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

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Condensation

Preeclampsia and COVID-19 exhibit a differential profile of circulating biomarkers with a similar end-stage in vitro induced endothelial dysfunction.

Short version of the title

Endothelial dysfunction in preeclampsia vs. COVID-19 in pregnancy

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.

What are the key findings?

Severe COVID-19 in pregnancy and preeclampsia share a similar end-stage *in vitro* induced p38MAPK phosphorylation in endothelial cells but a differential profile of circulating endothelial and angiogenic biomarkers. Severe COVID-19 is characterized by higher VCAM-1, sTNFR-I, HS, VWF antigen and NETS and reduced PlGF while preeclampsia is marked by increased VCAM-1, sTNFR-I, sFlt-1, Ang2, C5b9 and NETS and a reduction in VWF antigen, VWF activity, α2AP and PlGF.

What does this study add to what is already known?

Soluble biomarkers of coagulopathy [VWF], endothelial inflammation [sTNFRI], barrier damage [HS] and angiogenesis [sFlt1] seem to be highly specific to differentiate preeclampsia from severe COVID-19 in pregnancy. These findings improve our understanding of the

78 pathophysiological pathways in preeclampsia and COVID-19 and may help in the differential
79 diagnosis of these disorders during pregnancy.

ABSTRACT

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.

Methods: Plasma and sera samples were obtained from pregnant women with COVID-19 infection classified into mild (n=10) or severe (n=9) in addition to 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 (VCAM-1), soluble TNF-receptor I (sTNFRI), heparan sulfate (HS), von Willebrand factor (VWF) antigen, activity and multimeric pattern, α2-antiplasmin (α2AP), C5b9, neutrophil extracellular traps (NETS), placental growth factor (PlGF), fms-like tyrosine kinase-1 (sFlt-1) and angiopoietin 2 (Ang2). Additionally, microvascular endothelial cells were exposed patient's serum, and changes in the cell expression of intercellular adhesion molecule 1 (ICAM-1) on cell membrane and VWF release to the extracellular matrix were evaluated through immunofluorescence. Changes in inflammation cell signaling pathways were also assessed by of P38MAPK phosphorylation. Statistical analysis included univariate and multivariate methods.

Results: Biomarker profiles in mild COVID-19 were similar to controls. Both preeclampsia and severe COVID-19 showed significant alterations in the majority of circulating biomarkers with distinctive profiles. While severe COVID-19 exhibited higher concentrations of VCAM-1, sTNFR-I, HS, VWF antigen and NETS with a significant reduction of PlGF as compared to controls; preeclampsia presented a marked increase in VCAM-1, sTNFR-I (significantly increased compared to controls and to severe COVID-19) with a striking reduction in VWF

antigen, VWF activity and α2AP. As expected, reduced PlGF, increased sFlt-1 and Ang2 and a very high sFlt-1/PlGF ratio were also observed in preeclampsia. In addition, a significant increase in C5b9 and NETS was also detected in preeclampsia compared to controls. The principal component analysis demonstrated a clear separation between preeclampsia and the rest of groups (first and second components explained 42.2% and 13.5% of the variance), mainly differentiated by variables related to VWF, sTNFRI, HS and sFlt-1. VWF multimeric analysis revealed the absence of VWF high-molecular-weight multimers in preeclampsia (similar profile to von Willebrand disease type 2A) whereas in healthy pregnancies and COVID-19 patients, VWF multimeric pattern was normal.

Sera from both preeclampsia and severe COVID-19 patients induced an overexpression of ICAM-1 and VWF in endothelial cells in culture compared to controls. However, the effect of preeclampsia was less pronounced than the one triggered by severe COVID-19. Immunoblots of lysates from endothelial cells exposed to mild and severe COVID-19, and preeclampsia sera showed an increase in p38MAPK phosphorylation. 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: While similar *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.

Keywords: angiogenic factors, angiopoietin, C5b9, COVID-19, endothelial dysfunction, heparan sulfate, hypertensive disorders of pregnancy, NETS, PlGF, preeclampsia, SARS-CoV-2, sFlt-1, sTNFRI, Von Willebrand factor.

INTRODUCTION

Preeclampsia is a pregnancy complication and a leading cause of maternal and perinatal morbi-mortality 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 multi-organ 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 severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), which causes coronavirus disease 2019 (COVID-19), suggest that 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 two 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 to disease severity in COVID-19[12,16]. Inflammatory effects on these damaged

153 endothelial cells activates the innate immune response and induces a hypercoagulable state with
154 impaired fibrinolysis and angiogenic imbalance[17,18]. On the other hand, angiogenesis
155 dysregulation has emerged as one of the main pathophysiological features in the development
156 of preeclampsia[19,20]. Finally, *in vitro* studies enabled us to describe the endothelial cell
157 proinflammatory and thrombogenic response in COVID-19[21–23].

158 The aim of the present study was to comprehensively investigate the endothelial and angiogenic
159 profiles in preeclampsia and SARS-CoV-2 infection in pregnancy using circulating biomarkers
160 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 serological 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 mmHg and/or diastolic blood pressure ≥ 90 mmHg on two occasions, at least four hours apart) developed after 20 weeks of gestation with proteinuria (≥ 300 mg/24 h or protein/creatinine ratio ≥ 0.3), thrombocytopenia (platelet count < 100 x 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 before 34 weeks of gestation[26]. Baseline and perinatal data were obtained by interviews and from electronic medical records. Gestational age was calculated based on 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 patient's sera. Details of the laboratory methodology used is detailed below and in the Supplementary material.

This study was approved by the ethics committee of Hospital Clinic (HCB/2020/0401) and conformed to the ethical guidelines of the Helsinki Declaration. All participants provided informed written consent before sample collection.

Maternal blood sample collection

Peripheral maternal blood was obtained by venipuncture within 24-48 hours of the onset of symptoms and before starting any treatment. Plasma and sera samples were obtained by centrifugation of blood anticoagulated with EDTA and by incubation for 30 min at room temperature to allow clotting and subsequently centrifuged at 1500×g for 10 min 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 TNF-α receptor I (sTNFRI) and heparan sulfate (HS) by ELISA (R&D systems, MN, USA; Biomatik Corporation, DE, USA and AttendBio Research, Spain, respectively). The kit used for the detection of HS do not show any significant cross-reactivity or interference between HS and analogs according to the manufacturer´s instructions.

Biomarkers for *coagulation/fibrinolysis* included von Willebrand antigen (VWF:Ag) and activity (VWF:GPIbM) and α2-antiplasmin (α2AP) evaluated by immunoturbidimetry (Atellica 180 360 COAG, Siemens Healthineers, Germany). Visualization of VWF multimers

was achieved using a commercially available enhanced chemiluminescence kit for detecting HRP-labeled antibodies on Western blots.[29] In addition, VWF factor-cleaving protease (ADAMTS-13) activity was assessed by fluorescence resonance energy transfer (Fluoroskan Ascent FL; Thermolab Systems, MA, USA). Plasminogen activator inhibitor antigen (PAI) and thrombomodulin (TM) were measured by ELISA (Imubind, Toronto, Canada; and Biomatik Corporation, MN, USA, respectively).

Activation of *innate immune response* was determined by circulating terminal complement complex (C5b9) and dsDNA for neutrophil extracellular traps (NETS) quantified by Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen, Thermo Fisher, MA, USA) on a fluorescence reader.

Angiogenic profile was assessed by sera concentrations of free Placental Growth Factor (PlGF) and soluble fms-like tyrosine kinase-1 (sFlt1) by ELISA (R&D Systems Europe Ltd, Abingdon, UK) and Angiopoietin-2 (Ang2) (R&D systems, MN, USA). The ratio sFlt1/PlGF 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 patient's sera in order to study cell response to: a) the expression of adhesion receptors at the cell surface (InterCellular Adhesion Molecule 1, ICAM-1), as an indicator of a proinflammatory response of cells; b) the presence or the adhesive protein VWF, involved in thrombogenicity, on the extracellular matrix generated by these cells; and c) the activation of the endothelial intracellular signaling pathway related to inflammation p38MAPK. Details of the laboratory methodology used is detailed in the Supplementary material.

Statistical analysis

Baseline and perinatal data were analyzed with the statistical software STATA 14.2 (StataCorp LLC, Texas, USA) and results are expressed as median and interquartile range or percentage as appropriate. Statistical analysis comprised the comparison of each group of complicated pregnancies vs. controls. Soluble markers are expressed as median (interquartile range). Further statistical analyses were performed in R (version 4.0.0) using Student's 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 < 0.05. Data were ordinated and plotted using principal component analysis. An additional unsupervised hierarchical clustering was performed based on the univariate results comparing severe COVID-19 vs. preeclampsia. A sub analysis comparing early- vs late-onset preeclampsia was performed using Student's t-test and Benjamini-Hochberg procedure for multiple pairwise comparisons and included in the Supplementary material.

RESULTS

Baseline and perinatal characteristics of the study populations

Baseline characteristics of the study populations are summarized in Table 1. Study groups were mainly similar in terms of maternal and perinatal characteristics. However, preeclamptic patients had higher rates of Asian ethnicity and a tendency to younger age. Chronic hypertension was present in three preeclamptic patients and systemic lupus erythematosus in one control. None of the patients included in this study had pregestational diabetes or previous respiratory disorders. All the pregnancies complicated by preeclampsia were proteinuric, four were early-onset cases that were treated with corticosteroids for fetal lung maturity and five preeclamptic patients had preeclampsia with severe features that was treated with magnesium sulfate. Preeclamptic patients showed an earlier gestational age at delivery with a trend to higher rates of small-for-gestational-age fetus and admissions to 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, two were detected by RT-PCR and the rest by positive serology. Since this study has been conducted at the beginning of the pandemic, convalescent subjects should have been infected during the four weeks preceding the blood analysis. Two cases of mild COVID-19 had hypertension and one 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 as none of them presented signs of placental insufficiency nor abnormal sFlt1/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 one case required invasive mechanical ventilation). Six

patients with severe COVID-19 were treated with low-molecular-weight heparin, three of them were additionally treated with hydroxychloroquine and azithromycin and one of these three has been 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 median (interquartile range) of 40.2 (38.9 -41) weeks in controls, 39.1 (38.7 – 39.6) weeks in mild COVID-19, 39.3 (34.9 – 41.1) weeks in severe COVID-19 and 39.1 (35.1 – 39.6) weeks in preeclampsia. 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 Table S1. 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, immune and angiogenic biomarkers were detected in **severe COVID-19** including significantly higher concentrations of VCAM-1, sTNFR-I, HS, VWF:Ag and NETS with a significant reduction of PlGF as compared to controls. No differences were observed in Ang2, sFlt1, C5b9, ADAMTS13, PAI nor TM between severe COVID-19 and controls. Pregnant women with preeclampsia exhibited also 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, sTNFR-I (significantly increased compared to controls and to severe COVID-19) 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/PlGF ratio were also observed in preeclampsia. In addition, a significant increase in C5b9 and NETS was also

292 detected in preeclampsia compared to controls. HS, ADAMTS13, PAI and TM remained
293 unchanged in preeclampsia.

294 Principal component analysis demonstrated a clear separation between preeclampsia and the
295 other study populations (controls and mild/severe COVID-19) (Figure 2A). The first and second
296 components explained 42.2% and 13.5% of the variance between groups. Unsupervised
297 hierarchical clustering also showed a complete separation between severe COVID-19 cases and
298 preeclampsia (Figure 2B) with the most remarkable differences observed in VWF:GPIbM,
299 VWF:Ag and VWF:Ag/ VWF:GPIbM followed by HS (significantly lower in preeclampsia)
300 and sTNFRI, sFlt1 and sFlt-1/PlGF ratio (significantly higher in preeclampsia).

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

305 A sub analysis revealed a similar pattern of endothelial damage, coagulopathy and angiogenic
306 imbalance in early- vs late-onset preeclampsia (Supplementary Table S2), with much
307 remarkable changes in early-onset cases. In contrast, C5b9 and NETS were more altered in late-
308 onset preeclampsia.

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

311 Endothelial cells incubation with sera from mild and severe COVID-19 patients induced a
312 significant overexpression of ICAM-1 and VWF compared with controls (Figure 4). Cells
313 exposed to preeclampsia sera showed also significantly increased ICAM-1 and VWF

314 expression although preeclampsia effect was less pronounced than the one caused by severe
315 COVID-19 ($p<0.05$).

316 Immunoblots of lysates from endothelial cells exposed to mild and severe COVID-19, and
317 preeclampsia sera showed an increase in p38MAPK phosphorylation. Severe COVID-19 and
318 preeclampsia were statistically different from controls (Figure 5), suggesting that both severe
319 COVID-19 and preeclampsia sera can activate inflammatory signaling pathways.

COMMENT

Principal findings of the study

A comprehensive *ex vivo* and *in vitro* study revealed distinct endothelial and angiogenic profiles of severe COVID-19 *versus* preeclampsia. While severe COVID-19 exhibited alterations in heparan sulfate (HS), neutrophil extracellular traps (NETS) and placental growth factor (PlGF), preeclampsia presented abnormal levels of vascular adhesion molecule-1 (VCAM-1), soluble TNF receptor type I (sTNFRI), von Willebrand factor (VWF), complement C5b9, angiopoietin 2 (Ang2) and soluble fms-like tyrosine kinase-1 (sFlt-1). Sera from both severe COVID-19 patients and preeclampsia induced an overexpression of intercellular adhesion molecule-1 (ICAM-1) and VWF and activation of p38MAPK phosphorylation in endothelial cells in culture even though the effect of preeclampsia was less pronounced than the one triggered by 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. Preeclamptic patients 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 pro-inflammatory 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 is in line with previous reports on non-pregnant COVID-19 patients showing a good correlation of VCAM-1 and sTNFRI with disease severity[12,16]. The increased levels of HS suggest endothelial glycocalyx barrier disruption and degradation. This finding is consistent with previous reports in critically ill non-pregnant COVID-19 patients as 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 VWF antigen and functionality

Interestingly, the most remarkable differences between preeclampsia and COVID-19 were observed in VWF concentrations and activity. Our data in 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 to this observation is acute VWF consumption due to endothelial cell exhaustion[37] in preeclampsia, as 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/VWF:Ag ratio and confirmed by the multimeric analysis of VWF. Since ADAMTS-13 activity was similar in preeclampsia and the other study groups, the loss of high-molecular-weight multimers might be due 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 preeclampsia 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 since α2AP was significantly reduced in preeclampsia compared to the other groups.

Innate immune dysregulation in preeclampsia vs. COVID-19 in pregnancy

Our data confirm the previously reported increase in the 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 in order to capture extracellular pathogens, limit the spread of infections and directly activate alternative complement pathway. Our results are consistent with the previously reported activation of NETS directly by SARS-CoV-2 in non-pregnant individuals[42]. Hyperactivation of NETS formation in preeclampsia has been proposed to be induced by placental 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 pathological cascade leading to endothelial damage in both SARS-CoV-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 as compared to controls and COVID-19 with very high levels of sFlt-1 and Ang2 together with reduced PlGF[48]. As previously described, angiogenic was more severely altered in early-onset preeclampsia[49]. Interestingly, COVID-19 cases showed also significantly low PlGF but normal concentrations of sFlt-1 and therefore preserved sFlt-1/PlGF ratio. PlGF is mainly synthesized in the endothelium, which might explain a reduction in any case of endothelial damage. In contrast, sFlt-1 and Ang2 seems to be distinctive of preeclampsia. These findings are consistent with angiogenesis dysregulation being proposed one of the main pathophysiological features in the development of preeclampsia.[19,30] These results are also in line with previous reports[50] proposing sFlt1/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 p38MAPK induced by both severe COVID-19 and preeclampsia sera, together 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] While it is known that SARS-CoV-2 infection activates p38MAPK 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 p38MAPK inhibition pointing out its potential

413 therapeutic effect[53] These data suggest that, despite their different pathophysiology, both
414 preeclampsia and COVID-19 finally activate common pathways of endothelial dysfunction
415 explaining similarities in the clinical scenario.

416

417 **Strengths and limitations**

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

428

429 **Conclusion, clinical and research implications**

430 In conclusion, this study suggests a differential profile of circulating biomarkers with a similar
431 end-stage *in vitro* induced endothelial dysfunction. Soluble biomarkers of coagulopathy
432 [VWF], endothelial inflammation [sTNFRI], barrier damage [HS] and angiogenesis [sFlt1]
433 seem to be highly specific to differentiate preeclampsia from severe COVID-19 in pregnancy.
434 These findings hold the potential to improve our understanding of the pathophysiological

pathways in preeclampsia and COVID-19 in pregnancy. We also identify circulating biomarkers that may be useful in the differential diagnosis of preeclampsia and SARS-CoV-2 infection in pregnancy. Given the difficulty of clinically differentiate some cases of preeclampsia and COVID-19, a panel of circulating biomarkers for the differential diagnosis could be of most help to optimize patient's management. Finally, this study also opens opportunities for new therapeutic targets that could improve the underlying endothelial damage observed in these entities.

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608 **Tables**

609 **Table 1. Baseline and perinatal characteristics of the study populations.**

610

	Controls n=10	**Mild COVID-19 n=9**	**Severe COVID-19 n=8**	**Preeclampsia n=13**
Maternal characteristics				
Age (years)	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) *
Pre-gestational 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) *
Preterm delivery#	1 (10)	1 (11.1)	2 (25)	4 (30.8)
Cesarean section	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	3 (30)	0 (0)	0 (0)	7 (53.8)

ageΨ				
APGAR score 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 n (%) as appropriate.

Preterm delivery defined as delivery occurring before 37 weeks of gestation.

Ψ Small for gestational age defined as birthweight below the 10th centile according to local standards.

* $p<0.05$ by Mann Whitney U test, Pearson χ^2 or Fisher exact tests as appropriate, as compared to controls.

FIGURE LEGENDS

Figure 1. 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 1st and 3rd quartiles. The whiskers point to the maximum and the minimum values of the sample. For a better visualization of data points 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's t-test, Benjamini-Hochberg procedure for multiple pairwise comparisons) are noted as *$p<0.05$ and ** $p<0.01$ vs. Control, $ $p<0.05$ and $$ $p<0.01$ vs. mild COVID-19, and # $p<0.05$ and ## $p<0.01$ 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).

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: 2A) 2-dimensional principal component analysis (to visualize the distribution in 2 dimensions of the variability in the different study groups) 2B) Unsupervised hierarchical clustering based on univariate analysis comparing severe COVID-19 vs. 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). VWFAg, VWF antigen; VWFGPIbM, VWF activity; alpha2AP, α2-antiplasmin; sFlt-1, soluble fms-like tyrosine kinase-1; PlGF, placental growth factor; Ang2, Angiopoetin 2; VCAM-1, vascular cell adhesion molecule-1, TNFRI, soluble TNF-α receptor I, HS, heparan sulfate; NETS, neutrophil extracellular traps; PAI, plasminogen activator inhibitor; TM, thrombomodulin.

643 **Figure 3. VWF multimeric analysis in preeclampsia and severe COVID-19 patients**. This
644 analysis was performed to confirm the qualitative defects of this protein suggested by the low
645 VWF:Ag/VWF:GPIb detected in PE patients. A normalized 1.2% multimer gel (A) and
646 densitometry (B) of plasma VWF multimers from normal control (C), patient with known von
647 Willebrand disease Type 2A as a positive control (VWD2A) (characterized by a loss of high
648 molecular weight multimers and an increase in the low molecular weight multimers), 4
649 preeclampsia patients (PE), and 2 pregnant women with severe COVID-19 (sCOVID-19). Of
650 note, each sample dilution was performed following the antigenic concentration (VWF:Ag)
651 previously obtained (additionally it was an indirect confirmation of the results). Sample
652 identification is followed by the dilution used to resolve VWF multimeric pattern.
653
654 **Figure 4. Expression of ICAM-1 and VWF in cultured endothelial cells: effect of COVID-**
655 **19 and preeclampsia sera**. Changes in inflammation and thrombogenic phenotypes induced
656 by the study conditions were explored through an *in vitro* approach consisting of the exposure
657 of endothelial cells in culture to patients' sera. Representative fluorescence micrographs
658 showing ICAM-1 expression (in green, on the left panel) on cell surface and VWF release (in
659 red, on the right panel) on endothelial cells in culture supplemented with serum from controls
660 (C) or mild and severe COVID-19 (m and sCOVID-19) and preeclampsia (PE). The boxplots
661 represent the quantitative assessment of ICAM-1 expression and VWF release. The line in the
662 boxes depicts the sample median and the boxes are the 1st and 3rd quartiles. The whiskers point
663 to the maximum and the minimum values of the sample ($n = 6$, $^{*}P < 0.05$ vs. Controls and #P
664 < 0.05 comparison between sCOVID-19 and PE).
665
666 **Figure 5. Inflammatory signaling pathways in endothelial cells exposed to mild and severe**
667 **COVID-19 and preeclampsia milieu.** The analysis of signaling pathways activation was

performed by exposing resting endothelial cells to the sera under study. In the present study, activation of 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 B-actin, and the boxplots represent the relative quantification of p38 MAPK / B-actin compared to controls (n=3, *P < 0.05 vs. controls).

675 **Glossary of terms**

676

677 • A disintegrin and metalloproteinase with thrombospondin type 1 motif, 13 (ADAMTS-
678 13): is primarily synthesized in the liver, and its main function is to cleave von
679 Willebrand factor (VWF) anchored on the endothelial surface, in circulation, and at the
680 sites of vascular injury.

681

682 • Angiopoetin 2 (Ang2): is produced by endothelial cells and acts as an autocrine
683 regulator mediating vascular destabilization and regulating vascular homeostasis.

684

685 • α2-antiplasmin (α2AP): is a serine protease inhibitor (serpin) responsible for
686 inactivating plasmin.

687

688 • Endothelium: composed by endothelial cells plays an important role in inflammation by
689 regulating vascular permeability for macromolecules and leukocytes, vascular tone and
690 hemostasis, and by binding and producing and inflammatory mediators such as
691 cytokines.

692

693 • *Ex vivo* approach: to quantify the degree of endothelial activation is of interest when
694 evaluating inflammation. Due to the localization of this type of cells, this evaluation
695 cannot be carried out directly and a number of indirect measures such as the
696 measurement of soluble molecules released by the endothelium has been employed
697 instead.

698

- 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 upregulated in preeclampsia.

- sFlt-1/PIGF ratio: an imbalance in these two biomarkers levels has been reported to be involved in preeclampsia pathogenesis. An elevated sFlt-1/PIGF seems to be highly predictive of preeclampsia.

- Heparan sulfate (HS): is 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 patient's sera in order 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 upregulated during endothelial activation and mediates lymphocyte binding. This molecule is not only released from 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 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 a smaller ones. This method preserves as much information as possible and the resulting data set become easier to explore and visualize than the original one.

- p38 mitogen-activated protein kinase (P38MAPK): plays a pivotal role 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 cell that leads to cell lysis and death.

- Soluble TNF receptor type I (sTNFRI): is one of the two soluble receptors of TNF-alpha (TNFα), a proinflammatory cytokine that plays a central role in inflammation, which act as physiological attenuator of TNFα activity.

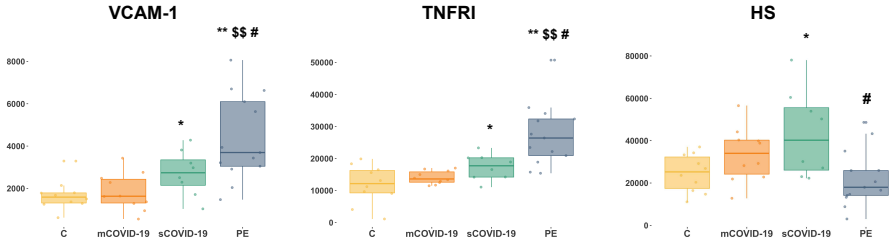
- Thrombomodulin (TM): is a thrombin receptor on endothelial cells that is involved in promoting activation of the anticoagulant protein C pathway during blood coagulation.

- Vascular Cell Adhesion Molecule -1 (VCAM-1): adhesion molecule that is upregulated during endothelial activation and mediates lymphocyte binding. Elevated levels of soluble VCAM-1 have been reported in preeclampsia.

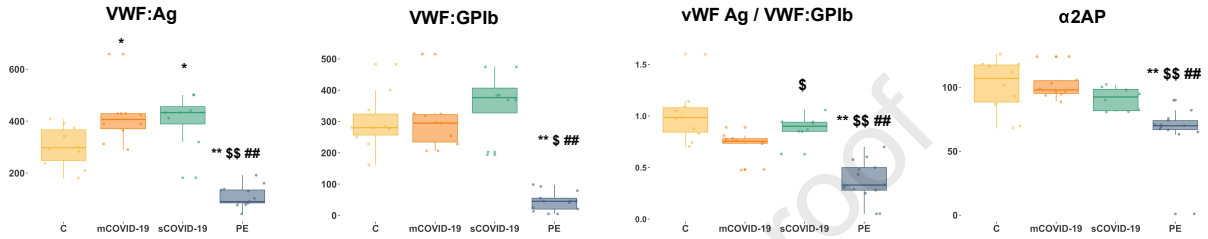
- von Willebrand Factor (VWF): a multimeric blood protein primarily synthesized, stored and secreted by endothelial cells. It constitutes a marker of acute and chronical inflammation. The analysis of this protein implies both antigen concentration (VWF:Ag) and functionality (VWF:GPIbM).

- von Willebrand Factor multimeric analysis: is a method carried out by electrophoresis of plasma samples using non-reducing agarose gels in the presence of different concentrations of sodium dodecyl sulphate. This analysis aims to identify qualitative defects of this protein and is usually performed after functional and immunological VWF assays indicate a potential abnormality.

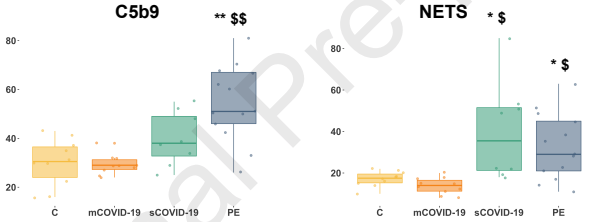
Biomarkers of endothelial damage



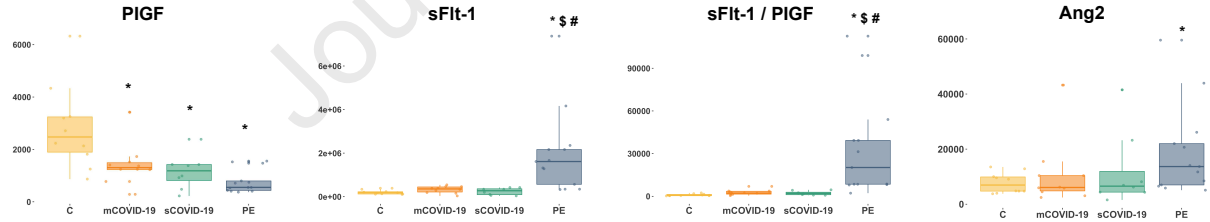
Biomarkers of coagulopathy/fibrinolysis



Biomarkers of innate immune response



Biomarkers of angiogenesis



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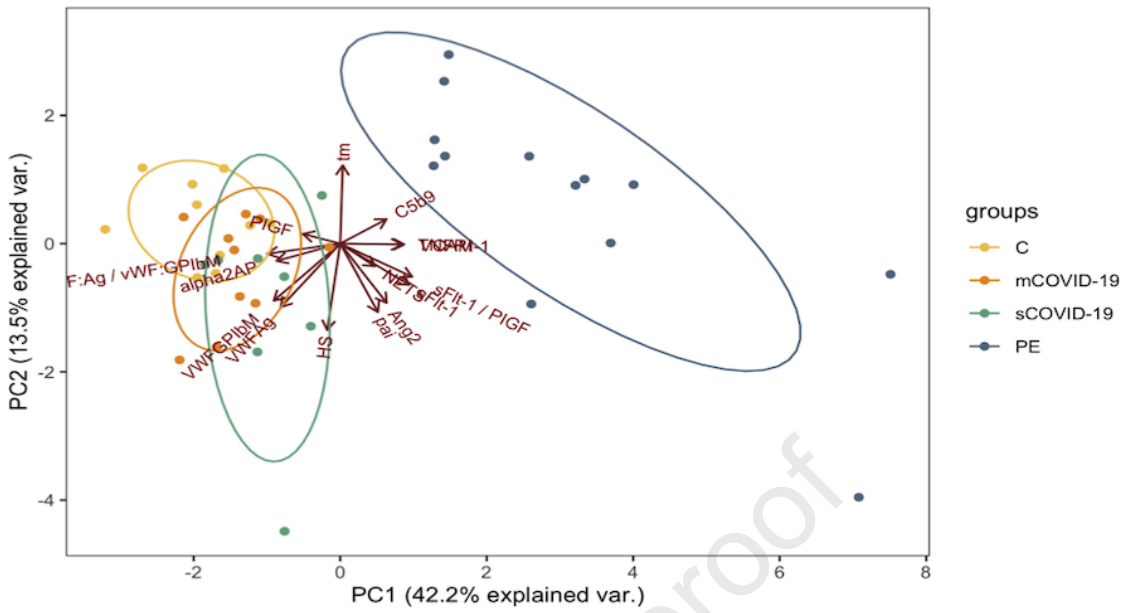

B