Greece). All experiments were licensed from the Greek Veterinary Directorate under the protocol numbers 471955/06.07.2020 and 846137/07.07.2023.

Conflict of Interest Statement

E.J.G.-B. has received honoraria from Abbott Products Operations AG, bioMérieux, Brahms GmbH, GSK, InflaRx GmbH, Sobi, and Xbiotech Inc.; independent educational grants from Abbott Products Operations AG, bioMérieux Inc., InflaRx GmbH, Johnson & Johnson, MSD, Sobi, and UCB; and funding from the Horizon2020 Marie Skłodowska-Curie International Training Network "the European Sepsis Academy" (granted to the National and Kapodistrian University of Athens), the Horizon 2020 European Grants ImmunoSep and RISCinCOVID, and the Horizon Health grant EPIC-CROWN-2 (granted to the Hellenic Institute for the Study of Sepsis). The other authors do not disclose any conflicts of interest.

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Author Contributions

G.R. contributed to animal experiments, wrote the manuscript, reviewed the final version for intellectual content, and gave approval for submission. S.F. and G.V. contributed in RNAseq, reviewed the final version for intellectual content, and gave approval for submission. T.A., V.-M.S., D.-E.D., and D.K. contributed in animal experiments, reviewed the final version for intellectual content and gave approval for submission. T.G. collected clinical data, reviewed the final version for intellectual content and gave approval for submission. G.D. contributed in lab measurements, reviewed the final version for intellectual content, and gave approval for submission. E.J.G.-B. designed the study, collected clinical data, wrote the manuscript, reviewed the final version for intellectual content, and gave approval for submission.

Data Availability Statement

RNA-seq raw and processed files have been deposited at GEO under accession number GSE222471. The data can be accessed using the edyjoiaqzhcjjar token. All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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