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Assessment of antiphospholipid antibodies and calprotectin as biomarkers for discriminating mild from severe COVID-19

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Abstract

Background: To explore the association of thrombo-inflammatory biomarkers with severity in coronavirus disease (COVID-19), we measured antiphospholipid antibodies (aPL) and calprotectin in sera of COVID-19 patients.

Methods: Anticardiolipin antibodies (aCL) and anti-β2-glycoprotein I antibodies were measured using enzyme-linked immunosorbent assay (ELISA) and multiplex flow immunoassay (MFIA) in hospitalized COVID-19 patients (N = 105) and healthy controls (N = 38). Anti-phosphatidylserine/prothrombin antibodies, calprotectin, and C-reactive protein (CRP) levels were also measured. We assessed the potential correlation between calprotectin levels and various laboratory parameters that were measured during the hospitalization period. After stratifying COVID-19 patients into two groups by their oxygenation status or acute respiratory distress syndrome presentation, the discriminatory performance of each biomarker was evaluated.

Results: A high proportion of COVID-19 patients (29.5%, 31/105) had low aCL IgM titers that were detectable by ELISA but mostly below the detection limit of MFIA. Calprotectin levels in severe groups of COVID-19 were significantly higher than those in non-severe groups, while CRP levels revealed no significant differences. Serum calprotectin levels showed strong to moderate degree of correlation with other routinely used parameters including peak levels of CRP, ferritin, procalcitonin, BUN, and neutrophil-to-lymphocyte ratio, but a negative correlation with minimal lymphocyte count and $CD4^+$ T cells. The discriminatory performance was highest for calprotectin in discriminating severe groups of COVID-19.

Conclusions: Serum calprotectin levels were significantly elevated in severe COVID-19 cases. The prevalence of clinically significant aPL did not differ. The link between calprotectin and inflammatory pathway in COVID-19 may help improve the management and outcomes of COVID-19 patients.

KEYWORDS
anticardiolipin antibodies, antiphospholipid antibodies, calprotectin, COVID-19, severity

1 | INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes coronavirus disease (COVID-19). Although COVID-19 most commonly presents with influenza-like illness and viral pneumonia, its critical cases often have a profound hypercoagulable state leading to coagulopathy, and this leads to acute respiratory distress syndrome (ARDS) or multi-organ failure.[1] Recently, some researchers reported an important role of antiphospholipid antibodies (aPL) in thrombotic events in critical cases of COVID-19,[2] while others suggested a poor correlation between aPL and thrombotic events; therefore, its association with thrombotic events remains controversial.[3]

Calprotectin (myeloid-related protein [MRP]8/14) is released under inflammatory conditions and is involved in neutrophil-related inflammatory processes.[4] Calprotectin levels in the blood are associated with poor clinical outcomes in COVID-19, especially in patients with severe pulmonary disease.[5]

In this study, the authors measured aPL (anticardiolipin antibody [aCL] and anti-β2-glycoprotein I antibody [aβ2GPI]) in the sera of hospitalized COVID-19 patients using two different solid-phase assays. While functional assays for aPL (i.e., lupus anticoagulant [LA]) were unavailable for this study, we performed anti-phosphatidylserine/prothrombin antibody (aPS/PT) assay as a surrogate test for LA.[6] We also assayed calprotectin and C-reactive protein (CRP) levels in the sera of hospitalized COVID-19 patients and assessed the possible relationship between oxygenation status and the lung involvement.

2 | MATERIALS AND METHODS

2.1 | Study population

Between July 4, 2020, and November15, 2020, 105 hospitalized COVID-19 patients (60 women and 45 men with a mean age of 68.0 ± 18.8 years) and 38 healthy controls (21 females and 17 males with a mean age of 53.1 ± 12.5 years) were enrolled in this retrospective study. Overview of the characteristics of COVID-19 cases, including demographic features, comorbidities, therapy received, complications, treatment, and final outcome, are summarized in Table 1. COVID-19 diagnosis was confirmed in all the patients based on a positive result of real-time reverse transcription polymerase chain reaction (RT-PCR) test of routine nasal and pharyngeal swab specimens. Remnant serum samples were collected after completing routinely ordered laboratory tests for COVID-19 or healthy controls and stored at −80°C until the time of research testing. This study complied with all relevant ethical regulations and was approved by the Inha University Hospital Institutional Review Board (IRB ID: 2020-12-018) and Seoul Clinical Laboratories IRB (ID: IRB-20-086), which waived the requirement for informed consent given the discarded nature of the samples.

TABLE 1 Overview of characteristics of COVID-19 cases

Demographic features	
Number	105
Age (years)[a]	68.0 ± 18.8 (56–83)
Female	60 (57.1%)
BMI (kg/m^2)[a]	24.31 ± 4.5 (21.8–26.5)
Comorbidities	
Diabetes	47 (44.8%)
Hypertension	60 (57.1)
Cardiovascular diseases	10 (9.5%)
Renal disease	1 (1.0%)
Cancer	6 (5.7%)
Autoimmune disease	1 (1.0%)
Dementia/mental retardation	16 (15.2%)
Pregnancy	2 (1.9%)
Hyperlipidemia	6 (5.7%)
Hepatitis B virus infection	1 (1.0%)
Obesity (BMI ≥ 30 kg/m^2)	11 (8.8%)
Medications or therapy	
Anticoagulant	78 (74.3%)
Antivirals	37 (35.2%)
Corticosteroids	53 (50.5%)
Intravenous immunoglobulin	4 (3.8%)
Renal replacement therapy	2 (1.7%)
Oxygen therapy	
Room air	70 (66.7%)
Low flow oxygen therapy (flow rates <10 L/min)	5 (5.7%)
High flow oxygen therapy (flow rate ≥10 L/min)	24 (22.9%)
Mechanical ventilation	19 (18.1%)
Extracorporeal membrane oxygenation	5 (4.8%)
Complications	
Lung	26 (24.8%)
Renal	12 (11.4%)
Systemic infection	6 (5.7%)
Disseminated intravascular coagulation	1 (0.9%)
In-hospital thrombosis	2 (1.6%)
Final outcome	
Discharged alive	92 (87.6%)
Died	9 (8.6%)
Transfer to other hospital	4 (3.8%)
Length of hospital stay (days)[a]	21.8 ± 14.7 (13.0–27.0)
Sampling time (days)[a,b]	5.7 ± 3.6 (3.0–7.8)

Abbreviations: ARDS, acute respiratory distress syndrome; BMI, body mass index; COVID-19, coronavirus disease; IQR, interquartile range.

[a]Mean ± standard deviation (IQR).

[b]Time from first symptom onset to sampling.

2.2 | Data collection

The date of symptom onset, previous history, clinical features including comorbidities, laboratory findings, treatment, respiratory status, complications, and final outcome were obtained from the hospital's electronic medical records according to previously designed standardized data collection forms. The highest results of CRP, procalcitonin (PCT), ferritin, D-dimer, fibrinogen, and blood urea nitrogen (BUN) for each patient during hospital admission were defined as the peak levels and were selected as representative of each parameter; in contrast, the lowest values of platelet count and estimated glomerular filtration rate (eGFR) were defined as the minimum levels and were selected as representative data.

The data for neutrophil count, lymphocyte count, neutrophil-to-lymphocyte ratio (NLR), and $CD4^+$ T cell count values were selected within 48 h from the research sample collection time.

2.3 | Stratifying COVID-19 patients by oxygen therapy or clinical severity

COVID-19 patients were stratified into two groups based on the degree of oxygen therapy during hospitalization as follows: Group R1 ($N = 75$) which included patients who were breathing in room air and patients requiring low flow oxygen therapy (flow rates < 10 L/min); and Group R2 ($N = 30$) which included patients requiring high flow oxygen therapy (flow rate ≥ 10 L/min), mechanical ventilation, or extracorporeal membrane oxygenation (ECMO). In addition, we stratified COVID-19 patients based on the presence or absence of ARDS during hospitalization into two groups, including ARDS− ($N = 79$) and ARDS+ ($N = 26$) groups. ARDS was defined according to the 2012 Berlin Definition.[7]

2.4 | Laboratory assays

2.4.1 | Measurement of aPL

aCL and aβ2GPI IgG/IgM/IgA were measured by a multiplex flow immunoassay (MFIA) using BioPlex 2200® (Bio-Rad, Laboratories, CA, USA) and the APLS IgG, IgM, and IgA kits (Bio-Rad, Laboratories, CA, USA) (cutoff: 20 U/ml). To compare the aPL results by MFIA, aCL IgG/IgM/IgA and aβ2GPI IgG/IgM were also assayed by different methods as follows: aCL IgG/IgM by enzyme-linked immunosorbent assay (ELISA) using semiquantitative test kits (Corgenix, Colorado, USA) (cutoff: IgG, 23 GPL; IgM, 11 MPL); aCL IgA by ELISA using ORG 515A Anti-Cardiolipin IgA® (Orgentec Diagnostika, Mainz, Germany) (cutoff: 10 APL U/ml); and aβ2GPI IgG/IgM by ELISA using IgG/IgM aβ2GPI semiquantitative test kits (Corgenix, Colorado, USA) (cutoff: IgG, 20 G unit; IgM, 10 M unit). aPS/PT IgG/IgM were quantified by ELISA using QUANTA Lite® aPS/PT IgG/IgM (Inova Diagnostics, CA, USA) (cutoff: 30 unit). All assays were performed according to the manufacturer's instructions and interpreted based on the manufacturers' cutoff values, which had been locally validated.

2.4.2 | Quantification of serum calprotectin and CRP

Serum calprotectin levels were measured by ELISA using the MRP8/14 (S100A8/S100A9) (BÜHLMANN® Laboratories AG, Switzerland) (reference interval, <2.9 μg/ml) according to the manufacturer's instructions. Serum CRP was quantified by particle-enhanced turbidimetric immunoassay using Modular P800 (Roche®, Basel, Switzerland) and interpreted based on the manufacturers' cutoff value (<0.5 mg/dl), which had been locally validated.

2.5 | Statistical analysis

The data of the groups were expressed as medians and interquartile range (IQR), wherever appropriate. When two groups were compared, normally distributed data were analyzed using a two-sided *t* test, and skewed data were analyzed using the Mann–Whitney *U* test. For three or more groups, one-way analysis of variance (ANOVA) or Kruskal–Wallis test with correction by Dunn's test for multiple comparisons was performed. Correlations between calprotectin levels and other biomarkers were estimated using Spearman's correlation analysis. The clinical performance of the biomarkers was tested by the receiver operating characteristic (ROC) curve analysis (Hanley and McNeil method), considering the area under the curve (AUC) and pairwise comparison between laboratory parameters. The Youden index optimal cutoff, sensitivity, and specificity were also evaluated. Data were analyzed using the MedCalc Statistical Software ver. 20.006 (Ostend, Belgium), and statistical significance was defined as $p < 0.05$.

3 | RESULTS

3.1 | Prevalence and concentration of aPL in COVID-19 patients

The overall prevalence of aPL (any aCL IgG/IgM/IgA or aβ2GPI IgG/IgM) by ELISA was significantly higher in COVID-19 cases than in controls: 29.5% (31/108) versus 10.5% (4/38), respectively ($p = 0.020$). On the contrary, the prevalence of aPL (any positive result of aCL IgG/IgM/IgA or β2GPI IgG/IgM/IgA) by MFIA was similar between COVID-19 cases and controls: 1.9% (2/105) versus 2.6% (1/38), respectively ($p = 0.790$). The prevalence of aPS/PT IgG/IgM was also similar between COVID-19 cases (2.9% [3/105]) and controls (2.5% [1/38]) ($p = 0.941$).

TABLE 2 Prevalence of aPL in COVID-19 cases (N = 105) and healthy controls (N = 38)

	MFIA				ELISA					
	aCL		aβ2GPI		aCL		aβ2GPI		aPS/PT	
	COVID-19	Control	COVID-19	Control	COVID-19	Control	COVID-19	Control	COVID-19	
IgG	0.7 (1)	2.5 (1)	0.7 (1)	2.5 (1)	1.9 (2)	2.5 (1)	3.8 (4)	5.3 (2)	0 (0)	0 (0)
IgM	0.7 (1)	0 (0)	0.7 (1)	0 (0)	27.6 (29)*	2.5 (1)*	3.8 (4)	2.5 (1)	2.9 (3)	2.5 (1)
IgA	1.9 (2)	0 (0)	0.7 (1)	0 (0)	0.7 (1)	0 (0)	ND	ND	ND	ND

Note: Values are expressed as percentage (n) of positive patients.

Abbreviations: aCL, anticardiolipin antibodies; aPL, antiphospholipid antibodies; aPS/PT, anti-phosphatidylserine/prothrombin antibodies; aβ2GPI, anti-β2 glycoprotein I antibodies; COVID-19, coronavirus disease; ELISA, enzyme-linked immunosorbent assay; MFIA, multiplex flow immunoassay; ND, not determined.

*p = 0.002 by chi-square test.

For the aPL assay by ELISA, the prevalence of aCL IgM was significantly higher in COVID-19 cases than in controls: 27.6% (29/105) and 2.5% (1/38), respectively (p = 0.002) (Table 2); however, most of the positive cases had low titers (Figure 1A). Although the background level of aCL IgM (MFIA) in COVID-19 patients was also higher than that of controls (Figure 1B), the difference was not statistically significant (p = 0.243) and most values were below the cutoff value. A low degree of positive correlation (r = 0.3163, p = 0.001) was observed between MFIA and ELISA for aCL IgM (Figure 1C). However, the prevalence of aCL IgG/IgA (ELISA), β2GPI IgG/IgM (ELISA), and aPS/PT IgG/IgM (ELISA) was similar in COVID-19 cases and controls ($p > 0.05$) (Table 2). We did not find any association between aPL positivity and thrombotic events in COVID-19 patients.

3.2 | Serum calprotectin and CRP levels in COVID-19 patients

The median (IQR) calprotectin levels in COVID-19 cases (N = 105) were significantly higher than those in healthy controls (N = 38) (4.10 [1.98–8.80] versus 1.85 [1.30–3.60] μg/ml, respectively [p = 0.002]) (Table 3). Qualitative analysis revealed that 59.0% (62/105) of COVID-19 cases and 36.8% (14/38) of healthy controls had above the reference interval (≥2.9 μg/ml) (p = 0.019). The median (IQR) CRP levels in COVID-19 cases (1.18 [0.38–4.60] mg/dl, N = 105) were significantly higher than those in healthy controls (0.03 [0.01–0.07] mg/dl, N = 38) ($p < 0.001$) (Table 3). Qualitative analysis revealed that 69.5% (73/105) of COVID-19 cases and 7.9% (3/38) of healthy controls had above the reference interval (≥0.5 mg/dl) ($p < 0.001$). Three healthy controls with CRP levels above the reference interval were considered to have a minor degree of CRP elevation (0.705, 1.303, and 1.809 mg/dl, respectively).

3.3 | Serum calprotectin and CRP levels by stratified groups of COVID-19 patients

When comparing calprotectin levels between oxygen therapy groups (R1 versus R2) or lung involvement groups (ARDS− versus ARDS+), calprotectin levels in group R2 or group ARDS+ were significantly higher than those in group R1 or group ARDS−, respectively (Figure 2A,B) (Table 4). When comparing CRP levels between oxygen therapy groups (R1 versus R2) or lung involvement groups (ARDS− versus ARDS+), CRP levels did not reveal significant differences between groups R1 and R2 or groups ARDS− and ARDS+ (Figure 2C,D) (Table 4).

3.4 | Correlation of serum calprotectin with CRP and other routinely used laboratory parameters

Subsequently, we assessed the correlation between serum calprotectin and CRP levels, which were assayed using the same samples for measuring calprotectin. Calprotectin levels demonstrated a moderate positive correlation with CRP (r = 0.5294, $p < 0.001$). Similarly, we assessed the potential correlation between calprotectin levels and various laboratory parameters, which were measured during each patient's hospital admission period. Calprotectin levels were significantly positively correlated with the neutrophil count (r = 0.3697, $p < 0.001$), NLR (r = 0.4827, $p < 0.001$), peak CRP (r = 0.7199, $p < 0.001$), peak ferritin (r = 0.5056, $p < 0.001$), peak PCT (r = 0.4397, p = 0.001), and peak BUN (r = 0.4625, $p < 0.001$). Calprotectin levels were significantly negatively correlated with the lymphocyte count (r = −0.3838, $p < 0.001$) and $CD4^+$ T cell count (r = −0.3501, p = 0.001) (Table 5).

3.5 | Laboratory parameters in stratified groups of COVID-19 patients

When comparing laboratory parameters between oxygen therapy groups (R1 and R2) in the patients with COVID-19, the neutrophil count, NLR, peak CRP, peak ferritin, peak PCT, peak D-dimer, and peak BUN were significantly higher in the R2 group than in the R1 group. On the contrary, lymphocyte count and $CD4^+$ T cell count were lower in the R2 group than in the R1 group (Table 6). When comparing the same parameters between lung

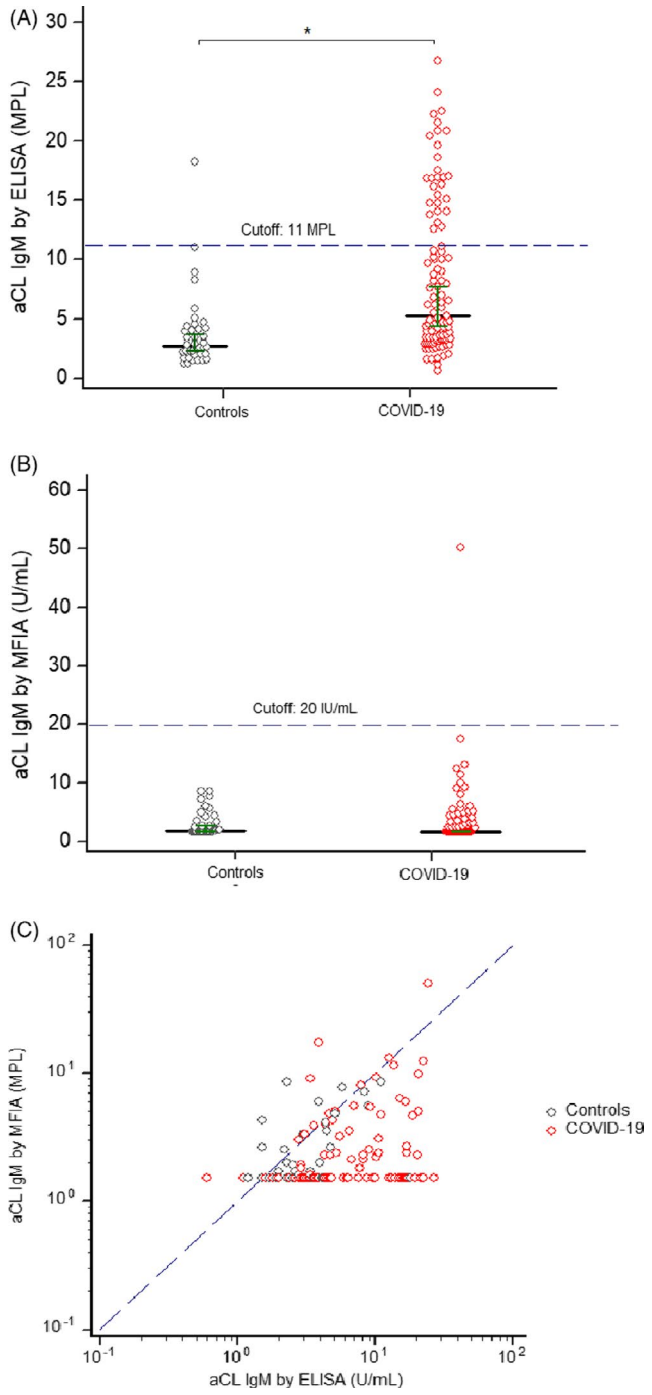

FIGURE 1 Concentrations of aCL IgM in COVID-19 patients ($N = 105$) and controls ($N = 35$) determined by two solid-phase assays. (A) Concentrations of aCL IgM by ELISA. Black line indicates the median value, while green line indicates 95% confidence interval for median. *$p < 0.001$ (by Mann–Whitney test for independent samples). (B) Concentrations of aCL IgM by MFIA. (C) Correlation between MFIA and ELISA for aCL IgM ($r = 0.3163$, $p = 0.001$). aCL, anticardiolipin antibodies; COVID-19, coronavirus disease; ELISA, enzyme-linked immunosorbent assay; MFIA, multiplex flow immunoassay

involvement groups (ARDS− and ARDS+), similar patterns were observed for each parameter between ARDS− and ARDS+ groups (Table 6).

TABLE 3 Median (IQR) concentrations of calprotectin and CRP in COVID-19 patients and controls

	COVID-19 ($N = 105$)	Control ($N = 38$)	p*
Calprotectin (μg/mL)	4.10 (1.988–80)	1.85 (1.30–3.60)	0.002
CRP (mg/dl)	1.18 (0.38–4.60)	0.03 (0.01–0.07)	<0.001

Abbreviations: COVID-19, coronavirus disease; CRP, C-reactive protein; IQR, interquartile range.

*by Mann–Whitney test (independent samples).

3.6 | Discriminatory performance of calprotectin and other laboratory parameters

When assessing the discriminatory performance of each parameter between R1 and R2 groups using ROC curves, the highest AUC was observed for serum calprotectin (AUC 0.91), followed by peak CRP, peak ferritin, and NLR with AUCs of 0.88, 0.80, and 0.79, respectively. The AUC for discriminating ARDS− and ARDS+ groups was highest with peak CRP (AUC 0.91), followed by calprotectin, NLR, and $CD4^+$ T cell count with AUCs of 0.89, 0.86, and 0.81, respectively. Calprotectin level > 3.6 μg/ml discriminated the R2 group from the R1 group with a sensitivity of 100.0% and a specificity of 68.0% (AUC 0.91; 95% confidence interval [CI] = 0.84–0.96), while calprotectin level > 6.9 μg/ml discriminated the ARDS+ group from the ARDS− group with a sensitivity of 88.5% and a specificity of 88.3% (AUC 0.89; 95% CI = 0.82–0.94) (Table 7). The multiple comparisons of ROC curves for serum calprotectin and the five other biomarkers (CRP, lymphocyte count, NLR, neutrophil count, and $CD4^+$ T cell count) for discriminating between stratified groups (R1 versus R2, and ARDS− versus ARDS+) are shown in Figure 3 and Table 8 and are based on data from 85 COVID-19 patients.

4 | DISCUSSION

aPL (LA, aCL, and/or aβ2GPI) are a heterogeneous group of antibodies that underlie the pathogenesis of antiphospholipid syndrome (APS) via their interactions with phospholipid-binding plasma proteins. APS is a complex thrombo-inflammatory disease with a broad clinical spectrum.[8]

Approximately 57% of COVID-19 patients have prolonged activated partial thromboplastin time (aPTT). Yet, only a minimal proportion of COVID-19 patients have aCL and aβ2GPI antibodies. This suggests that other factors are responsible for the prolonged aPTT phenomenon and likely for the LA activity.[9] LA may be affected by the concomitant heparin treatment[10] and the high CRP levels.[11] Since aPS/PT can be associated with a prolonged aPTT and with the presence of LA,[12] aPS/PT antibodies were included as a partial surrogate for LA in this study.

The threshold for clinically relevant levels of aPL for the diagnosis of APS remains debatable. Defining cutoff reference values for aPL solid-phase assays is a determining factor for the diagnosis of APS.

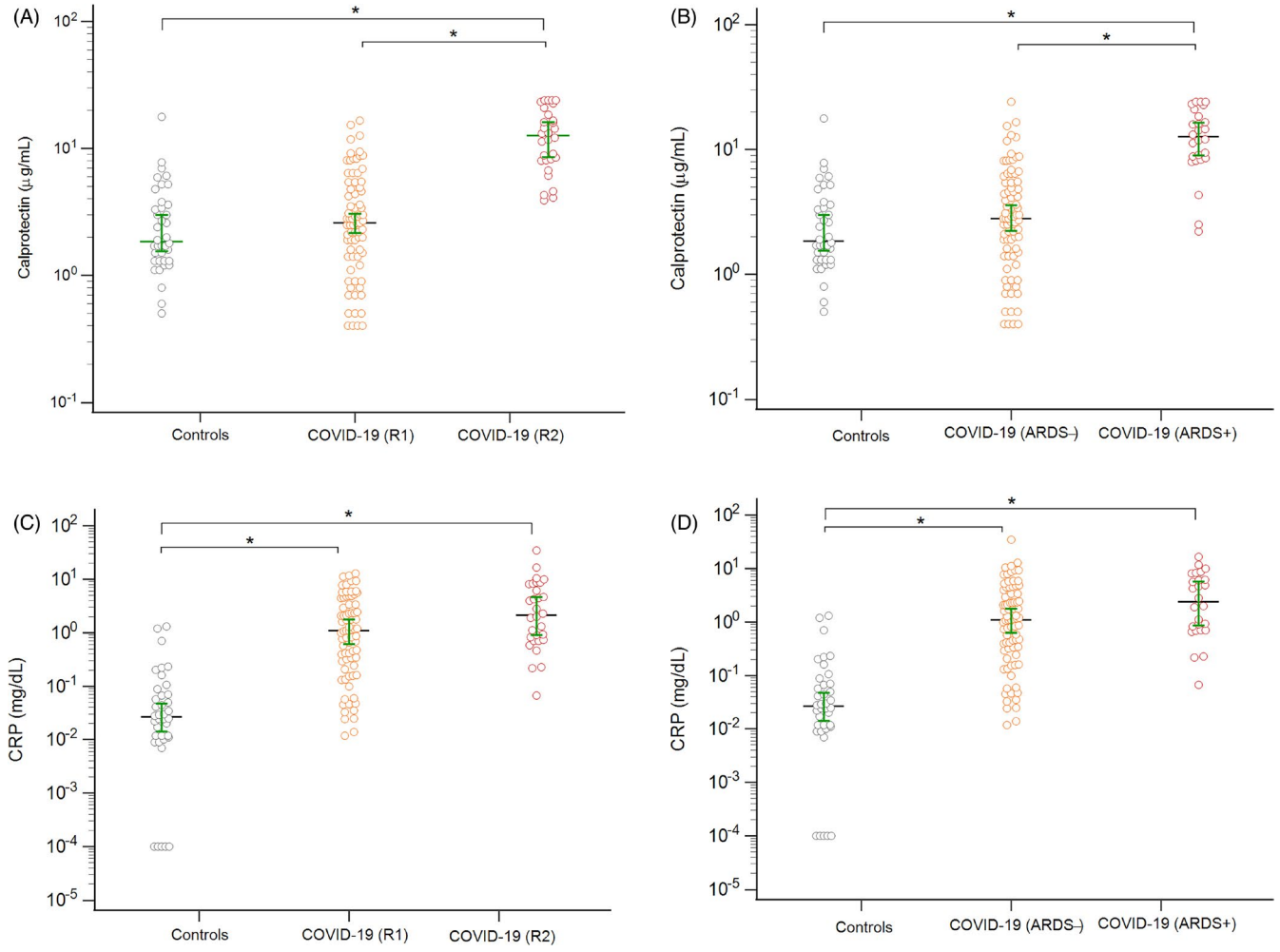

FIGURE 2 Levels of serum calprotectin and CRP by stratified groups of COVID-19 patients. Group R1 ($N = 75$) included patients who were breathing in room air and patients requiring low flow oxygen therapy (flow rates < 10 L/min). Group R2 ($N = 30$) included patients requiring high flow oxygen therapy (flow rates ≥ 10 L/min), mechanical ventilation, or extracorporeal membrane oxygenation. In addition, COVID-19 patients were stratified into two groups by the presence or absence of ARDS during hospitalization, including ARDS− ($N = 79$) and ARDS+ ($N = 26$) groups. (A) Serum calprotectin levels by stratified groups of oxygen therapy (R1 versus R2). (B) Serum calprotectin levels by stratified groups of lung involvement (ARDS− versus ARDS+). (C) Serum CRP levels by stratified groups of oxygen therapy (R1 versus R2). (D) Serum CRP levels by stratified groups of lung involvement (ARDS− versus ARDS+). Levels of calprotectin and CRP were compared by Mann–Whitney test (independent samples); $^*p < 0.05$. CRP, C-reactive protein; COVID-19, coronavirus disease; ARDS, acute respiratory distress syndrome

However, aCL and aβ2GPI results are not expressed in International Units because of the lack of an international reference standard; rather, they are expressed in arbitrary units according to the calibration curve used in the method.[13] The Scientific and Standardization Committee (SSC) on lupus anticoagulant/antiphospholipid antibodies (SSC-aPL) of the International Society of Thrombosis and Haemostasis (ISTH) states that the nonparametric 99th percentile cutoff appears to be more specific than the cutoff value for >40 GPL. Additionally, it recommends that only medium and high aPL levels are included as diagnostic criteria for APS.[14]

In this study, positive cases of aCL IgM (≥11 MPL) by ELISA in COVID-19 patients mostly had low to medium titers; however, MFIA revealed that they majorly had below the cutoff values for aCL IgM. Therefore, we concluded that they did not meet the laboratory criteria (medium or high titer of aCL IgG/IgM or aβ2GPI IgG/IgM [i.e., ≥40 GPL/MPL]) for the diagnosis of APS. According to the manufacturer's information, the cutoff value (11 MPL) of aCL IgM for ELISA was derived from the 95th percentile of the sera of 94 healthy blood donors, while aCL IgM for MFIA (20 U/ml) was derived from the 99th percentile using the sera of 300 blood bank donors. The number of 120 which SSC-aPL of ISTH recommends as an optimal sample size for calculating 99th percentile[14] is actually derived from the number needed to determine the 90% CIs of the 2.5th and 97.5th percentiles of a population using nonparametric statistics,[13] whereas the minimum sample size required for reliably estimating the 99th percentile is at least 300.[15]

Additionally, we found no significant difference in the prevalence of "non-criteria aPL," including that between aCL IgA and aβ2GPI IgA, as well as aPS/PT IgG/IgM, in COVID-19 patients. Thus, our research determined an extremely low overall prevalence of aPL in COVID-19

TABLE 4 Serum calprotectin and CRP levels in median (IQR) by stratified groups of COVID-19 patients

	By oxygenation status		By lung involvement		
	R1 (*N* = 75)	R2 (*N* = 30)	ARDS− (*N* = 79)	ARDS+ (*N* = 26)	Control (*N* = 38)
Calprotectin (μg/mL)	2.60 (1.40–5.28)	12.60 (8.10–18.50)	2.80 (1.43–5.48)	12.65 (8.50–18.50)	1.85 (1.30–3.60)
CRP (mg/dl)	1.01 (0.25–3.41)	2.13 (0.73–8.05)	1.10 (0.30–3.41)	2.40 (0.71–6.10)	0.03 (0.01–0.07)

Note: Group R1 included patients who were breathing in room air and patients requiring low flow oxygen therapy (flow rates < 10 L/min). Group R2 included patients requiring high flow oxygen therapy (flow rates ≥ 10 L/min), mechanical ventilation, or extracorporeal membrane oxygenation. In addition, the COVID-19 patients were stratified into two groups by the presence or absence of ARDS during hospitalization.

Abbreviations: ARDS, acute respiratory distress syndrome; COVID-19, coronavirus disease; CRP, C-reactive protein; IQR, interquartile range.

TABLE 5 Correlation of calprotectin with other laboratory parameters

	Laboratory parameters	*r*	95% CI	*p**	*n*
Calprotectin (log) versus	CRP (log)	0.5294	0.3759 to 0.6546	<0.001	105
	Peak CRP (log)	0.7199	0.6115 to 0.8017	<0.001	105
	Peak ferritin (log)	0.5056	0.3467 to 0.6362	<0.001	104
	Peak PCT (log)	0.4397	0.2691 to 0.5836	0.001	103
	Peak fibrinogen	0.1980	−0.01453 to 0.3933	0.068	95
	Peak D-dimer (log)	0.2722	0.0855 to 0.4409	0.005	105
	Peak BUN (log)	0.4625	0.3480 to 0.6382	<0.001	103
	Neutrophil count (log)	0.3697	0.1916 to 0.5242	<0.001	105
	Lymphocyte count (log)	−0.3838	−0.5403 to −0.2016	<0.001	99
	NLR (log)	0.4827	0.3154 to 0.6210	<0.001	99
	$CD4^+$ T cell count (log)	−0.3501	−0.5241 to −0.1480	0.001	85
	Minimum eGFR (Log)	−0.2517	−0.435 to −0.0758	0.010	85
	Minimum platelet (Log)	−0.2315	−0.4092 to −0.0445	0.019	103

Note: The highest results of CRP, ferritin, PCT, fibrinogen, D-dimer, and BUN for each patient during hospital admission were defined as the peak levels; in contrast, the lowest values of eGFR and platelet count were defined as minimum levels.

Abbreviations: BUN, blood urea nitrogen; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; NLR, neutrophil-to-lymphocyte ratio; PCT, procalcitonin.

"*n*" represents specific patients with available data.

*by Spearman's correlation.

patients, and these results are in accordance with several other recent observations.[3] In the past few decades, common bacterial and viral infections have been implicated in the induction of APS. Many infections are accompanied by transient aPL; with aCL (usually the IgM isotype) being the most frequent during infections.[16,17] In some cases, both aPL and clinical manifestations resembling those of APS are triggered, while in others, aPL often does not have any pathological role.[3] Specifically, regarding aPL associated with COVID-19, several reports suggested a possible role of aPL in COVID-19-induced thrombotic complications.[18,19] However, some researchers have reported that aPL is not elevated in patients with severe COVID-19 and is poorly associated with thrombotic events or suggested a different epitope specificity from the antibodies in APS.[9]

Neutrophils have recently received attention as key perpetuators of arterial, venous, and microvascular thrombosis. Neutrophils and neutrophil extracellular trap (NET) formation have only recently been investigated, while many studies of general thrombosis research have revealed that activated neutrophils, particularly NET formation, contribute to the propagation of thrombi.[20] A relatively recent discovery in APS pathogenesis relates to the implication of NET in thrombin formation and the initiation of inflammatory cascades.[21,22] Regarding COVID-19, several investigators have recently demonstrated significantly elevated levels of NETs in COVID-19-induced ARDS and suggested that NETs negatively influence COVID-19 outcomes.[23] Some researchers have also suggested NETs as therapeutic targets in COVID-19.[24]

Calprotectin, which accounts for 45% of the cytoplasmic proteins in neutrophils, is released during a specific form of holocrine secretion referred to as NETosis[25] and has been found to bind to NETs.[26] Although fecal calprotectin measurement represents a well-established and reliable biomarker for the diagnosis of inflammatory bowel disease, the role of serum calprotectin in the pathogenesis of diseases is not well established.[27] Serum calprotectin has recently gained attention as a marker of disease activity and a predictor of response to methotrexate in rheumatoid arthritis.[28] High levels of serum calprotectin have also been found in many types of infectious and inflammatory diseases, including bacterial sepsis,[29] lupus,[30] acute respiratory infections,[31] community-acquired

TABLE 6 Laboratory parameters in median (IQR) by stratified groups of COVID-19 patients

	By oxygen therapy					By lung involvement				
Parameters	**R1 (*N* = 75)**	***n***	**R2 (*N* = 30)**	***n***	***p****	**ARDS− (*N* = 79)**	***n***	**ARDS+ (*N* = 26)**	***n***	***p****
Peak CRP (mg/dl)	1.70 (0.30–4.73)	75	10.30 (5.35–14.05)	28	<0.001	1.70 (0.30–4.75)	77	10.10 (5.80–14.70)	26	<0.001
Peak ferritin (ng/ml)	345.0 (127.0–751.0)	74	1164.0 (518.5–1684.0)	29	<0.001	345.0 (178.2–802.5)	79	1000.0 (645.8–1421.4)	25	0.001
Peak PCT (ng/mL)	0.06 (0.04–0.08)	74	0.30 (0.07–0.46)	29	<0.001	0.06 (0.04–0.09)	77	0.16 (0.07–0.39)	26	0.001
Peak D-dimer (μg/ml)	0.70 (0.43–1.80)	75	1.44 (0.83–2.38)	30	0.003	0.84 (0.44–1.90)	75	1.15 (0.69–1.89)	30	0.086
Peak BUN (mg/dl)	11.9 (19.7–21.4)	75	23.1 (16.3–38.9)	28	<0.001	12.4 (9.9–22.6)	77	24.8 (13.5–36.2)	26	<0.001
Peak fibrinogen (mg/dl)	424.0 (305.8–500.3)	69	470.0 (367.0–555.0)	17	0.313	426.0 (314.0–496.0)	72	478.0 (297.0–631.0)	14	0.413
Lymphocyte count (10^9/L)	1.09 (0.77–1.56)	75	0.59 (0.38–0.80)	24	0.001	1.10 (0.70–1.52)	77	0.59 (0.49–0.76)	22	<0.001
Neutrophil count (10^9/L)	3.69 (2.43–4.86)	75	5.78 (3.45–10.74)	30	0.001	3.68 (2.38–4.83)	79	6.25 (3.86–11.07)	26	<0.001
NLR	3.20 (1.93–5.50)	75	9.45 (4.45–15.60)	24	<0.001	3.10 (1.90–4.90)	77	11.25 (6.40–20.30)	22	<0.001
$CD4^+$ T cell count (10^9/L)	0.44 (0.26–0.67)	71	0.23 (0.14–0.33)	14	0.001	0.44 (0.26–0.65)	73	0.23 (0.15–0.28)	12	<0.005
Minimum eGFR (L/min/1.73 m^2)	87.0 (66.8–107.8)	75	81.5 (56.0–100.0)	28	0.197	87.0 (72.8–108.0)	77	82.0 (48.0–103.0)	26	0.239
Minimum platelet count (10^9/L)	201.0 (154.3–239.8)	75	175.0 (123.0–233.0)	28	0.145	201.0 (154.5–243.3)	77	175.0 (145.0–220.0)	26	0.132

Note: Data are presented as median (IQR). "*N*" represents the total number of stratified patients, whereas "*n*" represents specific patients with available data. For each patient, the highest levels of CRP, ferritin, PCT, D-dimer, BUN, and fibrinogen were defined as the peak levels; in contrast, the lowest values of eGFR and platelet count were defined as minimum levels.

Abbreviations: ARDS, acute respiratory distress syndrome; BUN, blood urea nitrogen; COVID-19, coronavirus disease; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; IQR, interquartile range; NLR, neutrophil-to-lymphocyte ratio; PCT, procalcitonin.

*by Mann–Whitney test (independent samples).

TABLE 7 The discriminatory performance of laboratory parameters by stratified groups of COVID-19 patients

	Between R1 and R2 groups					Between ARDS− and ARDS+ groups					
	ROC					ROC					
Parameters	AUC (95% CI)	*p*	Sensitivity (%)	Specificity (%)	Optimal cutoff (Youden)[a]	AUC (95% CI)	*p*	Sensitivity (%)	Specificity (%)	Optimal cutoff (Youden)[a]	*n*
Calprotectin	0.91 (0.84–0.96)	<0.001	100.0	68.0	3.6 μg/ml	0.89 (0.82–0.94)	<0.001	88.5	88.3	6.9 μg/ml	105
CRP	0.65 (0.55–0.74)	0.011	90.0	36.0	0.46 mg/dl	0.64 (0.54–0.73)	0.022	88.5	39.2	0.59 mg/dl	105
Peak CRP	0.88 (0.81–0.94)	<0.001	71.4	89.3	7.5 mg/dl	0.91 (0.84–0.96)	<0.001	72.7	100.0	3.6 mg/dl	103
Peak ferritin	0.80 (0.71–0.87)	<0.001	72.4	77.3	779 ng/ml	0.77 (0.68–0.85)	<0.001	80.0	67.1	445.0 ng/ml	104
Peak PCT	0.78 (0.68–0.85)	<0.001	62.1	83.8	0.10 ng/ml	0.71 (0.62–0.80)	0.001	85.7	61.5	0.12 ng/ml	103
Peak BUN	0.77 (0.68–0.85)	<0.001	92.9	54.7	12.6 mg/dl	0.72 (0.68–0.85)	0.001	88.5	51.9	12.6 mg/dl	103
Peak D-dimer	0.68 (0.60–0.77)	<0.001	93.3	44.0	0.55 μg/ml	0.61 (0.51–0.71)	0.042	40.5	92.3	0.54 μg/ml	105
Neutrophil count	0.70 (0.61–079)	0.002	56.7	85.3	5.53×10^9/L	0.77 (0.68–0.85)	<0.001	82.3	65.4	5.24×10^9/L	105
NLR	0.79 (0.69–0.86)	<0.001	75.0	74.7	4.9	0.86 (0.88–0.92)	<0.001	77.9	86.5	5.0	99
Lymphocyte count	0.77 (0.68–0.85)	<0.001	75.0	78.7	0.69×10^9/L	0.77 (0.68–0.85)	<0.001	74.0	77.4	0.76×10^9/L	99
$CD4^+$ T cell count	0.77 (0.67–0.86)	<0.001	92.9	56.3	0.39×10^9/L	0.81 (0.71–0.89)	<0.001	71.2	91.7	0.29×10^9/L	85

Note: For each patient, the highest levels of CRP, ferritin, PCT, BUN, and D-dimer were defined as the peak levels.

"*n*" represents specific patients with available data.

Abbreviations: ARDS, acute respiratory distress syndrome; AUC, area under curve; BUN, blood urea nitrogen; COVID-19, coronavirus disease; CRP, C-reactive protein; NLR, neutrophil-to-lymphocyte ratio; PCT, procalcitonin; ROC, receiver operating characteristic curve.

[a]For the calculation, the Youden index-derived cutoff for each biomarker was used.

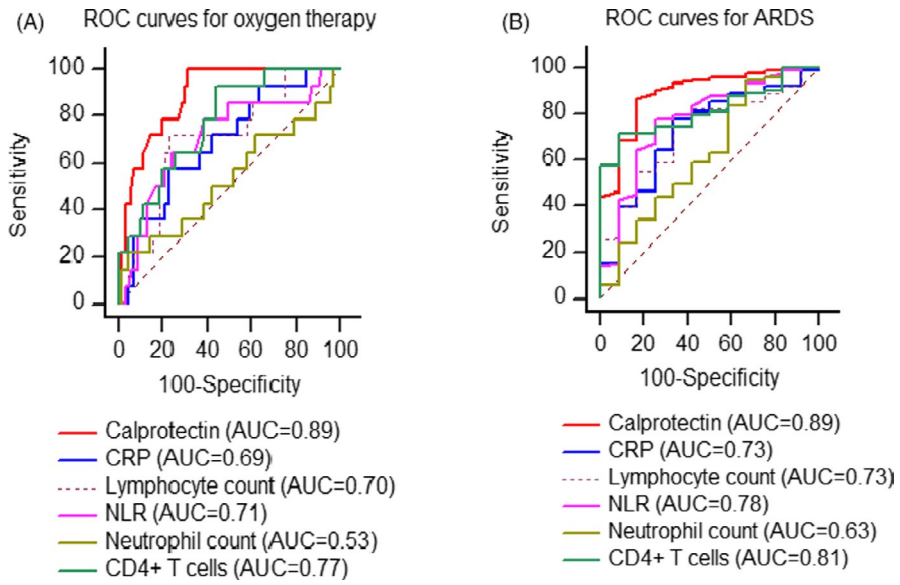

FIGURE 3 Multiple comparison of receiver operating characteristic (ROC) curve evaluation for the performance of laboratory parameters. Calprotectin and CRP levels were measured in this research. Lymphocyte count, neutrophil count, NLR, and $CD4^+$ T cells count were obtained from the hospital's electronic medical records, in which the data were selected within 48 h from the research sample collection time ($n = 85$). (A) ROC curves for discriminating between R1 and R2 groups. (B) ROC curves for discriminating between ARDS− and ARDS+ groups. CRP, C-reactive protein; NLR, neutrophil-to-lymphocyte ratio; ARDS, acute respiratory distress syndrome

TABLE 8 Multiple comparison of discriminatory performance between laboratory parameters by stratified groups of COVID-19 patients ($n = 85$)

	Between R1 and R2 groups			Between ARDS− and ARDS+ groups		
	ROC			ROC		
Parameters	AUC (95% CI)	Difference between AUC of calprotectin (95% CI)	*p*	AUC (95% CI)	Difference between AUC of calprotectin (95% CI)	*p*
Calprotectin	0.89 (0.80–0.95)	–	–	0.89 (0.80–0.95)	–	–
CRP	0.69 (0.58–0.78)	0.21 (0.07–0.34)	0.002	0.73 (0.63–0.82)	0.17 (0.03–0.30)	0.019
Lymphocyte count	0.70 (0.61–0.81)	0.19 (0.02–0.34)	0.021	0.73 (0.62–0.82)	0.16 (0.00–0.32)	0.045
Neutrophil count	0.53 (0.42–0.64)	0.36 (0.18–0.55)	<0.001	0.63 (0.52–0.73)	0.26 (0.08–0.44)	0.004
NLR	0.71 (0.60–0.80)	0.18 (0.02–0.34)	0.025	0.78 (0.67–0.86)	0.12 (−0.03–0.26)	0.129
$CD4^+$ T cells	0.77 (0.67–0.86)	0.12 (−0.01–0.25)	0.076	0.81 (0.71–0.89)	0.08 (0.04–0.19)	0.190

Abbreviations: ARDS, acute respiratory distress syndrome; AUC, area under curve; CI, confidence interval; COVID-19, coronavirus disease; CRP, C-reactive protein; NLR, neutrophil-to-lymphocyte ratio; ROC, receiver operating characteristic curve.

pneumonia,[32] and idiopathic pulmonary fibrosis,[33] wherein it correlates closely with disease severity. In addition to serving as an inflammatory biomarker, calprotectin may play a direct role in the self-amplifying thrombo-inflammatory storm that afflicts many COVID-19 patients.[5]

Our research showed that calprotectin levels positively correlated with several other biomarkers of inflammation, including neutrophil count, NLR, peak levels of CRP, PCT, ferritin, and D-dimer, but negatively correlated with lymphocyte count and $CD4^+$ lymphocyte count. Among the inflammatory biomarkers, calprotectin, peak CRP, and NLR were especially superior for discriminating severe clinical cases of COVID-19. These findings are consistent with those of other studies. Lymphopenia has been considered a cardinal laboratory finding with prognostic potential in COVID-19.[34] NLR has been reported to have prognostic value in determining severe cases.[35] During the disease course, the longitudinal evaluation of inflammatory indices revealed CRP, PCT, and ferritin as poor prognostic factors and may identify cases with poor prognosis to enable prompt intervention and improved outcomes.[1,36]

To estimate the severity of COVID-19, variable methods were developed and researched; scoring systems (such as sequential organ failure assessment [SOFA] score, a modified version thereof [qSOFA], and COVID-GRAM score), as well as classification based on clinical parameters (such as systolic blood pressure, multi-lobar chest radiography involvement, albumin level, respiratory rate, tachycardia, confusion, oxygenation status, mechanical ventilation,

and the presence of ARDS) or final outcomes (intensive care unit admission or death), have been used by various studies.[5] In this research, we first stratified COVID-19 patients based on severity into two groups by the degree of oxygen therapy, and such classification has been used in previous studies[37,38] that reported elevated plasma calprotectin level to discriminate severe from mild COVID-19 cases. Additionally, we stratified the severity of COVID-19 patients into two groups based on the presence or absence of ARDS, and this classification is similar to that used in the research by Ma et al.[39] that reported NLR as a predictive biomarker of moderate to severe ARDS in COVID-19 patients.

Due to the retrospective nature of our study design, there are some limitations to this study. First, because of the unavailability of longitudinal sample collection, aPL and calprotectin were measured using samples collected at a single point in time. Similarly, we could not arrange the time of sample collection evenly between samples. Although a considerable proportion of samples were collected in the early phase of COVID-19 illness after hospital admission, the sampling days from disease onset still varied among patients (5.7 ± 3.6 days from the first symptom onset) (Table 1). By measuring the longitudinally collected serial samples, the kinetics and exact performance of a new biomarker, calprotectin, could be clearly elucidated. Second, we could not validate the manufacturer's cutoff value for serum calprotectin, nor established a locally derived cutoff. The calprotectin levels of a considerable proportion of the healthy controls (36.8% [14/38]) were elevated above the manufacturer's cutoff value (≥2.9 μg/ml); however, most of the cases were distributed to a mild degree of elevation; the median (IQR) calprotectin level was 1.85 (1.30–3.60) μg/ml. Similarly, slight elevation in serum calprotectin levels related to pre-analytical problems in healthy controls cannot be completely excluded. Pre-analytical issues in calprotectin measurement have been suggested by several researchers, with some proposing that neutrophil activation, by either clotting or centrifugation, should be avoided during the pre-analytical process.[40] In serum, calprotectin levels have been shown to increase over time, reaching a maximum overestimation of 5–10 h.[41] Furthermore, there is a report suggesting distinct exercise intensity-dependent changes in calprotectin following extreme physical exertion.[42] Pre-analytical standardization in sample collection and processing, as well as proper validation of reference intervals, would be mandatory in future research.

In this study, representative data for conventionally used parameters were selected based on their peak levels (CRP, ferritin, PCT, and D-dimer) or minimal levels (platelet count and eGFR), with multiple measurements during each patient's hospital admission. Although serum calprotectin levels were measured at one point in sera collected during the relatively early phase of COVID-19 illness, calprotectin's performance surpassed other parameters' performances in predicting COVID-19 severity. Therefore, we suggest that serum calprotectin is a potentially valuable biomarker for predicting the clinical severity of COVID-19, independently or in combination with other conventional parameters. Nevertheless, further studies using multivariate analyses will be needed to identify independent predictors of severe disease and define the best combination of markers to facilitate risk stratification and management of COVID-19 cases.

In summary, the prevalence of clinically significant aPL among COVID-19 patients was low and was not associated with clinical severity. We observed significantly elevated levels of serum calprotectin in severe cases of hospitalized COVID-19 patients, requiring more intensive oxygen therapy. Our research suggests strong evidence supporting that neutrophils are potentially involved in severe COVID-19 cases. The link between calprotectin and the inflammatory pathway in COVID-19 may open new opportunities to improve its management and outcomes.

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