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OBSTETRICS

Differences and similarities in endothelial and angiogenic profiles of preeclampsia and COVID-19 in pregnancy

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Marta Palomo, Msc, PhD; Lina Youssef, MD, PhD; Alex Ramos, BS; Sergi Torramade-Moix, Msc;
Ana Belen Moreno-Castaño, MD; Julia Martinez-Sanchez, Msc; Laura Bonastre, BS; Marc Pino, BS;
Pilar Gomez-Ramirez, BS; Lidia Martin, BS; Estefania Garcia Mateos, BS; Pablo Sanchez, PhD; Sara Fernandez, MD;
Francesca Crovetto, MD, PhD; Ginés Escolar, MD, PhD; Enric Carreras, MD, PhD; Pedro Castro, PhD;
Eduard Gratacos, MD, PhD; Fàtima Crispi, MD, PhD; Maribel Diaz-Ricart, PhD

BACKGROUND: COVID-19 presents a spectrum of signs and symptoms in pregnant women that might resemble preeclampsia. Differentiation between severe COVID-19 and preeclampsia is difficult in some cases.

OBJECTIVE: To study biomarkers of endothelial damage, coagulation, innate immune response, and angiogenesis in preeclampsia and COVID-19 in pregnancy in addition to in vitro alterations in endothelial cells exposed to sera from pregnant women with preeclampsia and COVID-19.

STUDY DESIGN: Plasma and sera samples were obtained from pregnant women with COVID-19 infection classified into mild (n=10) or severe (n=9) and from women with normotensive pregnancies as controls (n=10) and patients with preeclampsia (n=13). A panel of plasmatic biomarkers was assessed, including vascular cell adhesion molecule-1, soluble tumor necrosis factor-receptor I, heparan sulfate, von Willebrand factor antigen (activity and multimeric pattern), α2-antiplasmin, C5b9, neutrophil extracellular traps, placental growth factor, soluble fms-like tyrosine kinase-1, and angiopoietin 2. In addition, microvascular endothelial cells were exposed to patients' sera, and changes in the cell expression of intercellular adhesion molecule 1 on cell membranes and von Willebrand factor release to the extracellular matrix were evaluated through immunofluorescence. Changes in inflammation cell signaling pathways were also assessed by of p38 mitogen-activated protein kinase phosphorylation. Statistical analysis included univariate and multivariate methods.

RESULTS: Biomarker profiles of patients with mild COVID-19 were similar to those of controls. Both preeclampsia and severe COVID-19 showed significant alterations in most circulating biomarkers with distinctive profiles. Whereas severe COVID-19 exhibited higher concentrations of vascular cell adhesion molecule-1, soluble tumor necrosis factor-α receptor I, heparan sulfate, von Willebrand factor antigen, and neutrophil extracellular traps, with a significant reduction of placental growth factor compared with controls, preeclampsia presented a marked increase in vascular cell adhesion molecule-1 and soluble tumor necrosis factor-α receptor I (significantly increased compared with controls and patients with severe COVID-19), with a striking reduction in von Willebrand factor antigen, von Willebrand factor

activity, and α2-antiplasmin. As expected, reduced placental growth factor, increased soluble fms-like tyrosine kinase-1 and angiopoietin 2, and a very high soluble fms-like tyrosine kinase-1 to placental growth factor ratio were also observed in preeclampsia. In addition, a significant increase in C5b9 and neutrophil extracellular traps was also detected in preeclampsia compared with controls. Principal component analysis demonstrated a clear separation between patients with preeclampsia and the other groups (first and second components explained 42.2% and 13.5% of the variance), mainly differentiated by variables related to von Willebrand factor, soluble tumor necrosis factor-receptor I, heparan sulfate, and soluble fms-like tyrosine kinase-1. Von Willebrand factor multimeric analysis revealed the absence of von Willebrand factor high-molecular-weight multimers in preeclampsia (similar profile to von Willebrand disease type 2A), whereas in healthy pregnancies and COVID-19 patients, von Willebrand factor multimeric pattern was normal.

Sera from both preeclampsia and severe COVID-19 patients induced an overexpression of intercellular adhesion molecule 1 and von Willebrand factor in endothelial cells in culture compared with controls. However, the effect of preeclampsia was less pronounced than the that of severe COVID-19. Immunoblots of lysates from endothelial cells exposed to mild and severe COVID-19 and preeclampsia sera showed an increase in p38 mitogen-activated protein kinase phosphorylation. Patients with severe COVID-19 and preeclampsia were statistically different from controls, suggesting that both severe COVID-19 and preeclampsia sera can activate inflammatory signaling pathways.

CONCLUSION: Although similar in in vitro endothelial dysfunction, preeclampsia and severe COVID-19 exhibit distinctive profiles of circulating biomarkers related to endothelial damage, coagulopathy, and angiogenic imbalance that could aid in the differential diagnosis of these entities.

Key words: angiogenic factors, angiopoietin, C5b9, COVID-19, endothelial dysfunction, heparan sulfate, hypertensive disorders of pregnancy, neutrophil extracellular traps, placental growth factor, preeclampsia, SARS-CoV-2, soluble fms-like tyrosine kinase-1, soluble tumor necrosis factor-α receptor I, von Willebrand factor

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Introduction

Preeclampsia is a pregnancy complication and a leading cause of maternal and perinatal morbimortality and iatrogenic prematurity.[1–3] Although its etiology is not completely understood,[4,5] it is accepted that this condition relies on placental insufficiency and maternal cardiovascular maladaptation underlined by angiogenic imbalance, endothelial dysfunction, coagulopathy, and complement dysregulation,[6–8] which lead clinically to hypertension and proteinuria that can progress to multiorgan dysfunction during pregnancy. The multifactorial nature of preeclampsia explains a variable clinical/laboratory presentation, mainly determined by gestational age at onset (early vs late).

Clinical and analytical data from patients infected by SARS-CoV-2, which causes COVID-19, suggest that

AJOG at a Glance

Why was this study conducted?
We conducted this study to characterize the profile of endothelial damage, coagulation, innate immune response, and angiogenesis in preeclampsia and COVID-19 in pregnancy, which are both considered disorders associated with endothelial dysfunction.

Key findings
Severe COVID-19 in pregnancy and preeclampsia share a similar end-stage in vitro-induced p38 mitogen-activated protein kinase phosphorylation in endothelial cells but a differential profile of circulating endothelial and angiogenic biomarkers. Severe COVID-19 is characterized by higher vascular cell adhesion molecule-1 (VCAM-1), soluble tumor necrosis factor-α receptor I (sTNFRI), heparan sulfate (HS), von Willebrand factor (VWF) antigen, and neutrophil extracellular traps (NETs) and reduced placental growth factor (PlGF), whereas preeclampsia is marked by increased VCAM-1, sTNFRI, soluble fms-like tyrosine kinase-1 (sFlt-1), angiopoietin-2, C5b9, and NETs and a reduction in VWF antigen, VWF activity, α2-antiplasmin, and PlGF.

What does this add to what is known?
Soluble biomarkers of coagulopathy (VWF), endothelial inflammation (sTNFRI), barrier damage (HS), and angiogenesis (sFlt-1) seem to be highly specific in differentiating preeclampsia from severe COVID-19 in pregnancy. These findings improve our understanding of the pathophysiological pathways in preeclampsia and COVID-19 and may help in the differential diagnosis of these disorders during pregnancy.

endothelial dysfunction plays an important role in the pathophysiology of this condition,[9−12] involving extrapulmonary manifestations of COVID-19 like hypertension, kidney disease, thrombocytopenia, and liver injury. Some of these clinical features overlap with those observed in preeclampsia. In addition, an increased incidence of preeclampsia has been reported in association with COVID-19.[13−15] Despite their clinical resemblance, the mechanisms underlying endothelial dysfunction might differ between COVID-19 and preeclampsia. Understanding endothelial and angiogenic profiles could enlighten the pathophysiological basis of these 2 entities.

The endothelium is a monolayer of cells that lines the interior of blood vessels, acting as a protective layer between circulating blood and other tissues. The endothelium is crucial for the regulation of vascular homeostasis, coagulation cascade, immune response, and angiogenesis. Circulating biomarkers related to endothelial activation and loss of barrier integrity seem to be associated with disease severity in COVID-19.[12,16] Inflammatory effects on these damaged endothelial cells activate the innate immune response and induce a hypercoagulable state with impaired fibrinolysis and angiogenic imbalance.[17,18] On the contrary, angiogenesis dysregulation has emerged as 1 of the main pathophysiological features in the development of preeclampsia.[19,20] Finally, in vitro studies enabled us to describe the endothelial cell proinflammatory and thrombogenic response in COVID-19.[21−23]

The aim of the present study was to comprehensively investigate the endothelial and angiogenic profiles in preeclampsia and SARS-CoV-2 infection in pregnancy using circulating biomarkers and in vitro studies.

Materials and Methods
Study populations and design
Pregnant women with laboratory-confirmed SARS-CoV-2 infection were selected from a large multicenter prospective population-based cohort study conducted from March 15 to May 31, 2020, in Barcelona, Spain, including consecutive cases detected during the study period.[24] SARS-CoV-2 infection was confirmed by a positive real-time polymerase chain reaction (RT-PCR) on nasopharyngeal swab or a positive serologic result. SARS-CoV-2−positive pregnancies were subdivided into mild (n=9) and severe disease (n=8) according to the presence of pneumonia or coexistence of fever, dry cough, and dyspnea. In addition, we also included SARS-CoV-2−negative pregnant women, including preeclampsia (n=13) and normotensive pregnancies as controls (n=10) who were matched to COVID-19 cases by gestational age at blood sampling. Preeclampsia was defined as high blood pressure (systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg on 2 occasions, at least 4 hours apart) developed after 20 weeks of gestation with proteinuria (≥300 mg/24 h or protein/creatinine ratio ≥0.3), thrombocytopenia (platelet count <100 × 10^9/L), renal insufficiency (serum creatinine concentrations >1.1 mg/dL), impaired liver function (elevated blood concentrations of liver transaminases to twice-normal concentration), pulmonary edema, or a new-onset headache unresponsive to medication and not accounted for by alternative diagnoses or visual symptoms.[25] Early-onset preeclampsia was defined by gestational age at delivery <34 weeks.[26] Baseline and perinatal data were obtained by interviews and from electronic medical records. Gestational age was calculated on the basis of the crown-rump length at first-trimester ultrasound.[27] Birthweight centiles were assigned according to local standards.[28] Pregnancies with chromosomal/structural anomalies or intrauterine infection were excluded. Endothelial and angiogenic profiles were studied in all participants by analyzing circulating molecules in maternal peripheral blood and by in vitro study of endothelial cells exposed to patients' sera. Details of the laboratory methodology used are included in the Supplemental Material.

This study was approved by the ethics committee of the Hospital Clínic de

Barcelona (HCB/2020/0401) and conformed to the ethical guidelines of the Declaration of Helsinki. All participants provided informed written consent before sample collection.

Maternal blood sample collection

Peripheral maternal blood was obtained by venipuncture within 24 to 48 hours after onset of symptoms and before starting any treatment. Plasma and sera samples were obtained by centrifugation of blood anticoagulated with ethylenediaminetetraacetic acid and by incubation for 30 minutes at room temperature to allow clotting, and subsequently centrifuged at $1500\times$ g for 10 minutes at 4°C to separate the serum from clots, respectively. All samples were aliquoted and stored at -80°C until used.

Assessment of circulating biomarkers

Endothelial damage was assessed by measuring plasmatic concentrations of vascular cell adhesion molecule-1 (VCAM-1), soluble tumor necrosis factor-α receptor I (sTNFRI), and heparan sulfate (HS) with enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Minneapolis, MN; Biomatik Corporation, Wilmington, DE; and AttendBio Research, Barcelona, Spain, respectively). The kit used for the detection of HS did not show any significant cross-reactivity or interference between HS and analogs according to the manufactureŕs instructions.

Biomarkers for coagulation/fibrinolysis included von Willebrand factor (VWF) antigen (VWF:Ag) and activity (VWF:GPIbM) and α2-antiplasmin (α2AP) evaluated by immunoturbidimetry (Atellica 180 360 COAG, Siemens Healthineers, Erlangen, Germany). Visualization of VWF multimers was achieved using a commercially available enhanced chemiluminescence kit for detecting horseradish peroxidase-labeled antibodies on Western blots.[29] In addition, VWF-cleaving protease (ADAMTS13) activity was assessed by fluorescence resonance energy transfer (Fluoroskan Ascent FL, Thermo Fisher Scientific, Waltham, MA). Plasminogen

activator inhibitor antigen (PAI) and thrombomodulin (TM) were measured by ELISA (Imubind, BioMedica Diagnostics, Windsor, Canada and Biomatik Corporation, respectively).

Activation of innate immune response was determined by circulating terminal complement complex (C5b9) and double-stranded DNA (dsDNA) for neutrophil extracellular traps (NETs) quantified by Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Thermo Fisher Scientific) on a fluorescence reader.

Angiogenic profile was assessed by sera concentrations of free placental growth factor (PlGF) and soluble fms-like tyrosine kinase-1 (sFlt-1) with ELISA (R&D Systems Europe Ltd, Abingdon, United Kingdom) and angiopoietin-2 (Ang2) ELISAs (R&D Systems, Minneapolis, MN). The sFlt-1 to PlGF ratio was calculated as previously described.[30]

In vitro studies

For the in vitro studies, human dermal microvascular endothelial cells (ATCC, CRL-3243, Lot: 62630587) in culture were exposed to patients' sera to study cell response to: (1) the expression of adhesion receptors at the cell surface (intercellular adhesion molecule 1 [ICAM-1]) as an indicator of a proinflammatory cell response; (2) the presence of the adhesive protein VWF, involved in thrombogenicity, on the extracellular matrix generated by these cells; and (3) the activation of the endothelial intracellular signaling pathway related to inflammation—p38 mitogen-activated protein kinase (p38 MAPK). Details of the laboratory methodology used are included in the Supplemental Material.

Statistical analysis

Baseline and perinatal data were analyzed with the statistical software STATA 14.2 (StataCorp LLC, College Station, TX) and results are expressed as median and interquartile range or percentage as appropriate. Statistical analysis comprised the comparison of each group of complicated pregnancies with controls. Soluble markers are expressed as median (interquartile range). Further

statistical analyses were performed in R version 4.0.0 (R Foundation for Statistical Computing, Vienna, Austria) using the Student t test with the Benjamini-Hochberg correction for multiple comparisons after checking data normality and homoscedasticity. Results were considered statistically significant when adjusted P value was $<.05$. Data were ordinated and plotted using principal component analysis. An additional unsupervised hierarchical clustering was performed on the basis of the univariate results comparing severe COVID-19 with preeclampsia. A subanalysis comparing early- with late-onset preeclampsia was performed using the Student t test and Benjamini-Hochberg procedure for multiple pairwise comparisons and included in the Supplemental Material.

Results
Baseline and perinatal characteristics of the study populations

Baseline characteristics of the study populations are summarized in the Table. Study groups were mainly similar in terms of maternal and perinatal characteristics. However, patients with preeclampsia had higher rates of Asian ethnicity and a tendency to younger age. Chronic hypertension was present in 2 patients with preeclampsia and systemic lupus erythematosus in 1 control. None of the patients included in this study had pregestational diabetes mellitus or previous respiratory disorders. All the pregnancies complicated by preeclampsia were proteinuric, 4 were early-onset cases that were treated with corticosteroids for fetal lung maturity, and 5 patients had preeclampsia with severe features that was treated with magnesium sulfate. Patients with preeclampsia showed an earlier gestational age at delivery, with a trend toward higher rates of small-for-gestational-age fetuses and admissions to the neonatal intensive care unit. Three cases of preeclampsia were complicated by peripartum hemorrhage. Severe COVID-19 cases were all detected by RT-PCR. Among the mild cases, 2 were detected by RT-PCR and the rest by positive serology. Given that this study

TABLE
Baseline and perinatal characteristics of the study populations

	Controls n=10	Mild COVID-19 n=9	Severe COVID-19 n=8	Preeclampsia n=13
Maternal characteristics				
Age (y)	36.9 (31.6—38.7)	36 (30.6—37.7)	35.2 (24.7—39.1)	29 (26—35.9)
Ethnicity				
White	8 (80)	5 (55.6)	4 (50)	4 (30.8)
African	0 (0)	0 (0)	1 (12.5)	2 (15.4)
Latin	2 (20)	3 (33.3)	2 (25)	2 (15.4)
Asian	0 (0)	1 (11.1)	1 (12.5)	5 (38.5)[a]
Pregestational body mass index (kg/m^2)	22.4 (21.1—25.6)	22.7 (20.3—28.7)	21.8 (21—23.9)	25.9 (21.9—28.4)
Nulliparity	7 (70)	5 (55.6)	2 (25)	7 (53.8)
Use of assisted reproductive technologies	2 (20)	0 (0)	0 (0)	0 (0)
Smoking during pregnancy	0 (0)	0 (0)	1 (12.5)	0 (0)
Perinatal outcomes				
Gestational age at delivery (weeks)	40.2 (38.9—41)	39.1 (38.7—39.6)	39.2 (38.3—41.1)	39.1 (35.1—39.6)[a]
Preterm delivery[b]	1 (10)	1 (11.1)	2 (25)	4 (30.8)
Cesarean delivery	1 (10)	3 (33.3)	2 (25)	5 (38.5)
Female gender	4 (40)	4 (44.4)	5 (62.5)	6 (46.1)
Birthweight (g)	2975 (2780—3220)	3280 (2940—3335)	3290 (2780—3670)	2558 (2010—3268)
Small-for-gestational age[c]	3 (30)	0 (0)	0 (0)	7 (53.8)
APGAR score at 5 min <7	0 (0)	0 (0)	1 (12.5)	1 (7.7)
Umbilical artery pH	7.21 (7.15—7.23)	7.18 (7.12—7.21)	7.17 (7.12—7.2)	7.22 (7.17—7.24)
Admission to neonatal intensive care unit	1 (10)	0 (0)	1 (12.5)	5 (38.5)

Data are median (interquartile range) or number (percentage) as appropriate.

[a] $P<.05$ by Mann—Whitney U, Pearson chi square, or Fisher exact tests as appropriate, compared with controls; [b] Preterm delivery defined as delivery occurring before 37 weeks of gestation; [c] Small-for-gestational-age defined as birthweight below the 10th centile according to local standards.

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was conducted at the beginning of the pandemic, convalescent subjects should have been infected during the 4 weeks preceding the blood analysis. Two cases of mild COVID-19 had hypertension, and 1 of them had associated proteinuria. None of the COVID-19 patients (mild or severe) had thrombocytopenia, elevated liver enzymes, or elevated creatinine. All COVID-19 cases were followed up to 40 days postpartum to exclude the diagnosis of evolving preeclampsia. The diagnosis of atypical preeclampsia in COVID-19 cases was excluded because none of them presented signs of placental insufficiency nor abnormal sFlt-1 to PlGF ratio (according to our institutional protocol for the differential diagnosis of hypertensive disorders in pregnancy). Severe COVID-19 cases were not critically ill (no mortality and only 1 case required invasive mechanical ventilation). Six patients with severe COVID-19 were treated with low-molecular-weight heparin, 3 of them were additionally treated with hydroxychloroquine and azithromycin, and 1 of these 3 was also given lopinavir/ritonavir and corticosteroids. As mentioned earlier, maternal blood samples were obtained before starting any treatment. Gestational age at sampling was similar between the study groups at a median (interquartile range) of 40.2 (38.9—41) weeks in controls, 39.1 (38.7—39.6) weeks in mild COVID-19 cases, 39.3 (34.9—41.1) weeks in severe COVID-19 cases, and 39.1 (35.1—39.6) weeks in preeclampsia cases. No cases of perinatal mortality were observed in the study population.

Endothelial and angiogenic circulating biomarkers are differentially altered in COVID-19 vs preeclampsia

Results on soluble biomarkers in the study populations are displayed in Figure 1 and Supplemental Table 1. Most soluble biomarkers were similar in mild COVID-19 and controls with the exception of a significant increase in VWF:Ag. In contrast, profound alterations in endothelial, coagulation,

Scattered boxplots showing the levels of soluble endothelial damage and immune response markers in the study populations
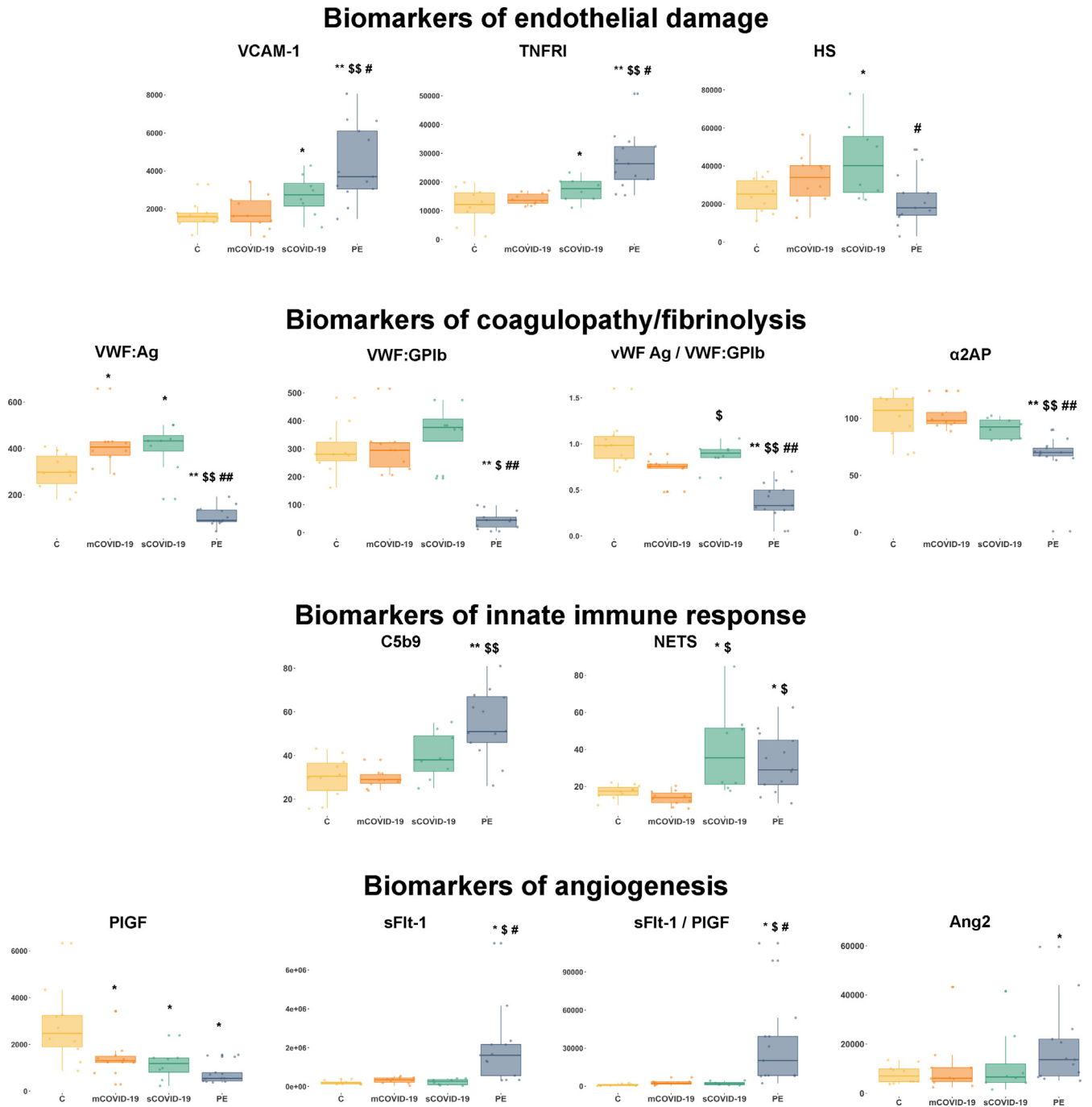

The *line in the boxes* depicts the sample median and the boxes are the first and third quartiles. The *whiskers* point to the maximum and the minimum values of the sample. For a better visualization of data point distribution and to show possible outliers, a second layer of information is included in the figure, with all data points scattered along the y axis. Significant differences of adjusted *P* values (Student *t* test, Benjamini-Hochberg procedure for multiple pairwise comparisons) are noted as *asterisk P<*.05 and *double asterisks P<*.01 vs controls, *dollar P<*.05 and *double dollar P<*.01 vs mild COVID-19, and *hashtag P<*.05 and *double hashtag P<*.01 vs severe COVID-19. Controls (C, n=10), mild COVID-19 (mcovid-19, n=9), severe COVID-19 (scovid-19, n=8), preeclampsia (PE, n=13).

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FIGURE 2
Analysis of the differential profile of soluble biomarkers among the study groups
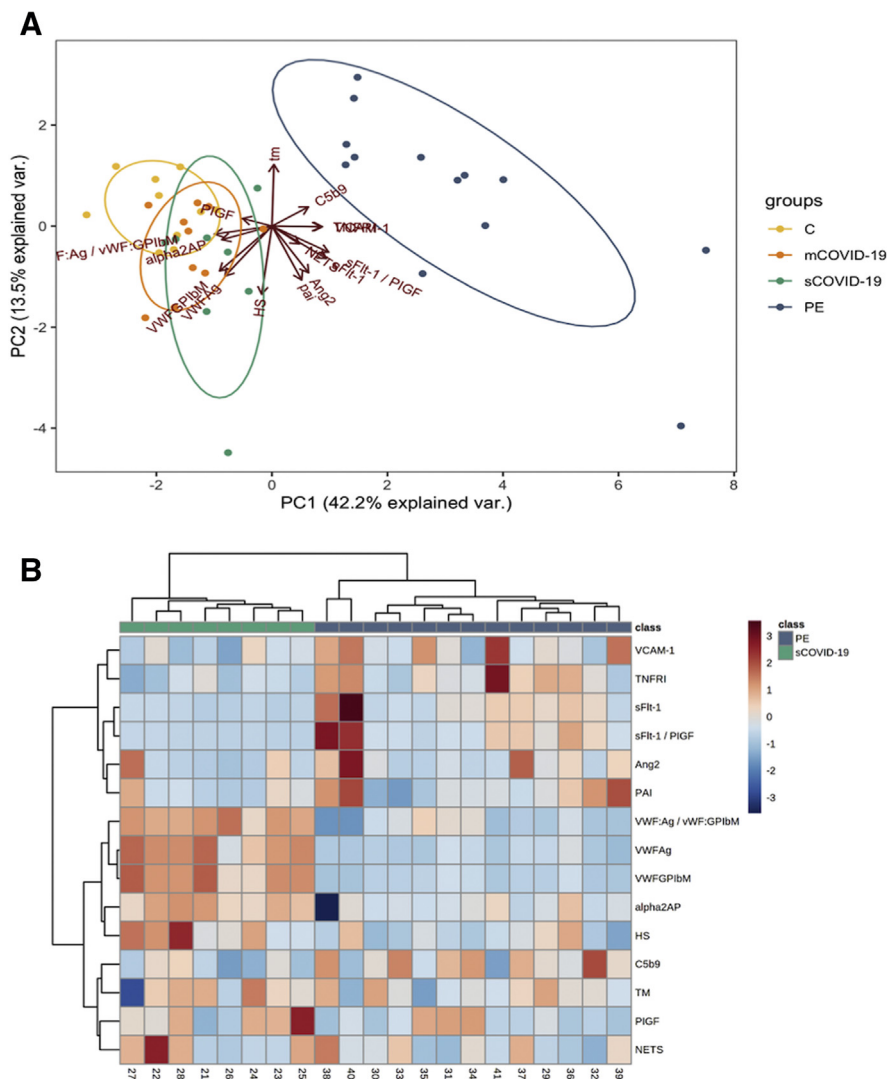
Through statistical methods previously described, the variability of all the soluble markers analyzed was transformed into the following: (**A**) 2-dimensional principal component analysis (to visualize the distribution in 2 dimensions of the variability in the different study groups); (**B**) Unsupervised hierarchical clustering based on univariate analysis comparing severe COVID-19 with preeclampsia. In this analysis, a z-score transformation was performed on the intensity of each biomarker across all samples, and each sample z-score is displayed in the heatmap. Biomarkers (in rows) and samples (in columns) are clustered by Euclidean distance and Ward linkage. Controls (C, n=10), mild COVID-19 (mcovid-19, n=9), severe COVID-19 (scovid-19, n=8), preeclampsia (PE, n=13).

Ang2, angiopoetin 2; *α2AP*, α2-antiplasmin; *HS*, heparan sulfate; *NETs*, neutrophil extracellular traps; *PAI*, plasminogen activator inhibitor; *PlGF*, placental growth factor; *sFlt-1*, soluble fms-like tyrosine kinase-1; *sTNFRI*, soluble tumor necrosis factor-α receptor I; *TM*, thrombomodulin; *VCAM-1*, vascular cell adhesion molecule-1; *VWF*, von Willebrand factor; *VWF:Ag*, VWF antigen; *VWF:GPIbM*, VWF activity.

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immune, and angiogenic biomarkers were detected in severe COVID-19 including significantly higher concentrations of VCAM-1, sTNFRI, HS, VWF:Ag, and NETs, with a significant reduction of PlGF compared with controls. No differences were observed in Ang2, sFlt-1, C5b9, ADAMTS13, PAI, nor TM between patients with severe COVID-19 and controls. Pregnant women with preeclampsia exhibited remarkable alterations in soluble biomarkers in a distinct profile from the one observed in COVID-19. Cases of preeclampsia showed a marked increase in VCAM-1 and sTNFRI (significantly increased compared with controls and severe COVID-19 cases), with a striking reduction in VWF:Ag, VWF:GPIbM, VWF:Ag/VWF:GPIbM, and α2AP. As expected, reduced PlGF, increased sFlt-1 and Ang2, and a very high sFlt-1 to PlGF ratio were also observed in preeclampsia. In addition, a significant increase in C5b9 and NETs was also detected in preeclampsia compared with controls. HS, ADAMTS13, PAI, and TM remained unchanged in preeclampsia.

Principal component analysis demonstrated a clear separation between preeclampsia and the other study populations (controls and mild and severe COVID-19 cases) (Figure 2, A). The first and second components explained 42.2% and 13.5% of the variance between groups. Unsupervised hierarchical clustering also showed a complete separation between severe COVID-19 cases and preeclampsia (Figure 2, B), with the most remarkable differences observed in VWF:GPIbM, VWF:Ag, and VWF:Ag/VWF:GPIbM followed by HS (significantly lower in preeclampsia) and sTNFRI, sFlt-1, and sFlt-1 to PlGF ratio (significantly higher in preeclampsia).

VWF multimeric analysis revealed the absence of VWF high-molecular-weight multimers in preeclampsia, comparable to a diagnosis of von Willebrand disease type 2A, with an accumulation of low-molecular-weight multimers (Figure 3). In healthy pregnancies and SARS-CoV-2—positive patients, VWF multimeric pattern was normal.

A subanalysis revealed a similar pattern of endothelial damage, coagulopathy, and angiogenic imbalance in early- vs late-onset preeclampsia (Supplemental Table 2), with remarkable changes in early-onset cases. In contrast, C5b9 and NETs were more altered in late-onset preeclampsia.

FIGURE 3
VWF multimeric analysis in preeclampsia and severe COVID-19 patients
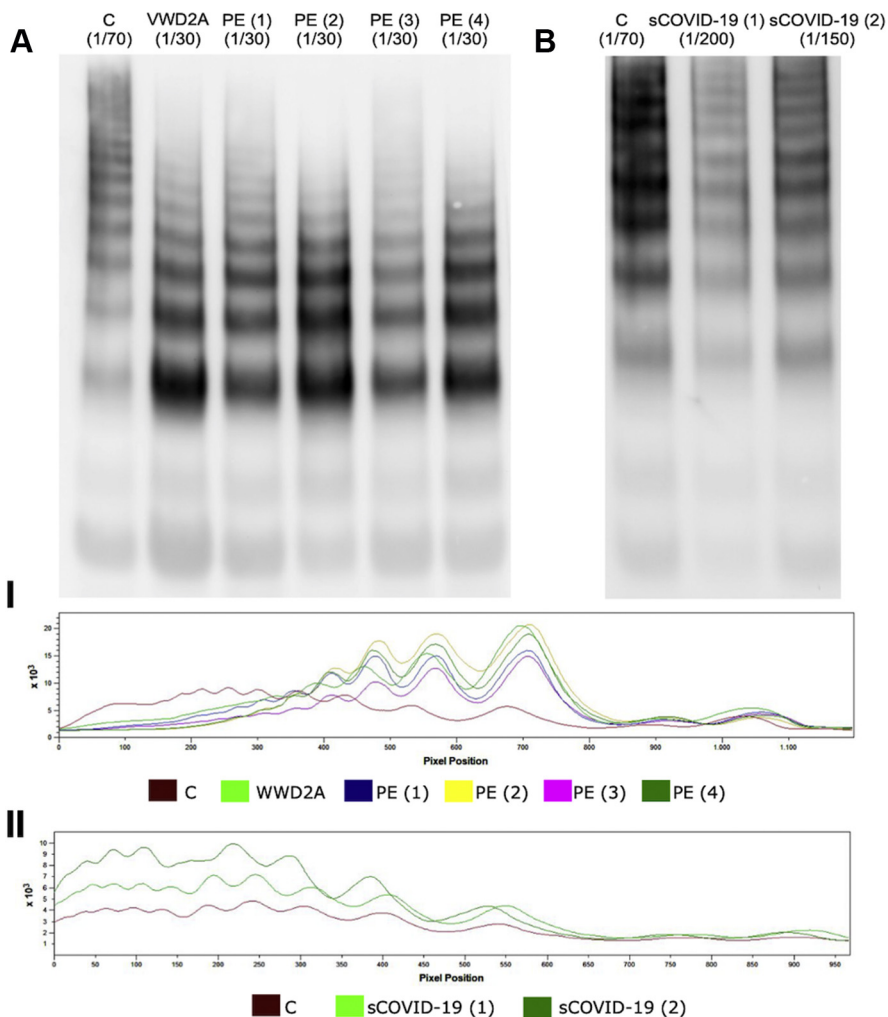
This analysis was performed to confirm the qualitative defects of this protein suggested by the low VWF:Ag/VWF:GPIb detected in preeclampsia patients (PE). **A,** A normalized 1.2% multimer gel and **B,** densitometry of plasma VWF multimers from normal control, and **C,** patient with known von Willebrand disease type 2A as a positive control (VWD2A) (characterized by a loss of high-molecular-weight multimers and an increase in the low-molecular-weight multimers), 4 PEs, and 2 pregnant women with severe COVID-19 (sCOVID-19). Of note, each sample dilution was performed following the antigenic concentration (VWF:Ag) previously obtained (in addition, it was an indirect confirmation of the results). Sample identification is followed by the dilution used to resolve VWF multimeric pattern.

VWF, von Willebrand factor; *VWF:Ag*, VWF antigen; *VWF:GPIbM*, VWF activity.

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Severe COVID-19 and preeclampsia sera induce similar endothelial damage and inflammation in vitro

Endothelial cell incubation with sera from mild and severe COVID-19 patients induced a significant overexpression of ICAM-1 and VWF compared with controls (Figure 4). Cells exposed to preeclampsia sera also showed significantly increased ICAM-1 and VWF expression, although preeclampsia effect was less pronounced than the one caused by severe COVID-19 (*P*<.05).

Immunoblots of lysates from endothelial cells exposed to mild and severe COVID-19 and preeclampsia sera showed an increase in p38 MAPK phosphorylation. Severe COVID-19 and preeclampsia were statistically different from controls (Figure 5), suggesting that both severe COVID-19 and preeclampsia sera can activate inflammatory signaling pathways.

Comment
Principal findings

A comprehensive ex vivo and in vitro study revealed distinct endothelial and angiogenic profiles of severe COVID-19 vs preeclampsia. Whereas severe COVID-19 exhibited alterations in HS, NETs, and PlGF, preeclampsia presented abnormal levels of VCAM-1, sTNFRI, VWF, complement C5b9, Ang2, and sFlt-1. Sera from patients with both severe COVID-19 and preeclampsia induced an overexpression of ICAM-1 and VWF and activation of p38 MAPK phosphorylation in endothelial cells in culture, although the effect of preeclampsia was less pronounced than that of severe COVID-19.

Preeclampsia vs COVID-19: a distinct profile of circulating endothelial damage biomarkers

Both preeclampsia and severe COVID-19 showed signs of endothelial damage, but with a differential pattern. Patients with preeclampsia presented a very significant increase in VCAM-1 and sTNFRI, with preserved HS. These results are consistent with previous reports demonstrating elevated VCAM-1.[31,32] The presence of sTNFRI has been only anecdotally described.[33] sTNFRI is the soluble receptor of tumor necrosis factor alpha, a proinflammatory cytokine that triggers the expression of inflammatory molecules, including cell adhesion molecules, such as VCAM-1 and ICAM-1,[34] resulting in inflammation, apoptosis, reactive oxygen species generation, cell proliferation, and cell survival. In contrast, severe COVID-19 cases showed a milder increase in VCAM-1 and sTNFRI, with a significant alteration of HS. These data are in line with previous reports on nonpregnant COVID-19 patients showing a good correlation of VCAM-1 and sTNFRI with disease severity.[12,16]

FIGURE 4
Expression of ICAM-1 and VWF in cultured endothelial cells: effect of COVID-19 and preeclampsia sera
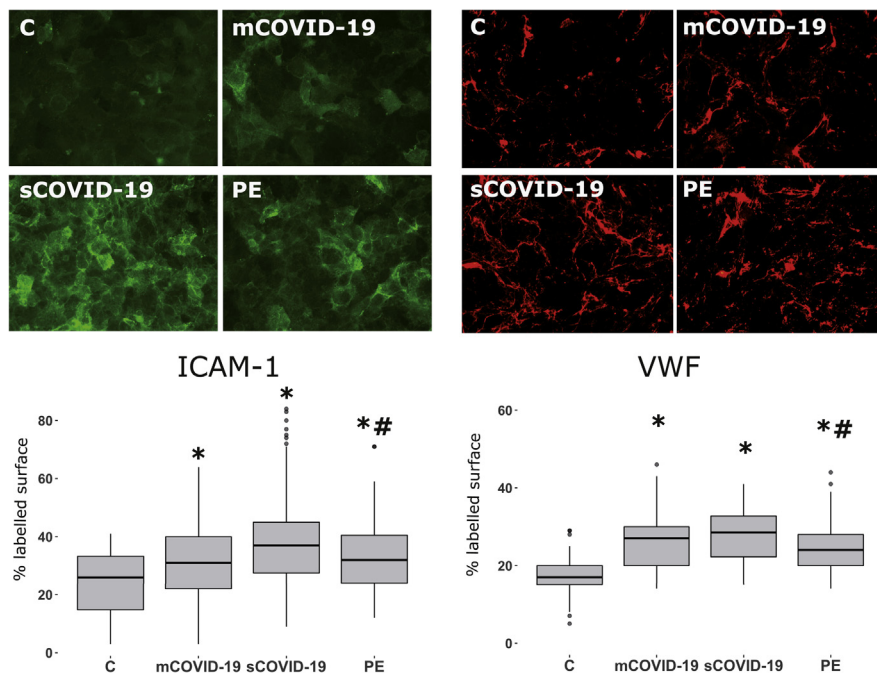
Changes in inflammation and thrombogenic phenotypes induced by the study conditions were explored through an in vitro approach consisting of the exposure of endothelial cells in culture to patients' sera. Representative fluorescence micrographs showing ICAM-1 expression (in *green*, on the left panel) on cell surface and VWF release (in *red*, on the right panel) on endothelial cells in culture supplemented with serum from controls (C) or patients with mild and severe COVID-19 (m and sCOVID-19) and preeclampsia (PE). The boxplots represent the quantitative assessment of ICAM-1 expression and VWF release. The *line in the boxes* depicts the sample median and the *boxes* are the first and third quartiles. The whiskers point to the maximum and minimum values of the sample (n=6, *asterisk denotes P*<.05 vs controls and *hashtag* denotes *P*<.05 comparison between sCOVID-19 and PE).

ICAM-1, intercellular adhesion molecule 1; *VWF*, von Willebrand factor.

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The increased levels of HS suggest endothelial glycocalyx barrier disruption and degradation. This finding is consistent with previous reports in critically ill nonpregnant COVID-19 patients demonstrating that HS is used by SARS-CoV-2 to interact with endothelial cells through its receptor-binding domain, leading to a damaged endothelial barrier.[35]

Preeclampsia is associated with remarkable alterations in von Willebrand factor antigen and functionality

Interestingly, the most remarkable differences between preeclampsia and COVID-19 were observed in VWF concentrations and activity. Our data on COVID-19 pregnancies are consistent with the previously described positive correlation of VWF with disease severity.[12] Conversely, in preeclampsia, we observed a striking decrease in VWF levels contrary to the increase reported formerly in the literature.[36] Interestingly, these changes were more pronounced in more severe early-onset cases. A potential explanation of this observation is acute VWF consumption owing to endothelial cell exhaustion[37] in preeclampsia given that, indeed, the in vitro exposure of endothelial cells to preeclampsia sera resulted in a relevant increase in VWF release. Other potential explanations could be bleeding or drug interaction (with corticosteroids given to ensure lung maturity). Moreover, our results suggest a qualitative VWF defect in preeclampsia manifested by low VWF:GPIbM to VWF:Ag ratio and confirmed by the multimeric analysis of VWF. Given that ADAMTS-13 activity was similar in preeclampsia and the other study groups, the loss of high-molecular-weight multimers might be owing to the lysis by other proteases such as plasmin. In fact, the degradation of VWF by plasmin has been described in hyperfibrinolytic states,[38] and pre-eclampsia is known to be a hypercoagulable and hyperfibrinolytic state.[39] Thus, it is plausible that a fibrinolytic imbalance might be underlying VWF proteolysis, specifically an imbalance in plasmin regulation given that α2AP was significantly reduced in patients with preeclampsia compared with the other groups.

Innate immune dysregulation in preeclampsia vs COVID-19 in pregnancy

Our data confirm the previously reported increase in soluble C5b9 in preeclampsia.[40,41] Damaged endothelial cells in preeclampsia seem to activate the innate immune response including the complement system. In addition, we also report the formation of NETs both in preeclampsia and severe COVID-19 in pregnancy. NETs are large structures of chromatin and antimicrobial proteins released by dying neutrophils to capture extracellular pathogens, limit the spread of infections, and directly activate alternative complement pathways. Our results are consistent with the previously reported activation of NETs directly by SARS-CoV-2 in nonpregnant individuals.[42] Hyperactivation of NETs formation in preeclampsia has been proposed to be induced by placenta-derived factors.[43] Interestingly, these changes were more remarkable in cases of late-onset preeclampsia. Overall, dysregulation of innate immune response seems to play a role in the complex pathologic cascade leading to endothelial damage in both SARS-CoV-

Inflammatory signaling pathways in endothelial cells exposed to mild and severe COVID-19 and preeclampsia milieu
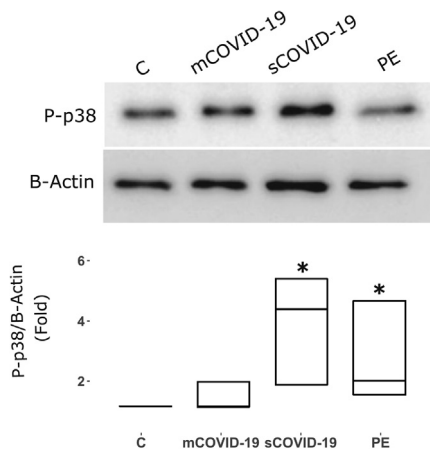
The analysis of signaling pathway activation was performed by exposing resting endothelial cells to the sera under study. In the present study, activation of p38 mitogen-activated protein kinase (p38 MAPK; a kinase with a key role in inflammatory cellular responses to injurious stress) in endothelial cells in culture exposed to sera from controls (C), mild and severe COVID-19 (m and sCOVID-19), and preeclampsia (PE) patients for 5 minutes. Immunoblot image shows phosphorylated p38 MAPK and β-actin, and the boxplots represent the relative quantification of p38 MAPK / β-actin compared with controls (n=3, *asterisk* denotes $P<.05$ vs controls).

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2 infection and preeclampsia.[43,44] Interestingly, certain aspects of the complement cascade and NETs facilitate coagulation and interfere with anticoagulation.[45] Therefore, the crosstalk between the complement and coagulation cascades, along with endothelial damage, may create the prothrombotic environment associated with adverse outcomes in COVID-19 and preeclampsia.[46,47]

Preferential angiogenic imbalance in preeclampsia vs COVID-19
Finally, our results show a profound disruption of the angiogenic balance in preeclampsia compared with controls and COVID-19, with very high levels of sFlt-1 and Ang2 and reduced PlGF.[48] As previously described, angiogenic profile was more severely altered in early-onset preeclampsia.[49] Interestingly, COVID-19 cases also showed significantly low PlGF but normal concentrations of sFlt-1 and therefore preserved sFlt-1 to PlGF ratio. PlGF is mainly synthesized in the endothelium, which might explain a reduction in any case of endothelial damage. In contrast, high sFlt-1 and Ang2 levels seem to be specific to preeclampsia. These findings are consistent with angiogenesis dysregulation being proposed as 1 of the main pathophysiological features in the development of preeclampsia.[19,30] These results are also in line with previous reports[50] proposing sFlt-1 to PlGF ratio for the differential diagnosis of preeclampsia and COVID-19 in pregnancy.

Similar in vitro-induced endotheliopathy in preeclampsia and SARS-CoV-2 infection
Our in vitro results demonstrate a strong activation of p38 MAPK induced by both severe COVID-19 and preeclampsia sera, along with a potent induction of ICAM-1 and VWF expression. This functional approach reflects the direct deleterious effect of both sera inducing microvascular endothelial damage in vivo. The slightly superior effect of severe COVID-19 sera could be attributed not only to the soluble factors present in the sera but to a direct viral infection. The observed activation of ICAM-1 and VWF is consistent with the known mechanism of activating adhesion molecules to recruit neutrophils and platelets in response to endothelial damage.[51] Although it is known that SARS-CoV-2 infection activates p38 MAPK and the downstream signaling, possibly leading to cell death,[52] the pathways leading to this activation in preeclampsia remain to be elucidated. Indeed, a preclinical study in a SARS-CoV-2 mouse model showed protective effects of p38 MAPK inhibition, pointing out its potential therapeutic

effect.[53] These data suggest that, despite their different pathophysiology, both preeclampsia and COVID-19 ultimately activate common pathways of endothelial dysfunction, explaining the similarities between their clinical scenarios.

Strengths and limitations
The main strength of this study is the prospective recruitment of well-characterized COVID-19 cases in pregnant women that were matched for baseline characteristics with both normotensive and preeclamptic SARS-CoV-2−negative pregnancies. In addition, a large panel of endothelial damage markers has been investigated. The small sample size should be considered a limitation of the present study. Indeed, it hindered the detection of heterogeneity, if present, between early- and late-onset preeclampsia. Conversely, we acknowledge that longitudinal changes in the studied biomarkers were not explored in the current study. Given the complexity and clinical heterogeneity of these conditions, future studies are warranted to confirm the similarities and differences in the endothelial and angiogenic profiles of these entities.

Conclusion, clinical and research implications
In conclusion, this study suggests a differential profile of circulating biomarkers with a similar end-stage in vitro-induced endothelial dysfunction. Soluble biomarkers of coagulopathy (VWF), endothelial inflammation (sTNFRI), barrier damage (HS), and angiogenesis (sFlt-1) seem to be highly specific in differentiating preeclampsia from severe COVID-19 in pregnancy. These findings hold the potential to improve our understanding of the pathophysiological pathways in preeclampsia and COVID-19 in pregnancy. We also identified circulating biomarkers that may be useful in the differential diagnosis of preeclampsia and SARS-CoV-2 infection in pregnancy. Given the difficulty of clinically

differentiating some cases of preeclampsia and COVID-19, a panel of circulating biomarkers for differential diagnosis could be of great help in optimizing patient management. Finally, this study also opens opportunities for new therapeutic targets that could improve the underlying endothelial damage observed in these entities. ∎

GLOSSARY

- A disintegrin and metalloproteinase with thrombospondin type 1 motif, 13 (ADAMTS-13): is primarily synthesized in the liver, and its main function is to cleave von Willebrand factor (VWF) anchored on the endothelial surface, in circulation, and at the sites of vascular injury.
- Angiopoetin 2 (Ang2): is produced by endothelial cells and acts as an autocrine regulator mediating vascular destabilization and regulating vascular homeostasis.
- α2-antiplasmin (α2AP): is a serine protease inhibitor (serpin) responsible for inactivating plasmin.
- Endothelium: composed by endothelial cells, it plays an important role in inflammation by regulating vascular permeability for macromolecules and leukocytes, vascular tone, and hemostasis, and by binding and producing inflammatory mediators such as cytokines.
- Ex vivo approach: to quantify the degree of endothelial activation is of interest when evaluating inflammation. Because of the localization of this type of cells, this evaluation cannot be carried out directly, and a number of indirect measures such as the measurement of soluble molecules released by the endothelium has been employed instead.
- Soluble fms-like tyrosine kinase-1 (sFlt-1): is a circulating antiangiogenic protein synthesized by the placenta, which acts as an antagonist of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) and is up-regulated in preeclampsia.
- sFlt-1/PlGF ratio: an imbalance in the levels of these 2 biomarkers has been reported to be involved in preeclampsia pathogenesis. An elevated sFlt-1/PlGF seems to be highly predictive of preeclampsia.
- Heparan sulfate (HS): is the glycosaminoglycan from endothelial glycocalyx used by viral pathogens such as SARS-CoV-2 for the initial interaction with host cells.
- In vitro approach: consists in a well-characterized in vitro model of endothelial dysfunction, in which endothelial cells in culture are exposed to patients' sera to assess its capacity to modulate the endothelial phenotype. This analysis is performed through the quantification of changes in inflammatory and thrombogenicity markers together with the activation of certain intracellular signaling pathways.
- Intercellular adhesion molecule-1 (ICAM-1): adhesion molecule that is up-regulated during endothelial activation and mediates lymphocyte binding. This molecule is not only released from the endothelium, but also from lymphocytes, monocytes, and eosinophils. Elevated levels of soluble ICAM-1 have been reported in preeclampsia.
- Neutrophil extracellular traps (NETs): are extracellular webs of chromatin, microbicidal proteins, and oxidant enzymes that are released by neutrophils to fight against infections and that, in elevated concentrations, have the potential to propagate inflammation and microvascular thrombosis.
- Placental growth factor (PlGF): is a member of the vascular endothelial growth factor (VEGF) family and is predominantly expressed in the placenta. The circulating levels of this molecule have been postulated as a useful screening tool in the prediction of preeclampsia.
- Plasminogen activator inhibitor (PAI): is a member of the serine protease inhibitor (serpin) superfamily and constitutes a central molecule linking pathogenesis and progression of thrombotic vascular events.
- Principal component analysis: is a statistical method that aims to reduce the dimensionality of large data sets by transforming them into smaller ones. This method preserves as much information as possible, and the resulting data set becomes easier to explore and visualize than the original one.
- P38 mitogen-activated protein kinase (P38 MAPK): plays a pivotal role in mediating cellular responses to injurious stress and immune signaling partly through the activation of gene expression.
- Soluble complement 5b-9 (C5b9): is also known as soluble membrane attack complex and constitutes a marker of complement activation. This molecule creates a transmembrane channel on the surface of targeted cells that leads to cell lysis and death.
- Soluble tumor necrosis factor-α receptor I (sTNFRI): is 1 of the 2 soluble receptors of TNF-alpha (TNFα), a proinflammatory cytokine that plays a central role in inflammation, which act as physiological attenuator of TNFα activity.
- Thrombomodulin (TM): is a thrombin receptor on endothelial cells that is involved in promoting activation of the anticoagulant protein C pathway during blood coagulation.
- Vascular cell adhesion molecule-1 (VCAM-1): adhesion molecule that is up-regulated during endothelial activation and mediates lymphocyte binding. Elevated levels of soluble VCAM-1 have been reported in preeclampsia.
- Von Willebrand factor (VWF): a multimeric blood protein primarily synthesized, stored, and secreted by endothelial cells. It constitutes a marker of acute and chronic inflammation. The analysis of this protein implies both antigen concentration (VWF:Ag) and functionality (VWF:GPIbM).
- Von Willebrand factor multimeric analysis: is a method carried out by electrophoresis of plasma samples using nonreducing agarose gels in the presence of different concentrations of sodium dodecyl sulfate. This analysis aims to identify qualitative defects of this protein and is usually performed after functional and immunologic VWF assays to indicate a potential abnormality.

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Author and article information

From the Josep Carreras Leukaemia Research Institute, Hospital Clinic, University of Barcelona, Barcelona, Spain (Dr Palomo, Mr Ramos, Ms Marinez-Sanchez, and Dr Carreras); Laboratory of Hemostasis and Eritropathology, Hematopathology, Pathology Department, Centre Diagnòstic Biomèdic, Hospital Clinic, University of Barcelona, Barcelona, Spain (Dr Palomo, Mrs Ramos and Torramade-Moix, Dr Moreno-Castaño, Mses Martinez-Sanchez and Bonastre, Mr Pino, Mses Gomez-Ramirez, Martin, and Garcia Mateos, and Drs Escolar and Diaz-Ricart); Barcelona Endothelium Team, Barcelona, Spain (Dr Palomo, Mr Ramos, Dr Moreno-Castaño, Ms Martinez-Sanchez, and Drs Carreras and Diaz-Ricart); BCNatal (Hospital Clínic and Hospital Sant Joan de Déu), Barcelona, Spain (Drs Youssef, Crovetto, Gratacos, and Crispi); Institut d'Investigacions Biomèdiques August Pi i Sunyer, University of Barcelona, Barcelona, Spain (Drs Moreno-Castaño, Castro, Gratacos, Crispi, and Diaz-Ricart); Department of Marine Biology and Oceanography, Institut de Ciències del Mar, Spanish National Research Council, Barcelona, Spain (Drs Sanchez, Escolar, and Castro); Medical Intensive Care Unit, Hospital Clinic, School of Medicine, Barcelona, Spain (Dr Fernandez); and Centre for Biomedical Research on Rare Diseases, Madrid, Spain (Drs Crovetto, Gratacos, and Crispi).

Supplemental Information

Methods

Laboratory tests for SARS-CoV-2 infection

Nasopharyngeal swab samples for SARS-CoV-2 RNA real-time polymerase chain reaction (RT-PCR) were collected in women attending for delivery. Samples were collected on Micronics tubes (Micronics, Chattanooga, TN) with Zymo DNA/RNA Shield Lysis Buffer (Zymo Research, Irvine, CA). RNA was extracted using the Quick-DNA/RNA Viral MagBead kit (Zymo Research) and the Tecan DreamPrep robot (Tecan Group Ltd., Männedorf, Switzerland). Five microliters of RNA solution were added to 15 μL of reverse RT-PCR master mix (Luna Universal Probe One-Step RT-qPCR Kit; New England Biolabs, Ipswich, MA) and used for amplification of SARS-CoV-2 N1 and N2 regions, and the human ribonuclease P (RNase P) gene as control, as described in the CDC-006-00019 CDC/DDID/NCIRD/Division of Viral Diseases protocol released on March 30, 2020. A SARS-CoV-2–positive result was considered if the cycle threshold (Ct) values for N1, N2, and RNase P were below 40. Samples discordant for N1 and N2 were repeated, and samples with a Ct $\geq$40 for RNase P were considered as invalid.

SARS-CoV-2 immunoglobulin (Ig) G and IgM/IgA antibodies were tested using COVID-19 VIRCLIA Monotest (Vircell, Granada, Spain). All indeterminate results were retested (VITROS Immunodiagnostic Products Anti-SARS-CoV2 Total Tests, Ortho Clinical Diagnostics, Raritan, NJ) and classified as positive or negative. Likewise, all samples that were positive for IgM+IgA but negative for IgG in women reporting no symptoms suggestive of COVID-19 during the 10 weeks before testing were retested by a quantitative suspension array assay based on the Luminex xMAP Technology (Luminex Corporation, Austin, TX) and classified as positive or negative. A positive serologic result was considered in the presence of any of the

following: (1) seropositivity for IgG, (2) seropositivity for IgM+IgA in women with symptomatic COVID-19, (3) seropositivity for IgM and/or IgA confirmed by 2 tests (Vircell and Luminex).

Multimeric profile of circulating von Willebrand factor

Analysis of von Willebrand factor (VWF) multimers was performed to discard or confirm potentially qualitative defects that can be suspected when a discrepancy between the VWF antigen and the VWF activity is appreciated.[1] In the present study, VWF multimers were resolved by sodium dodecyl sulfate-agarose (SDS) discontinuous gel electrophoresis (1.2%) followed by protein transfer to nitrocellulose membranes by Western blotting. Blots were probed using a primary antibody against VWF (rabbit, A0082, Dako, Agilent, Santa Clara, CA) followed by a horseradish peroxidase (HRP)-conjugated rabbit anti-VWF (P0047, Dako, Agilent).[1] Visualization of VWF multimers was achieved using a commercially available enhanced chemiluminescence kit for detecting HRP-labeled antibodies on Western blots.[2] A sample from a patient diagnosed with von Willebrand disease type 2A was included as a control to validate the potential loss of high-molecular-weight multimers. Chemiluminescence was read in an Image-Quant LAS 500 (GE Healthcare Europe GmbH, Freiburg, Germany).

Human endothelial cell culture

The human dermal microvascular endothelial cell line (ATCC, CRL-3243, Lot:62630587) was grown at 37 °C in a 5% CO_2 humidified incubator in MCDB 131 medium (Gibco, Life Technologies, Thermo Fisher Scientific, Waltham, MA) supplemented with 4% of L-glutamine, 1% of penicillin/streptomycin (Gibco, Life Technologies, Thermo Fisher Scientific), 1 μl/mL of hydrocortisone (Sigma-Aldrich Quimica SA, Madrid, Spain), 10 ng/mL of epidermal growth factor (BD Biosciences, Erembodegem, Belgium), and 10% fetal bovine sera (Gibco, Life Technologies,

Thermo Fisher Scientific). Cells between the 10th and 15th passage were used.[20]

Immunofluorescence detection of intercellular adhesion molecule 1 and van Willebrand factor

Cells were seeded into 8 Well μ-Slides (#80826, ibidi GmbH, Gräfelfing, Germany) and exposed to culture media supplemented with 20% of samples of sera patients for 48 hours. Then, cultures were washed, fixed (4% paraformaldehyde), and immunostaining for intercellular adhesion molecule 1 (ICAM-1) (#MAB2146, clone P2A4, MilliporeSigma, Temecula, CA, as primary antibody and antimouse IgG conjugated with Alexa 488; #A28175, Molecular Probes, New York, NY as secondary antibody) and VWF (antimouse IgG conjugated with Alexa 555, #A32727, Molecular Probes, Thermo Fisher Scientific, as secondary antibody) was performed as previously described.[21] Fluorescence micrographs were arbitrarily obtained from each preparation by fluorescent microscopy (DM4000B, Leica, Barcelona, Spain) through a video camera (Leica DFC310FX) and analyzed using Fiji (ImageJ, National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/)[22] by 2 independent and blinded investigators. The area covered by ICAM-1 and VWF was calculated for every sample and expressed as the average fold increase of each condition vs control sample. Cells incubated with lipopolysaccharide (1 μg/mL, #L4391, Sigma-Aldrich) and endothelial culture medium were used as a positive and negative control, respectively, as shown in the Supplemental Figure.

Activation of inflammation cell signaling pathways in endothelial cells

The effect of patient sera on phospho-P38 MAPK was evaluated in confluent cells grown in 6-well plates and starved 24 hours before experiments. After being exposed to patients' sera pool for 5 minutes, endothelial cells were lysed with

Laemmli buffer, sonicated to shear DNA and reduce viscosity (15 seconds), and heated to 90°C (5 minutes). Protein concentration in the supernatants was determined using Coomassie Plus (Pierce, Thermo Fisher Scientific). Samples were resolved by 8% SDS-PAGE and proteins transferred to nitrocellulose membranes and probed with specific antibodies against phospho-p38 MAPK and β-actin (#4511S and #4511S, respectively, Cell Signaling Technology, Danvers, MA). Membranes were incubated with a peroxidase-conjugated antirabbit IgG (#P0448, Dako, Agilent) for 1 hour at room temperature. Then, membranes were incubated with Clarity Western ECL Substrate (#170-5061, Bio-Rad Laboratories, Hercules, CA), and chemiluminescence was read in an ImageQuant LAS 500 for phospho-P38 MAPK and β-actin quantification.

Supplemental References

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SUPPLEMENTAL FIGURE
Negative and positive controls for the expression of ICAM-1 and VWF in cultured endothelial cells
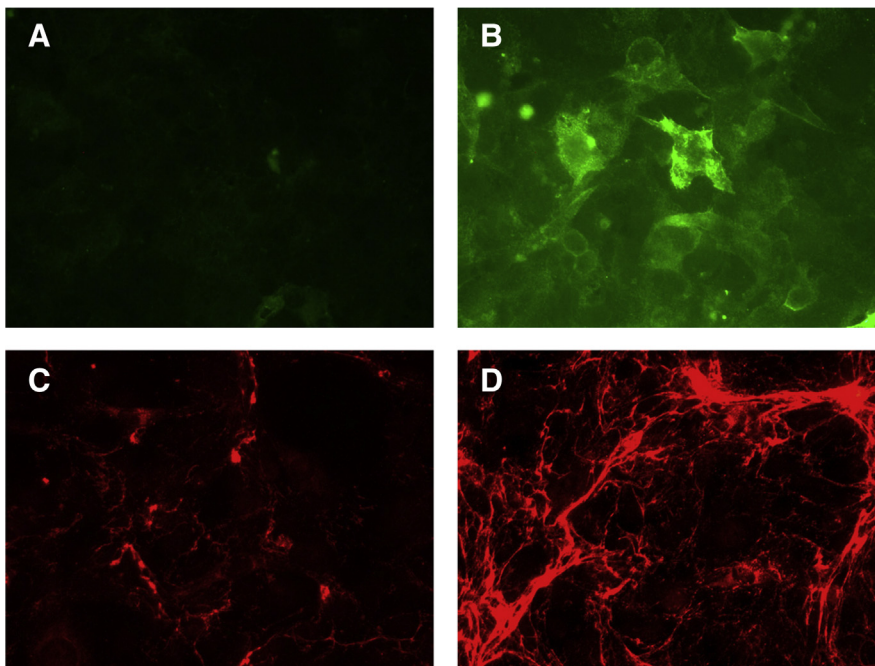
A, Negative control for ICAM-1; **B,** Positive control for ICAM-1; **C,** Negative control for VWF; **D,** Positive control for VWF.

ICAM-1, intercellular adhesion molecule 1; *VWF*, von Willebrand factor.

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SUPPLEMENTAL TABLE 1
Results of the circulating biomarkers in the study groups

Circulating biomarkers	Controls n=10	Mild COVID-19 n=9	Severe COVID-19 n=8	Preeclampsia n=13
Biomarkers of endothelial damage				
VCAM-1 (ng/mL)	158.7 (129.2–179)	162.9 (129.2–248.3)	273.9 (200–350.4)[a]	369.5 (304.6–610)[b,d,e]
sTNFRI (pg/mL)	1215.3 (910.5–1648.6)	1360.5 (1248.6–1610.5)	1772.4 (1417.6–2022.4)[a]	2639 (2091.4–3234.3)[b,d,e]
HS (ng/mL)	2524.8 (1647.3–3330.6)	3401.7 (2285.4–4034.6)	4021.7 (2505.8–5718.2)[a]	1799 (1414–2589.4)[e]
Biomarkers of coagulopathy/fibrinolysis				
VWF:Ag (%)	297.4 (237.2–375.4)	406.6 (364.3–428.5)[a]	432.7 (365.9–470.7)[a]	89.6 (84.5–135.3)[b,d,f]
VWF:GPIbM (%)	280.9 (250–337)	295.3 (228.4–324.3)	376.6 (285.5–429.5)	44.6 (20–54.5)[b,c,f]
VWF:Ag / VWF:GPIbM	0.99 (0.83–1.1)	0.76 (0.73–0.78)	0.9 (0.85–0.94)[c]	0.33 (0.28–0.5)[b,d,f]
α2AP (%)	107 (87–118)	98 (95–106)	92.5 (81.5–99)	70 (67–74)[b,d,f]
ADAMTS-13 activity (%)	100	100	100	100
PAI (ng/mL)	70.1 (66.7–85.3)	79.3 (64–106.2)	71.5 (59.1–89)	80.7 (68.7–136.4)
TM (ng/mL)	9.9 (9.5–10.1)	9.7 (9.6–10)	10.1 (9.6–10.4)	9.7 (9.5–10)
Immune response markers				
C5b9 (ng/mL)	31 (22–36.6)	29.1 (26.7–31.8)	38.1 (31.4–50)	51.1 (46.1–66.6)[b,d]
NETs (ugr/mL)	17.7 (15.2–19.9)	14 (11.5–17.3)	35.7 (20.7–52)[a,c]	28 (16.5–38)[a,c]
Biomarkers of angiogenesis				
sFlt-1 (pg/mL)	18,390.1 (12,014.2–23,187.4)	35,588.1 (19,006–46,271.4)	27,302.5 (8033.2–38,236.8)	161,368.9 (56,737.2–216,405.5)[a,c,e]
PIGF (pg/mL)	247.5 (181.7–325.6)	130.5 (122.6–152)[a]	118.4 (70.2–142.4)[a]	55.1 (43.6–79.7)[a]
sFlt-1 / PIGF	62.5 (44.3–150.8)	225 (135.2–372.9)[a]	202.5 (102.4 – 334)	2024.7 (850.3 – 3927.5)[a,c,e]
Ang2 (ng/mL)	6952.5 (4746–9996)	6078.1 (4496.3–10,396.3)	6552.5 (4365–15,690)	13,671.3 (6996.3–21,996.3)[a]

Data are expressed as median (interquartile range).

Ang2, angiopoetin 2; *α2AP*, α2-antiplasmin; *HS*, heparan sulfate; *NETs*, neutrophil extracellular traps; *PAI*, plasminogen activator inhibitor; *PIGF*, placental growth factor; *sFlt-1*, soluble fms-like tyrosine kinase-1; *sTNFRI*, soluble tumor necrosis factor-α receptor I; *TM*, thrombomodulin; *VCAM-1*, vascular cell adhesion molecule-1; *VWF*, von Willebrand factor; *VWF:Ag*, VWF antigen; *VWF:GPIbM*, VWF activity.

Significant differences of adjusted *P* values (Student *t* test, Benjamini-Hochberg procedure for multiple pairwise comparisons) are noted as [a]$P<.05$ and [b]$P<.01$ vs controls, [c]$P<.05$ and [d]$P<.01$ vs mild COVID-19, and [e]$P<.05$ and [f]$P<.01$ vs severe COVID-19.

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SUPPLEMENTAL TABLE 2
Results of the circulating biomarkers in early and late-onset preeclampsia

Circulating biomarkers	Early-onset preeclampsia n=4	Late-onset preeclampsia n=9
Biomarkers of endothelial damage		
VCAM-1 (ng/mL)	666.2 (612.8—738.2)[a]	321.8 (291.4—369.5)[b]
sTNFRI (pg/mL)	3498.6 (2489.1—4331.9)[a]	2343.8 (2091.4—2762.9)[b]
HS (ng/mL)	1859.1 (977.7—3194.2)	1799 (1414—2589.4)
Biomarkers of coagulopathy/fibrinolysis		
VWF:Ag (%)	82.8 (60.6—88.7)[a]	130.8 (86.8—138)[a]
VWF:GPIbM (%)	8.8 (5—16.3)[a]	45.9 (44.6—78.9)[a]
VWF:Ag / VWF:GPIbM	0.15 (0.05—0.27)[a]	0.48 (0.33—0.58)[a]
α2AP (%)	73.5 (36—79)[b]	69 (67—71)[b]
ADAMTS-13 activity (%)	100	100
PAI (ng/mL)	152.2 (108.7—169.1)[b]	78.3 (52.3—83.2)
TM (ng/mL)	9.5 (9.2—10)	9.8 (9.7—10)
Immune response markers		
C5b9 (ng/mL)	41.9 (29.4—59.6)	60.5 (60.5—66.6)[a]
NETS (ugr/mL)	33 (25.5—50.5)	21 (13.9—34.6)[b]
Biomarkers of angiogenesis		
sFlt-1 (pg/mL)	316,461.5 (125,464.1—567,831.6)[a]	132,000.9 (56,737.2—166,513.1)
PIGF (pg/mL)	47.9 (38.8—64.8)[b]	71.2 (53.1—147.4)[b]
sFlt-1 / PIGF	6911 (2388.9—10,575.9)[a]	866.1 (823.9—3135.8)
Ang2 (ng/mL)	24,052.5 (15,233.8—42,840)[b]	11,721.3 (6546.3—14,058.8)

Data are expressed as median (interquartile range).

Ang2, angiopoetin 2; *α2AP*, α2-antiplasmin; *HS*, heparan sulfate; *NETs*, neutrophil extracellular traps; *PAI*, plasminogen activator inhibitor; *PIGF*, placental growth factor; *sFlt-1*, soluble fms-like tyrosine kinase-1; *sTNFRI*, soluble tumor necrosis factor-α receptor I; *TM*, thrombomodulin; *VCAM-1*, vascular cell adhesion molecule-1; *VWF*, von Willebrand factor; *VWF:Ag*, VWF antigen; *VWF:GPIbM*, VWF activity.

Significant differences of adjusted *P* values (Student *t* test, Benjamini-Hochberg procedure for multiple pairwise comparisons) are noted as [b]$P<.05$ and [a]$P<.01$ preeclampsia vs controls.

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