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ORIGINAL RESEARCH

OPG and TNFR1 as Potential Biomarkers of Inflammation in Older Adults with Acute COVID-19 and Indicators of Frailty in Post-COVID-19: A Pilot Study

Lucero A Ramon-Luing[1,*], Julio Flores-Gonzalez[1,*], Daniela Josefina Cataneo-Piña[2], Ramcés Falfán-Valencia[1], Gloria Pérez-Rubio[1], Ivette Buendia-Roldan[1], Moisés Selman[1], Leslie Chavez-Galan[1]

[1]Research Unit, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Mexico City, 14080, Mexico; [2]Geriatrics, Palliative Care Clinic, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Mexico City, 14080, Mexico

*These authors contributed equally to this work

Correspondence: Leslie Chavez-Galan, Laboratorio de Inmunología Integrativa, Instituto Nacional de Enfermedades Respiratorias Ismael Cosio Villegas, Calzada de Tlalpan 4502, Col. Sección XVI, Tlalpan, Mexico City, 14080, Mexico, Tel +525554871700 ext. 5270, Email chavezgalan@gmail.com; lchavez_galan@iner.gob.mx

Background: Older individuals are at high risk for severe COVID-19 and often experience geriatric syndromes as post-COVID-19 sequelae associated with inflammation. Osteoprotegerin (OPG) and Tumor Necrosis Factor Receptor 1 (TNFR1) are emerging as promising biomarkers in inflammation-associated diseases. Here, these and other members of the tumor necrosis factor superfamily (TNFSF) were investigated as potential biomarkers to monitor older adults during acute COVID-19 and post-COVID-19 recovery.

Patients and Methods: This study included 75 patients with acute COVID-19, 26 post-COVID-19 (evaluated at 4 and 12 months), 35 healthy donors (HD), and 36 individuals with interstitial lung diseases (ILD), all aged over 60. Plasma levels of 14 soluble TNFSF members were measured using flow cytometry-based multiplex immunoassays and ELISA. Multiple logistic regression and ROC curve analyses were performed to assess the potential of TNFSF members as biomarkers.

Results: Flow cytometry revealed significantly higher levels of OPG, BAFF, and APRIL in acute COVID-19 patients than in HD and ILD ($p<0.001$). Through an ELISA, high levels of OPG ($p<0.0001$), APRIL ($p<0.05$), and BAFF ($p<0.0001$) were confirmed, and TNFR1 ($p<0.0001$) was also revealed. In this pilot study, OPG and TNFR1 had AUCs >0.90 and were predictive of COVID-19, independent of comorbidities, while BAFF levels were modified by diabetes. Persistently elevated OPG and TNFR1 levels were also observed in post-COVID-19 patients at 4 and 12 months. Notably, OPG levels were higher in frail versus non-frail individuals at both time points ($p<0.05$), while TNFR1 levels were higher only at 4 months ($p<0.05$).

Conclusion: This evidence indicates that OPG and TNFR1 are potential biomarkers of inflammation during acute COVID-19 and post-COVID-19 among older, mainly frail adults. These findings support their utility in managing post-COVID-19 geriatric frailty syndrome.

Keywords: SARS-CoV-2, aging, TNFSF, inflammaging, immunosenescence, frailty, biomarker

Introduction

The COVID-19 pandemic significantly impacted various aspects of life, including social structures, healthcare systems, and public health policies. Vaccines lower the risk of severe cases and death. However, post-COVID-related complications, such as damage to the respiratory, cardiovascular, cerebrovascular, and neurological/psychiatric systems, are now recognized as significant health concerns.[1]

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A notable feature of the COVID-19 disease was an abnormal immune response characterized by an uncontrolled systemic inflammatory reaction, called the "cytokine storm", where high levels of cytokines, including interleukin-1 (IL-1), 2, 6, 7, 8, 10, 12, 17, 18, tumor necrosis factor-alpha (TNF-α), and interferon gamma (IFN-γ), were observed.[2,3] Early reports on COVID-19 indicated that older patients (>65 years) were at a higher risk of severe illness and death.[4] The pandemic caused many deaths globally, with the oldest populations being the most vulnerable.[5]

Aging is associated with a decline in the immune system, known as immunosenescence, which fosters a phenomenon called "inflammaging."[6] This is a persistent, low-grade, chronic systemic inflammation that occurs under non-pathogenic conditions.[7] Aged individuals frequently exhibit this proinflammatory profile, which, due to its chronicity, affects both innate and adaptive immune responses. Recent studies have identified serum levels of tumor necrosis factor receptor 1 (TNFR1) as a reliable biomarker of chronic inflammation in older adults, suggesting systemic TNFR1 levels can predict adverse health outcomes in age-associated inflammation.[8]

Aging is also associated with a clinical condition called frailty, characterized by heightened vulnerability, stemming from age-related reductions in reserve capacity and functionality across multiple physiological systems. It may lead to functional decline and poor outcomes.[9] Reports identified that COVID-19 increases the risk of geriatric syndromes, even in cases of non-severe COVID-19. Older individuals with COVID-19 significantly raise the risk of pre-frailty and frailty, which affects the quality of life post-COVID-19.[10,11] Studies have suggested that both pre-frailty and frailty are associated with significantly elevated serum inflammatory markers, such as tumor necrosis factor alpha (TNF-α) and beta (TNF-β).[12,13]

Currently, frailty is regarded as a syndrome influenced by multiple factors, and chronic inflammation is considered a primary contributor.[14] TNFR1 is not the only member of the TNF superfamily (TNFSF) involved in inflammation and aging. Osteoprotegerin (OPG), a TNFSF member that inhibits the Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL) in bone tissue, has been found at higher levels in postmenopausal women with osteoporosis.[15] Additionally, a report suggested that elevated OPG levels in older individuals reflect progressive organ damage and are associated with frailty development.[16]

Thus, in the post-COVID-19 era, it is essential to focus on preventing or minimizing the development of these syndromes in older adults and to explore potential preventive and therapeutic interventions. Plasma biomarkers are valuable tools for identifying molecules that allow easy and quick monitoring of patients under specific conditions. This study aims to identify inflammatory biomarkers in the plasma of 75 COVID-19 patients and 26 post-COVID-19 patients, divided into Frail and Non-Frail, to provide a tool for early monitoring of older COVID-19 patients, which could positively impact the management of geriatric frailty syndrome associated with post-COVID-19.

Material and Methods

Ethics Statement

This study received ethical approval from the Institutional Ethics Committee of the Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas (Protocol Numbers B09-22, C39-14, and C15-22). It adhered to the principles outlined in the 1964 Helsinki Declaration. Patients or their responsible family members provided written informed consent following the ethical standards established by the Institutional Ethics Committee.

Study Populations

Seventy-five patients, aged 60 years and older (66, 61–73; median ± interquartile range [IQR]), diagnosed with acute COVID-19 (hereafter referred to as COVID-19) were enrolled at the Instituto Nacional de Enfermedades Respiratorias Ismael Cosio Villegas (INER). The diagnosis was confirmed by RT-PCR testing for SARS-CoV-2 on nasopharyngeal swabs and included only patients with severe disease. Based on our previous report, patients were classified using data obtained from the institutional PACS system, CARE and RALE scores, chest radiographs and tomography scans, and the

WHO definitions.[17] Blood samples were collected between May and September 2020. Upon hospital admission, clinical laboratory staff obtained nasopharyngeal swabs for diagnosis and blood samples for clinical testing.

Additionally, two groups were recruited at INER, matching the severe COVID-19 group in terms of age, gender, and comorbidities. The first group consisted of thirty-six asymptomatic respiratory volunteers older than 60 (67, 64–69; median ± IQR), hereafter referred to as healthy donors (HD), enrolled in our "Lung Ageing Programme" to evaluate the effect of aging on the expression of selected molecules. The second group included thirty-four patients older than 60 years (68, 65–70; median ± IQR) with interstitial lung disease (hereafter referred to as ILD). These patients were recruited at the time of diagnosis (before treatment initiation), subsequently attended the corresponding specialty clinics at INER, and received appropriate treatment. This group presented interstitial lung damage of diverse etiologies (Supplementary Figure S1), unrelated to COVID-19.

HD and ILD patients were recruited before the COVID-19 pandemic (between 2018 and 2019), and the COVID-19 group was enrolled during the first wave; then, all groups were naïve to COVID-19 vaccination at study time. Additional details are provided in Table 1.

A fourth group, recruited between 2022 and 2023, was included in this study: twenty-six patients older than 60 years (72, 66–85; median ± IQR) discharged from hospitalization due to severe COVID-19. These individuals sought follow-up care at the INER clinic dedicated to managing post-acute sequelae (hereafter referred to as the post-COVID-19 group). Blood samples were obtained in this group at 4 and 12 months after hospital discharge. Frailty status was determined in these patients through a modified frailty phenotype, validated in a Mexican population, and used in Americans who participated in the Spanish version of the epidemiological study, the National Health and Aging Study (ENASEM or MHAS). The scale includes the five domains proposed by Fried et al,[18,19] where a score of 3–5 points is indicative of frailty. Details of the frailty scale are provided in Supplementary Table 1.

At recruitment time (4 months post-COVID-19), eight patients were diagnosed as without frailty (hereafter called non-frail) and eighteen as frail (hereafter called Frail); at 12 months, eight patients of the Frail group did not attend the new call, and the remaining ten continued as Frail. All patients from the post-COVID-19 group had received the first anti-COVID-19 vaccination scheme. Additional details per group are provided in Table 2.

A summary of the included groups is shown in Supplementary Figure 1.

Table 1 Demographic and Clinical Characteristics of the Study Groups

Parameters **All, n= 145**	**HD** **n= 36**	**ILD** **n= 34**	**COVID-19** **n= 75**	***p*-value**
Age, median (IQR)	67 (64–69)	68 (65–70)	66 (61–73)	ns
Sex				
Male, n (%)	18 (50)	12 (35)	26 (35)	ns
Female, n (%)	18 (50)	22 (65)	49 (65)	
Body mass index, median (IQR)	26.5 (24–30)	27 (24–30)	26.8 (24–30)	ns
Comorbidities				
Diabetes mellitus type 2, n (%)	13 (36)	9 (27)	38 (51)	0.045
SAH, n (%)	17 (47)	12 (35)	35 (47)	ns
Smoking, n (%)	22 (65)	11 (31)	22 (29)	0.001

Notes: Data are shown as n (%) or median (IQR 25–75). Differences between groups were analyzed using Pearson's Chi-squared test.
Abbreviations: IQR, interquartile range; ns, no statistical difference; SAH, systemic arterial hypertension.

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Table 2 Demographic and Clinical Characteristics of Post-COVID-19 Patients are Divided into Non-Frail and Frail

Parameters All, n= 26	Non-Frail n= 8	Frail n= 18	*P* Value
Age, median (IQR)	69 (66–70)	76 (70–85)	0.01
Male, n (%)	3 (37)	12 (67)	ns
Female, n (%)	5 (63)	6 (33)	
Body mass index, median (IQR)	28 (27–30)	27 (26–31)	ns
Diabetes mellitus type 2, n (%)	4 (50)	10 (56)	ns
SAH, n (%)	4 (50)	16 (89)	0.03
Smoking (%)	4 (50)	5 (28)	ns

Notes: Data are shown as n (%) or median (IQR 25,75). Differences between groups were analyzed using Pearson's Chi-squared test.
Abbreviation: SAH, systemic arterial hypertension.

Blood Sample

All samples were collected and stored under the same pre-analytical conditions. Blood samples from patients with HD, ILD, COVID-19, and post-COVID-19 conditions (4 and 12 months post-COVID-19) were obtained in tubes with spray-coated EDTA (BD Vacutainer 367863, Franklin Lakes, NJ, USA). Plasma was kept at −20 °C until use, avoiding freezing and thawing processes; this means that samples were unfrozen until use.

Flow Cytometry-Based Multiplex Immunoassays (LEGENDplex^{TM} Assay)

Our study employed the LEGENDplex kit, a multiplex bead-based assay panel (LEGENDplex^{TM} Human TNFSF, Cat. N. 741308, BioLegend, San Diego, CA). These panels were explicitly chosen for quantifying the protein concentrations of TNF superfamily ligands, including OPG, RANKL, TNF-α, TNF-β, a proliferation-inducing Ligand (APRIL), Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL), Soluble CD40 Ligand (sCD40L), TNF-like Weak Inducer of Apoptosis (TWEAK), TNFSF14 or Ligand for Herpesvirus Entry Mediator (LIGHT), Fas Ligand (FasL), B-cell activating factor (BAFF), and CD30 Ligand (CD30L). The bead assays were conducted strictly following the manufacturer's guidelines. Data were acquired using flow cytometry (FACS Aria II, Becton Dickinson) and analyzed with the LEGENDplex™ Data Analysis Software Suite.

ELISA Sandwich Assays

Plasma levels of OPG (cat. MBS162588, MyBioSource, San Diego, CA), BAFF cat. 449704, BioLegend, San Diego, CA), APRIL (cat. 439307, BioLegend, San Diego, CA), and TNFR1 (cat. DY225 R&D systems, Minneapolis, MN) were evaluated using an Enzyme-Linked Immunosorbent Assay (ELISA). Quantifications were performed following the manufacturer's instructions using a standard curve. The optical density (450 nm) was measured using a microplate reader (Imark, Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Although samples were collected at different times, they were processed using the same reagent lot, equipment, and technician to keep these variables consistent, thus avoiding non-biological differences between samples.

The Shapiro–Wilk normality test was used to evaluate the data distribution, indicating that our data did not follow a normal distribution. Comparisons between two groups were performed using the Mann–Whitney *U*-test, while multiple comparisons were conducted using the Kruskal–Wallis test, followed by Dunn's and the original False Discovery Rate (FDR) method by Benjamini and Hochberg post-hoc test for correction. Statistical significance for dichotomous variables was assessed using the Chi-square (χ^2) test to analyze differences between groups of interest in their clinical characteristics.

To compare the two assay methods used in this study, we performed a Spearman correlation and Bland-Altman plots between OPG, BAFF, and APRIL molecules (by ELISA and LegendPlex).

Statistical power was performed using the post-hoc power online version (https://clincalc.com/stats/Power.aspx), considering the OPG and TNFR1 plasma levels as the primary endpoint. Comparisons were made between groups, for instance, severe COVID-19 (group 1) and HD (group 2). The percentage showed that the analysis was up to 95% reliable, even when comparing frail and non-frail individuals at 4 and 12 months.

A multiple logistic regression analysis was conducted based on the clinical variables that had statistical differences in the groups to identify associations between type 2 Diabetes mellitus (DM2) with COVID-19 disease, and OPG, TNFR1, APRIL, BAFF soluble levels (considering log2-transformation values to homogenize data), and comorbidities mainly observed in three patient groups, COVID-19, HD, and ILD. In addition, a multiple logistic regression analysis was performed with two-way interactions to identify associations with COVID-19 disease, with COVID-19 defined as the dependent variable and soluble levels of OPG, TNFR1, BAFF, DM2, and smoking as independent variables.

Data are presented as median and interquartile range (IQR, 25–75), and a p-value of <0.05 was considered statistically significant. All statistical analyses were conducted using GraphPad Prism 10 (GraphPad Software, Inc., San Diego, CA, USA).

Discrimination Performance of Potential Biomarkers

A multivariate exploratory receiver operating characteristic (ROC) curve analysis was done using the software MetaboAnalyst 6.0 (http://www.metaboanalyst.ca) to explore whether OPG, TNFR1, APRIL, and BAFF signatures could discriminate between controls and COVID-19 patients 60 years old or older than 60 years. The cutoffs were selected by the Youden index in each ROC analysis. Systematic differences among samples were adjusted and normalized by a median with log transformation on individual values. Finally, auto-scaling by a scaling factor computed based on the dispersion of the variable was done. ROC curve analyses were used to estimate the accuracy of the combined signatures model. They were performed using three multivariate algorithms: support vector machines (SVM), partial least squares discriminant analysis (PLS-DA), and random forests. We specify linear SVM as a classification method and selected PLS-DA built-in for the feature ranking method, specifying two latent variables.

Results

Description of COVID-19 and Control Groups

COVID-19 patients exhibited similar age (median of 66, 67, and 68 years corresponding to COVID-19, HD, and ILD, respectively), sex, body mass index, and systemic arterial hypertension (SAH) compared to both control groups (HD and ILD). However, there was a higher prevalence of DM2 among COVID-19 patients compared to ILD patients (p=0.045) but not to HD. Additionally, the HD group had a high smoking rate, 65% of these participants were smokers (p=0.001) (Table 1).

Flow Cytometry-Based Multiplex Immunoassays Showed High Levels of OPG, BAFF, and APRIL in COVID-19 Patients

Previous studies suggested that OPG and BAFF might be helpful biomarkers in severe COVID-19 cases.[20] Thus, we determined the systemic levels of many TNFSF members in COVID-19 patients and age-matched controls. The data showed that COVID-19 patients had significantly higher systemic levels of OPG (p<0.0001), BAFF (p<0.0001), and APRIL (p<0.001) compared to HD, and a similar increase was observed when they were compared with ILD patients (p<0.0001, p=0.0002, and p<0.0001, respectively) (Figure 1).

Other TNFSF members exhibited differences when compared to one or none of the control groups. sCD40L was higher in COVID-19 patients than in ILD patients, although ILD patients showed a lower median level than HD (Supplementary Figure 2). In contrast, RANKL, TNF-α, TRAIL, LIGHT, and TNF-β levels did not differ between groups (Supplementary Figure 2). These findings suggest that a highly sensitive assay like LEGENDplex™ can identify high levels of OPG, BAFF, and APRIL in response to COVID-19 in older patients, which is not observed in older patients with COVID-19-independent lung damage.

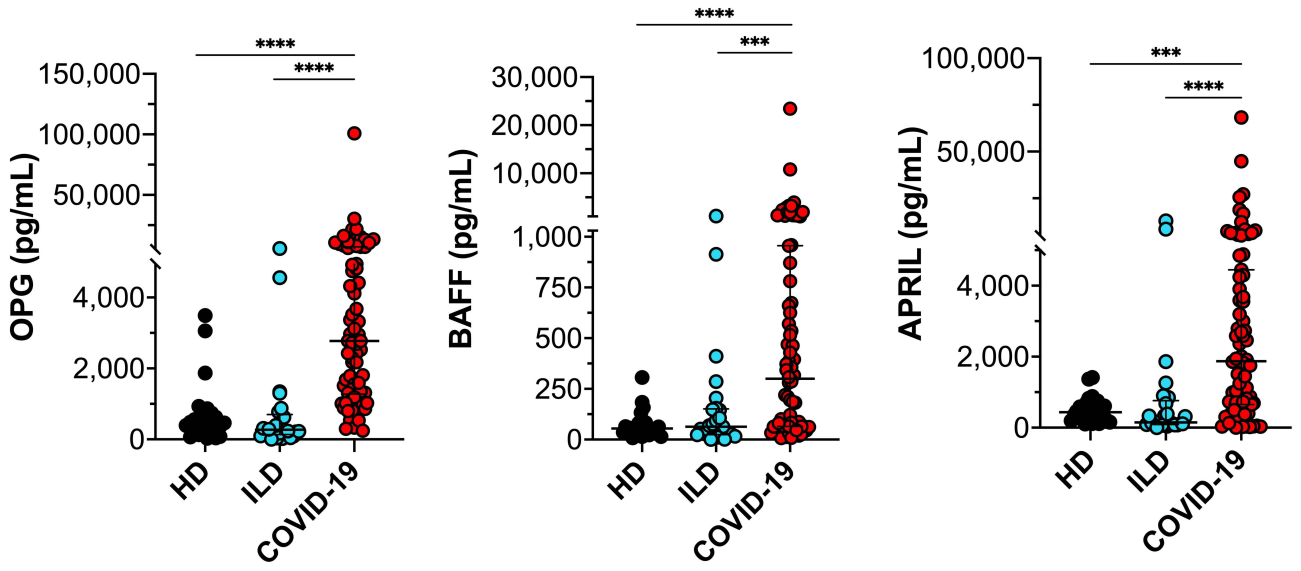

Figure 1 Systemic OPG, BAFF, and April levels are increased in COVID-19 patients. Flow cytometry-based multiplex immunoassays were used to evaluate plasma levels of OPG, BAFF, and APRIL. A comparison was performed between healthy donors (HD, n=36), individuals with interstitial lung disease (ILD, n=34), and those with COVID-19 (n=75), all of whom were older than 60 years. Data are presented as median and IQR (25–75); each symbol represents an individual subject. *p-values* were calculated by Kruskal–Wallis, followed by the Benjamin-Hochberg FDR test. ****p< 0.0001, ***p< 0.001.

High Levels of OPG, TNFR1, and APRIL in COVID-19 Patients Using ELISA: OPG and TNFR1 Show Optimal Sensitivity and Specificity

To corroborate our results, OPG, BAFF, and APRIL levels were assessed by ELISA. We also measured TNFR1, previously associated with severe COVID-19,[17] as it was not included in the flow cytometry-based multiplex immunoassay. ELISA analysis revealed significantly increased levels of OPG (p<0.0001) and TNFR1 (p<0.0001) in COVID-19 patients compared to both HD and ILD; APRIL levels were also higher compared to HD (p<0.01) and ILD (p<0.05), while BAFF levels were elevated compared to HD (p<0.0001) but not versus ILD (Figure 2A).

Compared to HD, OPG showed an AUC of 0.97, a cutoff of 7639 pg/mL, and sensitivity and specificity of 91% and 97%, respectively. TNFR1 had an AUC of 0.98, a cutoff of 973 pg/mL, and a sensitivity and specificity of 88% and 97%, respectively. APRIL showed an AUC of 0.70, with a cutoff of 861 pg/mL and sensitivity and specificity of 68% and 84%; BAFF showed an AUC of 0.70, a cutoff of 585 pg/mL, with sensitivity and specificity of 28% and 95% (Figure 2B). Compared to ILD, OPG exhibited an AUC of 0.96, a cutoff of 9254 pg/mL, and sensitivity and specificity of 88% and 97%. TNFR1, an AUC of 0.94, with a cutoff of 1865 pg/mL, sensitivity and specificity of 64% and 97%. APRIL had an AUC of 0.68, with a cutoff of 955 pg/mL, yielding a sensitivity and specificity of 67% and 80%, respectively (Figure 2B).

Predictive Value of OPG and TNFR1 as Biomarkers for COVID-19 Patients

Data suggest that elevated levels of OPG and TNFR1 are specifically associated with COVID-19, and it is not observed in COVID-19-unrelated lung damage disorders.[17,20] To further investigate their biomarker potential, a predictive analysis was conducted. First, individual comparisons were conducted between COVID-19 and each control group. OPG, BAFF, and TNFR1 effectively distinguished COVID-19 patients from HD (Supplementary Figure 3A), whereas OPG, TNFR1, and BAFF differentiated COVID-19 from ILD profiles (Supplementary Figure 3B). Notably, APRIL showed lower importance as a biomarker. Since OPG, BAFF, and TNFR1 were common to both discrimination panels, we then developed a combined predictive analysis including both reference groups to validate these findings.

Analysis of predicted class probabilities confirmed that these molecules effectively distinguished between controls and COVID-19 profiles (Figure 2C, left). A predictive model showed that using two variables yields a prediction accuracy of 82.4% to discriminate between COVID-19 and control (Figure 2C middle). The variable importance analysis

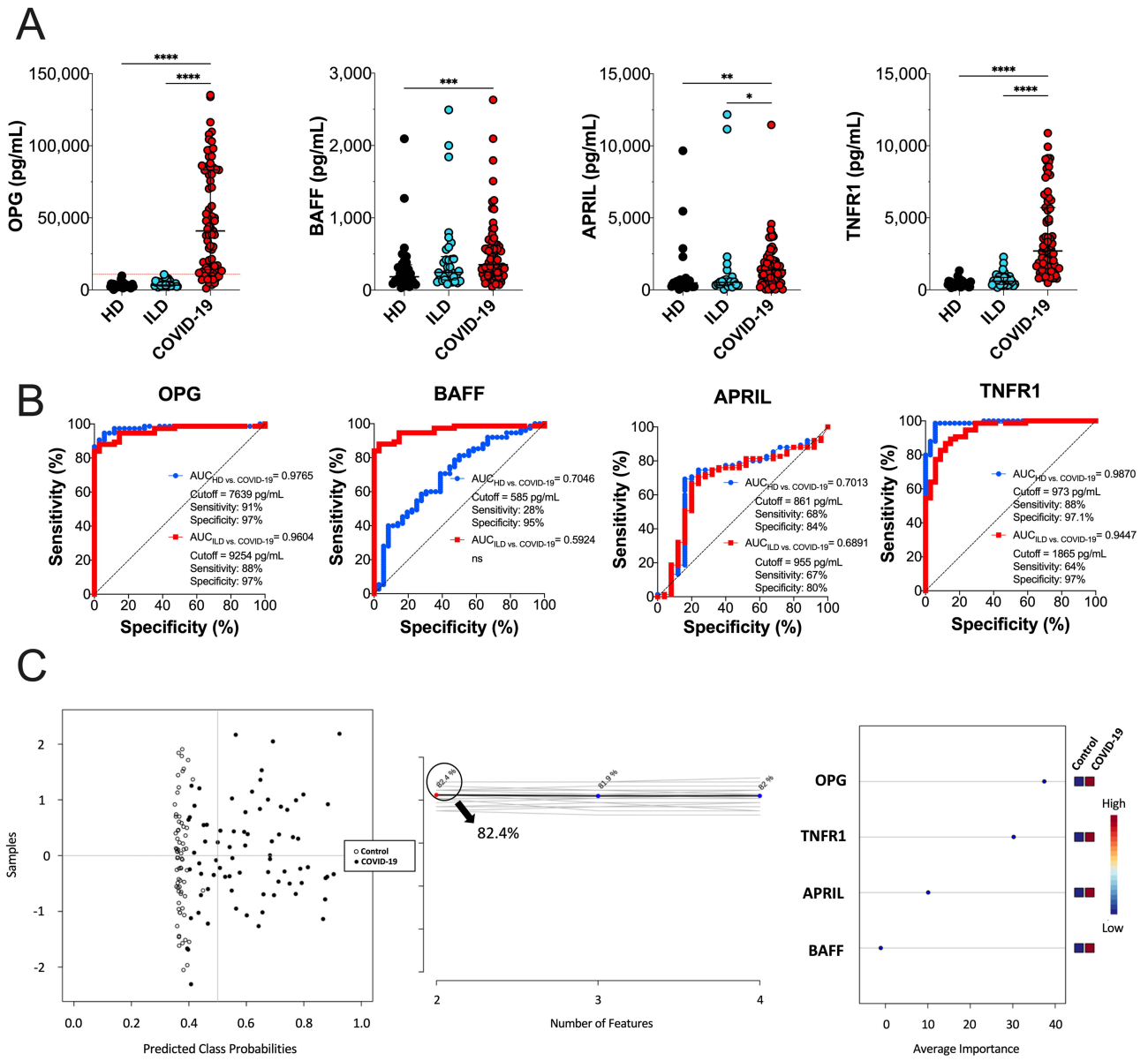

Figure 2 Systemic OPG, TNFR1, April, and BAFF levels are increased in older COVID-19 patients; nevertheless, only OPG and TNFR1 have a strong predictive value. Plasma levels of OPG, TNFR1, APRIL, and BAFF were evaluated by ELISA, and a comparison between healthy donors (HD, n=36), interstitial lung disease (ILD, n=34), and COVID-19 (n=75) was performed (**A**). OPG, TNFR1, APRIL, and BAFF levels were compared among COVID-19 and HD (dotted blue line) or ILD (dotted red line) to perform receiver operating characteristics (ROC) curves, which show the area under the curve (AUC); sensitivity, specificity, and cutoff value (pg/mL) were obtained using the Youden index. The AUC confidence intervals were for OPG 0.9484 to 1.000 (versus HD) and 0.9258 to 0.9950 (versus ILD), for BAFF 0.6010 to 0.8083 (versus HD) and 0.4744 to 0.7103 (versus ILD), for APRIL 0.5743 to 0.8283 (versus HD) and 0.5652 to 0.8129 (versus ILD), and for TNFR1 0.9708 to 1.000 (versus HD) and 0.9027 to 0.9867 (versus ILD) (**B**). OPG, TNFR1, APRIL, and BAFF levels were used to search for predictive markers for the COVID-19 group; HD and ILD were the reference group (**C**). The predicted class probability data plot (left), prediction accuracy (middle), and average importance plot (right) were performed. Data are presented as median and IQR (25–75); *p* values were calculated by Kruskal–Wallis, followed by Benjamin-Hochberg FDR test. ****p< 0.0001, ***p< 0.001, **p<0.01, *p<0.05.

indicated that OPG and TNFR1 are the most informative biomarkers for this purpose, followed by APRIL, with BAFF being the least informative (Figure 2C, right).

Multiple Logistic Regression Analysis Indicates That OPG and TNFR1 are Not Associated with DM2

Table 1 shows that DM2 was a common comorbidity in COVID-19. A multiple logistic regression analysis was conducted, including the four molecules and main comorbidities, to evaluate whether these comorbidities influenced the observed profile. The model demonstrated that COVID-19 and SAH were associated with DM2, which is consistent

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Table 3 Multiple Logistic Regression Analysis of the Association of DM2 or COVID-19 with Molecule Levels and Comorbidities

Dependent Variable	Independent Variable	*Z*	*p-value*	*OR*
DM2				
	Intercept	1.261	ns	0.01001 (6.691e-006 to 12.85)
	COVID-19	2.637	**	12.21 (2.037 to 87.36)
	OPG (pg/mL)	0.2229	ns	1.042 (0.7218 to 1.513)
	TNFR1 (pg/mL)	0.3069	ns	0.9266 (0.5617 to 1.503)
	APRIL (pg/mL)	0.3564	ns	0.9470 (0.6918 to 1.271)
	BAFF (pg/mL)	1.185	ns	1.288 (0.8531 to 1.986)
	Smoking	1.926	ns	3.005 (1.013 to 9.767)
	SAH	3.048	**	4.554 (1.759 to 12.56)
	Obesity	0.7809	ns	0.6678 (0.2389 to 1.850)
COVID-19				
	Intercept	2.847	**	1.106e-020 (1.170e-041 to 9.333e-011)
	OPG (pg/mL): BAFF (pg/mL)	0.6612	ns	1.064 (0.8813 to 1.324)
	OPG (pg/mL): TNFR1 (pg/mL)	2.730	**	1.395 (1.179 to 2.132)
	OPG (pg/mL): DM2	0.7244	ns	0.4598 (0.03329 to 3.837)
	OPG (pg/mL): Smoking	1.161	ns	3.559 (0.3737 to 56.32)
	BAFF (pg/mL): TNFR1 (pg/mL)	0.6691	ns	0.9312 (0.6891 to 1.131)
	BAFF (pg/mL): DM2	1.351	ns	25.47 (0.3988 to 9968)
	BAFF (pg/mL): Smoking	1.423	ns	0.02980 (7.739e-005 to 1.748)
	TNFR1 (pg/mL): DM2	0.5143	ns	0.3622 (0.009687 to 32.30)
	TNFR1 (pg/mL): Smoking	0.5861	ns	3.486 (0.02852 to 382.7)

Notes: A multiple logistic regression was conducted to examine the association between type 2 diabetes mellitus (DM2) and COVID-19, OPG, TNFR1, APRIL, BAFF levels, and clinical parameters (up). A second multiple logistic regression with two-way interactions was performed to investigate the association between COVID-19 and OPG, TNFR1, BAFF levels, DM2, and smoking, which showed statistically significant differences (down). The model included the three patient groups: COVID-19, HD, and ILD, and soluble levels in pg/mL of OPG, TNFR1, APRIL, and BAFF were calculated to determine the main effects on DM2. The absolute value of Z was computed as the coefficient estimate divided by its standard error. Asterisks indicate a significant difference (**p< 0.01).
Abbreviations: OR, odds ratio; ns, not significant p-value; SAH, systemic arterial hypertension.

with previous studies. In contrast, OPG, TNFR1, APRIL, and BAFF levels were not linked to DM2 (Table 3). However, a separate logistic regression confirmed the strong association of OPG and TNFR1 levels with COVID-19 (Table 3).

These findings indicate that elevated OPG and TNFR1 levels are specifically associated with COVID-19 in patients over 60 years of age, independently of the presence of other conditions such as DM2 or smoking. These data are in concordance with data from the predictive analysis; these two molecules could be potential biomarkers of COVID-19 in older adults, as shown in Figure 2.

Diabetes Influences BAFF Levels, but Not OPG and TNFR1

To confirm that elevated OPG and TNFR1 levels were specific to COVID-19 and independent of DM2, we compared COVID-19 patients to control groups (HD and ILD) stratified by diabetes status. The results showed that both diabetic and non-diabetic COVID-19 patients exhibited higher OPG and TNFR1 levels compared to their respective controls ($p<0.0001$) (Figure 3A and B, left). However, BAFF levels were influenced by diabetes status. Diabetic controls had

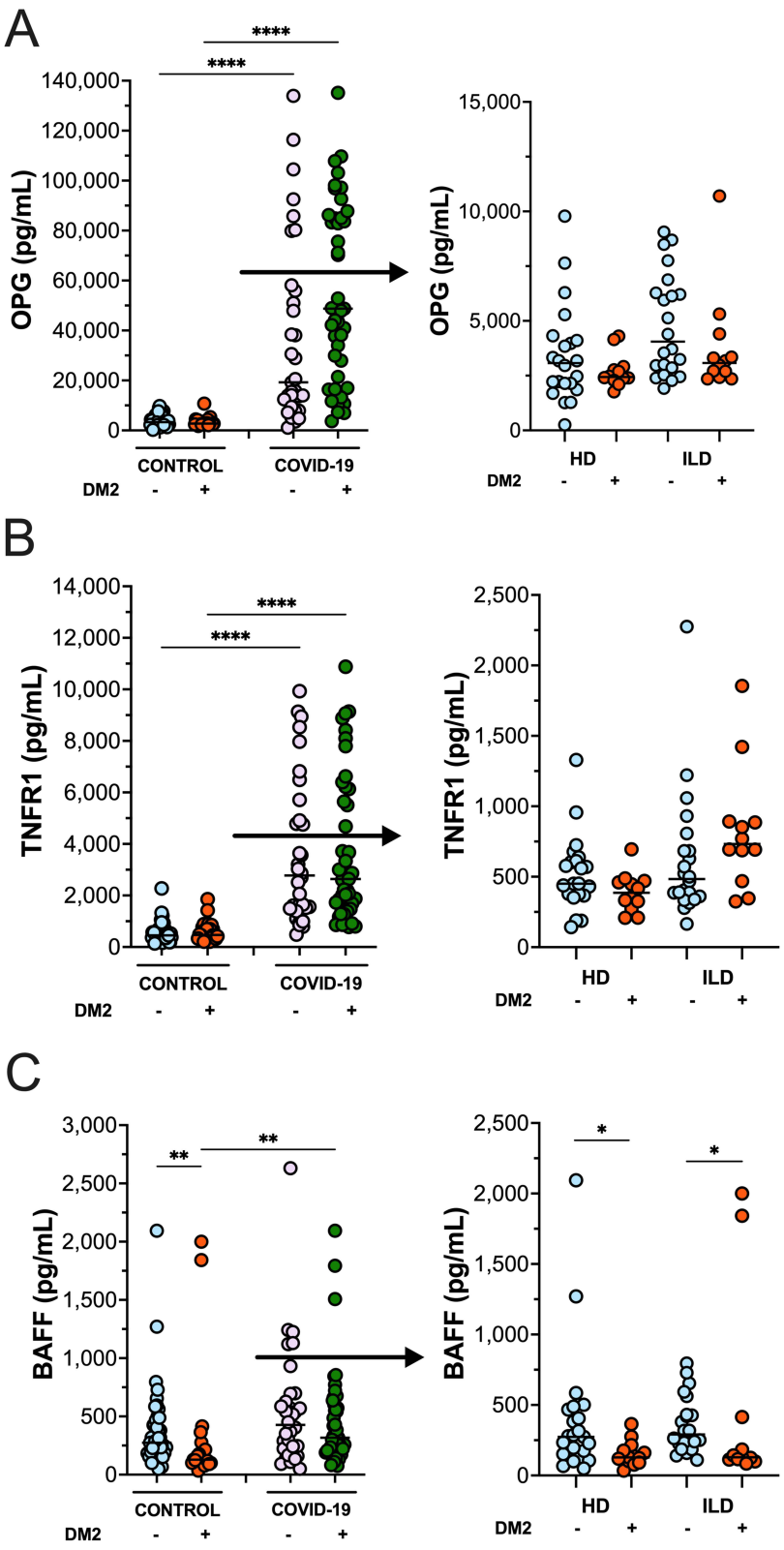

Figure 3 High OPG and TNFR1 levels in COVID-19 patients are independent of type 2 diabetes mellitus. Plasma levels of OPG, TNFR1, and BAFF were evaluated through ELISA. The control group consisted of mixed healthy donors (HD, n=36) and individuals with interstitial lung disease (ILD, n=34), while the second group comprised only COVID-19 patients (n=75). Both the control and COVID-19 groups were divided according to the status of type 2 diabetes mellitus (DM2) diagnosis. High OPG and TNFR1 levels are observed in COVID-19 patients, independent of DM2 status (**A** and **B**). BAFF levels are decreased in the control group with DM2 (**C**). Data are presented as median and IQR (25–75); each symbol represents an individual subject. *p*-values were calculated by Kruskal–Wallis, followed by Dunn's post-hoc test. ****p< 0.0001, **p<0.01, *p<0.05.

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lower BAFF levels compared to non-diabetic controls ($p<0.01$), whereas COVID-19 patients with DM2 did not exhibit decreased BAFF levels, unlike diabetic controls ($p<0.01$) (Figure 3C, left). To determine whether DM2 affects only one of the control groups, the HD and ILD groups were further divided by diabetes status. The data revealed that DM2 did not affect either OPG or TNFR1 levels (Figure 3A and B, right); in contrast, it decreased BAFF levels in both groups (Figure 3C, right).

Since OPG, APRIL, and BAFF were measured using both flow cytometry and ELISA, we assessed the agreement between platforms through a Spearman correlation and Bland–Altman analyses. Interestingly, OPG showed good concordance across methods, supporting its evaluation by either platform. In contrast, APRIL and BAFF did not show correlation, in line with their exclusion as potential biomarkers due to limited specificity and sensitivity (Supplementary Figure 4).

Together, these findings suggest that elevated OPG and TNFR1 levels are associated with COVID-19 infection independently of other lung pathologies or diabetes (the main comorbidity). Given its robust cross-platform agreement, ELISA may represent a practical and accessible method for quantifying OPG. Conversely, BAFF levels were influenced by DM2, limiting its utility as a biomarker in this context.

High OPG and TNFR1 Levels in Frail Post-COVID-19 Patients

Our data suggest that OPG and TNFR1 levels can be associated with COVID-19-specific damage, probably due to inflammation mediators. A previous report indicated that older patients have an increased risk of developing frailty after a COVID-19 infection, even if it was a non-severe status.[11] Our next aim was to clarify whether elevated OPG and TNFR1 levels were associated with frailty in post-COVID-19 patients.

In a different cohort of post-COVID-19 patients, frailty was assessed at 4 and 12 months after discharge due to COVID-19. Table 2 shows the demographic and clinical characteristics of post-COVID-19 patients; note that this patient group shows a similar profile to those patients of acute COVID-19, including the frequency of diabetes, although the Frail group exhibited a higher frequency of systemic hypertension as a second comorbidity.

ELISA evaluated OPG, TNFR1, and BAFF levels. Results indicated that frail patients had higher levels of OPG ($p < 0.05$) and TNFR1 ($p < 0.05$) at 4 months post-COVID-19. At 12 months, the Frail group still had higher levels of OPG ($p < 0.05$), whereas TNFR1 levels were similar between Non-frail and Frail (Figure 4). BAFF levels were not different between groups; the green dotted line in the graphics indicates the mean value of the control groups (Figure 4).

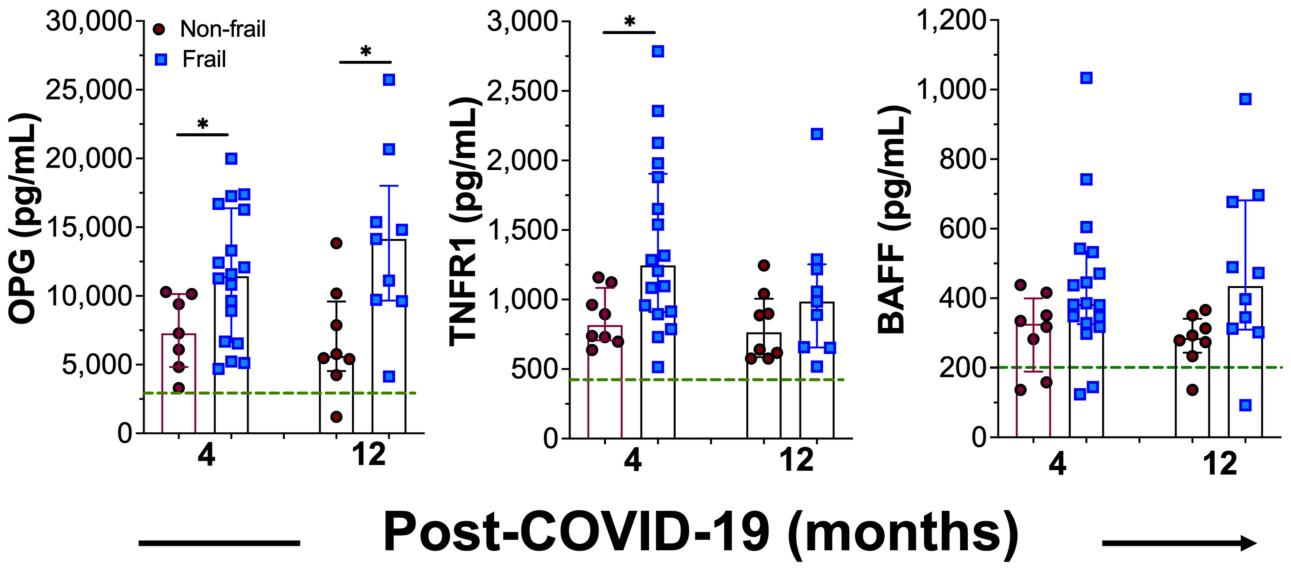

Figure 4 OPG and TNFR1 levels are increased in Frail patients post-COVID-19. A plasma sample was obtained from post-COVID-19 patients at 4 months and divided into non-frail (n=8) and frail (n=18), and a second sample was obtained at 12 months and divided into non-frail (n=8) and frail (n=10). OPG, TNFR1, and BAFF were evaluated using ELISA. The green dotted line represents the mean value of the control groups (taken from previous graphics). Data are presented as mean ± standard deviation; each symbol represents an individual subject. *p-values* were calculated by Kruskal–Wallis, followed by Dunn's post-hoc test. *p<0.05.

Our data indicate that post-COVID-19 patients with frailty exhibit elevated OPG and TNFR1 levels, while BAFF levels remain unchanged.

Discussion

The inflammatory response, mediated by cytokines such as IFN-γ and TNF-α, is essential to control viral infections, and COVID-19 is no exception.[21] However, in critical cases, an excessive inflammatory response in the lungs and bloodstream, mainly mediated by IFN-γ and TNF-α, plays a pathological role.[22] These cytokines trigger an inflammatory cell death and induce a cytokine storm, which is associated with a severe clinical complication referred to as acute respiratory distress syndrome (ARDS) and a markedly increased mortality rate among COVID-19 patients.[23,24]

Many serum proteins have been considered inflammation biomarkers in COVID-19; however, aging and frailty strongly influence the pathophysiological mechanisms in affected individuals.[25] Thus, it is necessary to investigate biomarkers further, specifically for the older adult population, to promote the early management of aging-associated syndromes.

TNFSF members regulate diverse cell functions, including immune responses, cell survival, and inflammation. Other members, such as TNFRs, have been implicated in poor outcomes during COVID-19.[17,26,27] Although this is a pilot study with a small sample size, specifically in the post-COVID-19 group, we found that TNFR1 and OPG show potential as biomarkers of inflammation in older COVID-19 patients and are not merely consequences of aging or COVID-19-independent lung damage. These findings are supported by strong statistical significance for the COVID-19 group and somewhat weaker significance for the post-COVID-19 group (Table 2).

Based on our data, we propose that OPG and TNFR1 serve as biomarkers of inflammation in older COVID-19 patients. Moreover, their elevated levels persist at 4 and 12 months post-COVID-19 compared to control groups. Outstandingly, both molecules are higher in frail patients than in non-frail patients, suggesting their utility in monitoring geriatric frailty syndrome associated with COVID-19.

Inflammaging, characterized by chronic low-grade inflammation associated with the aging process, has been found to interact with SARS-CoV-2 infections, thereby increasing the risk of severe COVID-19 among older adults.[28] TNFSF/TNFRSF axis is crucial in initiating specific signaling pathways that regulate the inflammatory immune response,[29] and the high levels expressed during this state of chronic inflammation may lead to the onset of frailty in the short and middle term after SARS-CoV-2 infection, predisposing older patients to worsen their functional reserve.

OPG has been described as a glycoprotein that regulates bone metabolism and immune responses, acting as a decoy receptor for RANKL, which is essential for the formation and activation of osteoclasts.[30] Inflammatory conditions can lead to changes in the expression of OPG during the transient inflammatory phase induced by bacterial or viral infections, and it has also been associated with chronic diseases such as rheumatoid arthritis, ankylosing spondylitis, and Crohn's disease.[31] It has been suggested that increased serum OPG levels can result from activating proinflammatory proteins, such as TNF-α.[32]

In the context of COVID-19, it has been shown that elevated levels of OPG may be associated with inflammation and responses to viral infections, potentially influencing the course of COVID-19.[33] SARS-CoV-2 can trigger prolonged inflammatory stimuli, leading to the secretion of proinflammatory cytokines that escalate with COVID-19 infection, potentially resulting in bone loss and resorption in seriously ill patients, particularly in older patients who are immobilized for extended periods.

OPG levels increase with age in both healthy men and women. OPG levels can be a marker of organ or system damage in older adults and may be a potential biomarker for geriatric frailty syndrome.[34] Our study has suggested a potential link between OPG levels and age-related susceptibility to SARS-CoV-2 disease and post-COVID frailty. As individuals age, their immune response becomes less robust, which could influence the severity of COVID-19. Elevated OPG levels in older adults reflect an ongoing inflammatory response that could exacerbate the severity of diseases.[35,36] Higher levels of OPG have been correlated with frailty in previous studies,[16] which may reflect the impairment in various organs contributing to frailty, which could be exacerbated after COVID-19.

While the influence of OPG on bone metabolism and vascular health is well-documented,[37] its full effect is not fully understood, underscoring the urgent need for further studies to evaluate the indices of bone metabolism and endothelial function during the transient inflammatory state and its long-term effects in the context of SARS-CoV-2 infection.

On the other hand, TNFR1 plays a crucial role in mediating the effects of TNF-α. When TNF-α binds to TNFR1, it triggers a cascade of intracellular signaling pathways that can lead to various cellular responses, including inflammation, apoptosis, and cell survival.[38] TNFR1 activation is crucial, as it can promote the expression of other proinflammatory cytokines, chemokines, and adhesion molecules.[39] This process enhances the recruitment of immune cells to sites of infection or injury, contributing to the inflammatory response. However, excessive or chronic activation of TNFR1 can lead to pathological inflammation and is implicated in several autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease, and psoriasis.[40,41]

In severe cases of COVID-19, there is often an exacerbated inflammatory response, sometimes referred to as a cytokine storm, where proinflammatory cytokines are released in excess. Research suggests that during COVID-19, TNFR1 may contribute to tissue damage because it mediates the effects of TNF, and this axis leads to increased inflammation associated with a hyperactivation status.[17,42]

Although additional studies are needed to clarify the precise role of elevated TNFR1 levels in older patients with COVID-19, our findings highlight the potential of selectively targeting TNFR1 signaling as a therapeutic approach in this inflammatory context, as previously suggested by other authors.[43] Identifying new inflammatory biomarkers in severe COVID-19, particularly in high-risk populations such as older adults, is essential for early disease monitoring and the development of new therapies.

While IL-6 and C-reactive protein (CRP) are widely used inflammation markers, CRP levels may be influenced by external factors, for example, unvaccinated patients often present with higher CRP levels than vaccinated individuals, and comorbidities such as DM2 can also impact these levels.[44,45] In contrast, our data suggest that OPG and TNFR1 levels are not affected by DM2, indicating that they have an additional value beyond existing biomarkers. To note, the severe COVID-19 group was naïve to SARS-CoV-2 vaccination and the post-COVID-19 group had been vaccinated, yet both displayed elevated OPG and TNFR1 levels indicative of inflammation and frailty; probably these molecule levels are not influenced by vaccination status. Furthermore, logistic regression analysis indicated that these levels were unrelated to DM2.

Our findings identified that DM2 influences the BAFF levels, but we did not clarify the pathway involved in the BAFF/DM2 axis. In the context of gestational diabetes mellitus, previous reports have suggested high BAFF levels as an early predictor, and other data have suggested that metformin, an anti-diabetic drug, has great potential to prevent excessive BAFF-induced cell activation.[46–48] Despite these reports, the specific role that BAFF plays in DM2 remains unclear.

This study is not free of limitations. First, since OPG levels may be influenced by physical activity,[49] future studies should assess OPG levels in healthy older adults (65+) with varying levels of routine exercise. Second, as a pilot study, our sample size was limited, particularly in the post-COVID-19 group; therefore, larger, multicenter studies with vaccine/unvaccinated patients are necessary to confirm these results. Third, although we conducted follow-ups at 4 and 12 months post-COVID-19, whether OPG and TNFR1 contribute to long-term biomarker dynamics or frailty progression remains uncertain.

Extended longitudinal studies are essential for assessing the potential of these targets as therapeutic interventions for reducing hyperinflammation and for understanding the impact of vaccination status on plasma levels of TNFSF molecules.

Conclusion

High OPG and TNFR1 levels could be potential biomarkers of inflammation during acute COVID-19 and frailty in post-COVID-19 among older adults; this pilot study supports the utility of measuring plasma levels of these molecules for monitoring inflammation and geriatric frailty syndrome associated with COVID-19.

Abbreviations

APRIL, Proliferation-inducing ligand; AUC, Area under the ROC curve; BAFF, B cell activating factor; CD30L, CD30 Ligand; DM2, Type 2 Diabetes mellitus; HD, Healthy donors; IQR, Interquartile range; ILD, Interstitial lung disease; LIGHT, Homologous to lymphotoxin; OPG, Osteoprotegerin; PLS-DA, Partial least squares discriminant analysis; RANKL, Receptor activator of nuclear factor Kappa B ligand; ROC, Receiver-operator characteristic; RT-PCR, Reverse transcription-polymerase chain reaction; SAH, Systemic arterial hypertension; sCD40L, Soluble CD40 ligand; SVM, Support vector machines; TNF-α, Tumor necrosis factor alpha; TNFR1, TNF receptor 1; TNFSF, Tumor necrosis factor superfamily; TRAIL, TNF-related apoptosis inducing ligand; TWEAK, TNF-like weak inducer of apoptosis.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical Approval and Consent to Participate

This study received ethical approval from the Institutional Ethics Committee of the Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas (Protocol Numbers B09-22, C39-14, and C15-22). All procedures performed on human participants followed the 1964 Helsinki Declaration. Patients or the responsible family member provided written informed consent following the ethical standards established by the Institutional Ethics Committee.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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