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Vascular endothelial damage in the pathogenesis of organ injury in severe COVID-19

Annabelle Dupont,^{1*} PharmD,PhD; Antoine Rauch,^{1*} MD,PhD; Senna Staessens,¹ MSc; Mouhamed Moussa,¹ MD; Mickael Rosa,¹ PhD; Delphine Corseaux,¹ PhD; Emmanuelle Jeanpierre,¹ PharmD; Julien Goutay,² MD; Morgan Caplan,² MD; Pauline Varlet,³ PharmD; Guillaume Lefevre,³ MD,PhD; Fanny Lassalle,¹ PharmD; Anne Bauters,⁴ PharmD; Karine Faure,⁵ MD,PhD; Marc Lambert,⁶ MD,PhD; Alain Duhamel,⁷ MD,PhD; Julien Labreuche,⁷ BST; Delphine Garrigue,⁸ MD; Simon F De Meyer,⁹ PhD; Bart Staels,¹ PhD; Flavien Vincent,¹ MD,PhD; Natacha Rouse,¹⁰ MD; Eric Kipnis,¹¹ MD,PhD; Peter Lenting,¹² PhD, Julien Poissy,¹³ MD,PhD; Sophie Susen,¹ MD,PhD; and the Lille Covid Research Network (LICORNE)

¹ Univ. Lille, Inserm, CHU Lille, Institut Pasteur de Lille, U1011- EGID, F-59000 Lille, France

² CHU Lille, Intensive Care Department, Pôle de Réanimation, F-59000 Lille, France

³ Univ. Lille, Inserm, CHU Lille, U1286 - INFINITE - Institute for Translational Research in Inflammation, F-59000 Lille, France

⁴ CHU Lille, Institut d'Hématologie-Transfusion, F-59000 Lille, France

⁵ Univ. Lille, Inserm, CHU Lille, Institut Pasteur de Lille, UMR1019-CIIL, 59000, Lille, France

⁶ Univ. Lille, Inserm, CHU Lille, INSERM U 1167, Institut Pasteur, 59000 Lille, France

⁷ Univ. Lille, CHU Lille, ULR 2694 - METRICS: Évaluation des technologies de santé et des pratiques médicales, F-59000 Lille, France

⁸ CHU Lille, Surgical Critical Care, Department of Anesthesiology and Critical Care, F-59000 Lille, France

⁹ Laboratory for Thrombosis Research, KU Leuven Campus Kulak Kortrijk, Kortrijk, Belgium
CHU Lille,

¹⁰ CHU Lille, Service de Chirurgie Cardiaque, F-59000 Lille, France

¹¹ Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 - UMR 9017 - CIIL - Center for Infection and Immunity of Lille, F-59000 Lille, France

¹² Inserm, UMR_1176, Université Paris-Saclay, 94276 Le Kremlin-Bicêtre, France

¹³ Univ. Lille, Inserm U1285, CHU Lille, CNRS, UMR 8576 - UGSF - Unité de Glycobiologie Structurale et Fonctionnelle, F-59000, Lille, France

* Pr Dupont and Dr Rauch contributed equally to this article.

Corresponding author:

Sophie Susen, sophiesusen@aol.com, Heart and Lung Institute, Hemostasis Department, Bd du Pr Leclercq, CHU Lille, 59037 Lille cedex, France.
Phone +33320444891, Fax +33320446504

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Abstract

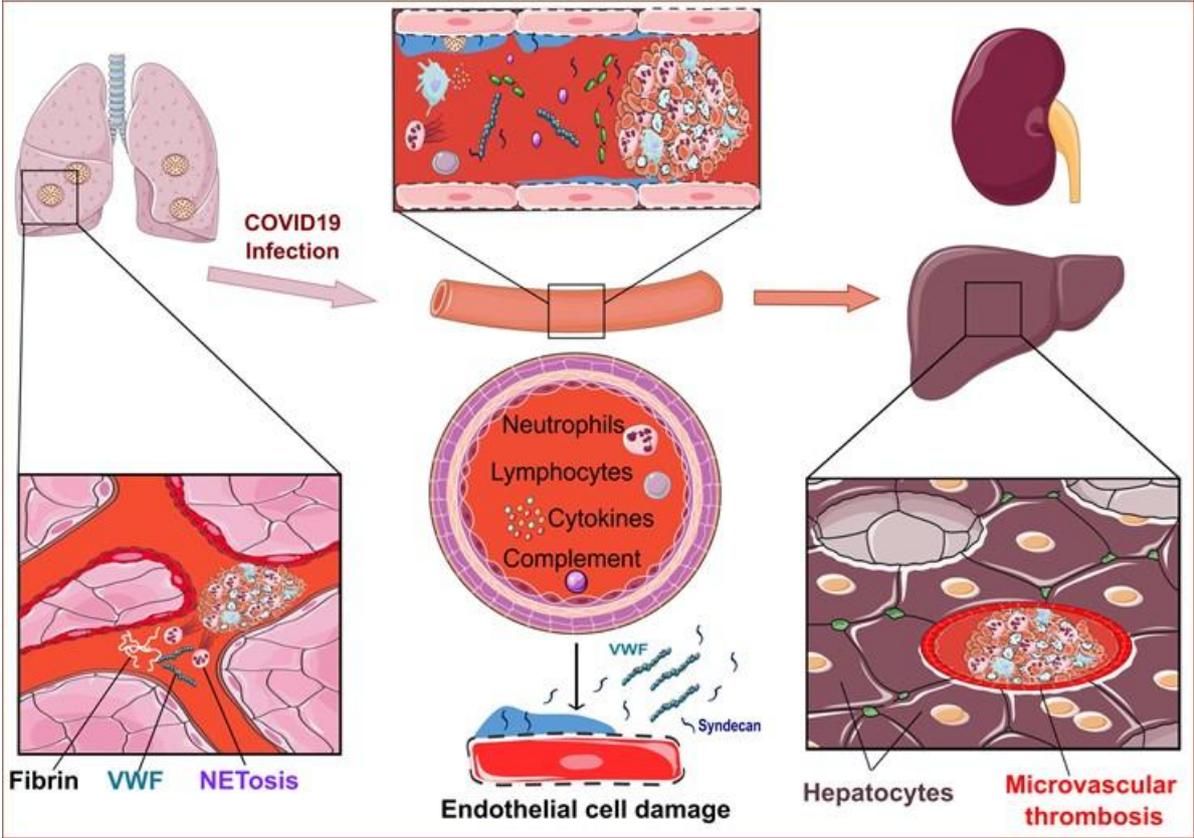
Objective: Whether endotheliopathy only mirrors COVID-19 severity or plays an intrinsic role in microvascular thrombosis and organ failure remains unanswered. We assessed whether markers of endothelial damage and immune dysregulation were associated with organ failure and death.

Approach: Markers of endothelial damage (VWF:Ag, PAI-1, syndecan-1, TFPI and soluble thrombomodulin), complement activation (C5a and C5b-9), cytokines (IL-6, TNF α , IL-2R) and NETs (cell-free DNA, nucleosomes, myeloperoxidase-DNA) were measured at intensive care unit admission in 82 COVID-19 patients. We also analyzed the histological composition of thrombi collected in living-patients successfully weaned from extracorporeal membrane oxygenation.

Results: Beside respiratory failure and death, VWF:Ag, PAI-1, TFPI and syndecan-1 were independently associated with liver injury and multi-organ failure development (all $p < 0.01$), underlining the direct role of endotheliopathy in organ failure. Moreover, dysregulated immune response including cytokines, complement and NETs was associated with markers of endothelial damage, respiratory failure and liver injury and COVID-19 thrombi contained a large accumulation of neutrophils, VWF and NETs.

Conclusion: This study establishes a link in severe COVID-19 patients between dysregulated immune response and respiratory and liver failure due to endothelial and microvascular damage and provide new evidence for a major role of VWF, a binding partner of NETs, as a scaffold for thrombus formation in these patients.

Graphic abstract



Abbreviations

BMI: body-mass-index

ICU: intensive care unit

NET: neutrophil extracellular trap

PAI-1: plasminogen activator inhibitor-1

TFPI: tissue factor pathway inhibitor

VV-ECMO: veno-venous extracorporeal membrane oxygenation

VWF:Ag: von Willebrand factor antigen

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that causes coronavirus disease 2019 (COVID-19), preferentially infects respiratory epithelial cells, but can also result in extra-pulmonary manifestations such as myocardial, hepatocellular or acute kidney injury.^{1,2} COVID-19 is also associated with a pro-thrombotic phenotype, elevated laboratory markers of coagulopathy and an increased risk of thrombosis first identified through manifestations of large vessels thrombosis.³⁻⁸ There is also growing evidence suggesting that microvascular thrombosis is likely a major pathophysiologic event in COVID-19.^{9,10} The angiotensin-converting enzyme 2 (ACE2) receptor, essential for SARS-CoV-2 uptake by host cells, is highly expressed on endothelial cells. Post-mortem lung histological analyses have reported the presence of vascular inflammation and severe endothelial injury as a direct consequence of SARS-CoV-2 intracellular infection.^{11,12} Although direct viral tissue damage is a plausible mechanism of endothelial injury, a dysregulated immune response could also be involved.² Using cultured human pulmonary microvascular endothelial cells, we demonstrated that plasma collected from critically-ill COVID-19 patients triggers endothelial damage *in vitro*.¹³ Interestingly, this plasma-induced endothelial cytotoxicity was associated in patients with a more pronounced hypoxemia and higher circulating levels of biomarkers of endothelial damage and a dysregulated immune response. This *in vitro* response could be a marker of *in vivo* microvascular damage. Once injured, endothelial cells can activate coagulation leading to widespread microvascular thrombi and promote multi-organ failure. To date, reports assessing the presence of endothelial damage and microvascular thrombosis in COVID-19 patients focused on respiratory failure or death as main outcomes.¹⁴⁻¹⁸

To address the question whether endothelial damage and microvascular thrombosis could occur in other organs, we assessed whether the degree of endotheliopathy at intensive care unit (ICU)-admission is an early indicator of organ failure (hepatic, renal, hemodynamic or multi-organ failure) in addition to respiratory failure by investigating a large panel of endothelial damage markers (von Willebrand factor [VWF], plasminogen activator inhibitor-1 [PAI-1], tissue factor pathway inhibitor [TFPI], soluble thrombomodulin [sThrombomodulin] and endothelial-glycocalyx disruption [syndecan-1]). As a dysregulated immune response in COVID-19 has also been proposed to mediate organ dysfunction through several pathways inducing endothelial damage and microvascular thrombosis, we evaluated if endotheliopathy and organ failure are associated with a dysregulated immune response.^{19,20} Finally, we investigated the composition of thrombi collected in living critically-ill COVID-19 patients.

Material and methods

Patients

Consecutive patients admitted for COVID-19 were recruited from the intensive-care-unit (ICU) of Lille-University-Hospital between March 21th and April 16th, 2020. Inclusion criteria were: individuals aged 18 years or older with a positive SARS-CoV-2 RT-PCR. Patients received enoxaparin or unfractionated heparin according to their renal status and the need for invasive procedures. In overweight and obese patients, the dosing regimen was adapted according to the European Society of Cardiology proposals.²¹ From April 6th onwards, patients received thromboprophylaxis according to the GFHT/GIHP proposals.²² This study was approved by the French institutional authority for personal data protection (Commission Nationale de l'Informatique et des Libertés (CNIL), registration number DEC20-086), and ethics committee (ID-CRB-2020-A00763-36) and informed consent was obtained from all participants. The protocol was registered as a clinical trial (registration number NCT-04327180).

Outcomes

The outcomes were the occurrence of any organ injury, respiratory failure and/or development of renal, hemodynamic, liver injury or multi-organ injury within 14 days of ICU-admission or all-cause 28-day mortality. Secondary respiratory failure was defined by the need for mechanical ventilation. Kidney, liver and shock components of the SOFA score were used to assess the presence of renal, liver or hemodynamic injuries. Renal-failure was defined as presence or any aggravation of renal SOFA and/or KDIGO score from ICU admission; liver-injury as presence or any aggravation of hepatic SOFA score from ICU admission or alanine aminotransferase (ALT) or aspartate transaminase (AST) values more than thrice the upper limit of normal; Hemodynamic dysfunction as a need for epinephrine or norepinephrine support at a dose > 0.1 µg/kg/min. SOFA scores were evaluated at ICU-admission and on ICU days 4, 7 and 14. If the patient was discharged from ICU before day 14, the last SOFA score recorded during ICU stay was used. Multi-organ injury was defined by the presence of more than one dysfunction.

Follow-up period was limited to 14 days to minimize the contribution of long-term ICU complications unspecific to COVID-19 as confounding event in outcome assessment.

Laboratory testing

Blood was collected at ICU-admission for each patient on 0.109M trisodium citrate tube (BD Vacutainer®, BD Diagnostics, Sparks, MD, USA). All analysis were performed on platelet-poor plasma obtained after a double centrifugation at 2500g for 15 min at room temperature. ELISA assays were performed to measure sThrombomodulin, syndecan and nucleosomes using Human Thrombomodulin/BDCA-3 Quantikine® ELISA kit (R&D Systems, Minneapolis, MN, USA), Human sCD138 ELISA kit (Diacclone, Besançon, France) and Cell Death Detection ELISAPLUS kit (Roche Diagnostics, Bâle, Suisse), respectively. TFPI was measured with Asserachrom total TFPI (Diagnostica Stago, Asnières sur Seine, France) and PAI-1 with Asserachrom PAI-1 (Diagnostica Stago). C5a and C5b-9 were measured using standard ELISA kits (Quidel, San Diego, CA, USA). Prothrombin time (PT) was measured on a STA R Max analyser (Diagnostica Stago) using STA Neoplastin R (Diagnostica Stago). D-dimers levels were measured in µg/ml Fibrinogen Equivalent Units (FEU) using an immunoturbidimetric latex-particle assay (Liatest DDI-Plus, Diagnostica Stago) on the STA R Max analyser (Diagnostica Stago). VWF:Ag was measured using an immunoturbidimetric assay (LIAPHEN vWF:Ag, HYPHEN BioMed, Andresy, France), both on a CS 2400 analyser (Sysmex, Kobe, Japan). ADAMTS-13 activity was measured with the Technozym® ADAMTS13 activity ELISA kit (Technoclone, Vienna, Austria). Cell-free DNA was measured by spectrofluorimetry at 520 nm after excitation at 480 nm with Quant-iT PicoGreen dsDNA assay (Thermo fischer Scientific, Waltham, MA, USA) on a SAFAS spectrophotometer (Monaco, France). For MPO-DNA complexes quantification, we

used the Cell Death Detection Elisa kit (Roche, Basel, Switzerland). All samples were analysed in duplicate.

Interleukin-6 (IL-6), TNF α and soluble interleukin-2 receptor (IL-2R) concentrations were assessed using the Ella Automated Immunoassay System (ProteinSimple, San Jose, CA, USA).

Other laboratory blood dosages including a complete blood count, C-reactive protein (CRP), liver transaminases, bilirubin and creatinine by standard methods as part of patient's care in the Biology and Pathology Center of Lille University Hospital.

Thrombi analysis

Histological analysis was performed on thrombi collected immediately after veno-venous extracorporeal membrane oxygenation (VV-ECMO) removal from 8 COVID-19 patients with successful weaning of ECMO and 3 non-COVID-19 patient requiring VV-ECMO (Supplementary 1 for description). Thrombi were washed in saline, incubated in 4% paraformaldehyde for 24 hours at room temperature and analyzed as previously described.^{23,24}

Statistical analysis

Quantitative variables were expressed as means (\pm standard deviation (SD)) or medians (interquartile range, IQR) and categorical variables as frequencies (percentage). We assessed the association of the endothelial markers and nucleosomes with the occurrence of organ injury (censored at 14 days) and death during ICU-stay using Student test and ANCOVA for adjusted analysis on age, sex and body-mass-index (BMI). A log-transformation of original markers data was performed if required. For each outcome, p-values were adjusted for multiple comparisons using false discovery rate (FDR) procedure. We computed the adjusted effect sizes of the 6 markers using the Cohen's d formula for ANCOVA with the 95% confidence intervals (CI). Spearman correlation coefficient was used to test the association between variables and Mann-Whitney or Kruskal-Wallis test for univariate analysis of continuous variables. Statistical analyses were performed using the SAS software package version 9.4 (SAS Institute, Cary, NC, USA).

Results

Patients characteristics

A total of 82 COVID-19 patients were admitted in ICU (Table 1). At admission, median PaO₂/FiO₂ was 146 mmHg and median SOFA score was 5.9 [2-9]. All patients had increased oxygen requirements with 48 patients (59%) requiring mechanical ventilation, 19 (23%) high-flow oxygen therapy and 15 (18%) low-flow oxygen therapy. Respiratory failure requiring mechanical ventilation was observed in 12 more patients in the 14 days after ICU-admission. Eleven patients (13%) presented with hemodynamic failure while hepatic or renal dysfunction unrelated to pre-existing liver or renal disease were observed in 15 (18%) and 34 (41%) patients respectively. Worsening of the hemodynamic, hepatic or renal status was observed in 12 (15%), 16 (20%) and 6 (7%) patients, respectively. Hepatic dysfunction and hemodynamic failure occurred at a significantly later stage than renal or respiratory failure ($p < 0.001$). Ten patients required a VV-ECMO support and their characteristics are presented in Supplementary Table 1. Eleven patients were receiving heparin (unfractionated heparin, $n=8$; low molecular weight heparin, $n=3$).

Endotheliopathy and dysregulated immune response are associated with altered baseline markers of respiratory and liver function

Baseline levels of VWF:Ag ($p=0.001$), syndecan-1 ($p=0.0005$), and TFPI ($p=0.03$) were significantly higher in patients requiring high-flow oxygen therapy or mechanical ventilation at admission than in patients with lower oxygen requirements (Supplementary Figure 1).

Bilirubin, AST and ALT correlated with most of the endothelial damage biomarkers (VWF:Ag, syndecan-1, TFPI and PAI-1) (Table 2). We also observed a correlation between either PaO₂/FiO₂ ratio, ALT or bilirubin and markers of complement activation (C5a) and NETosis. Bilirubin was also correlated with IL-6. AST was correlated with C5a, cytokines and markers of NETosis (cell-free DNA and nucleosomes). Among cytokines, NETs and complement activation markers, nucleosomes were the immune variable that was the most consistently and significantly associated with impaired liver and respiratory functions. No correlation was observed between endothelial markers, cytokines, complement or NETosis markers with creatinine levels.

Furthermore, a severe imbalance between VWF and ADAMTS13 concentrations (ADAMTS13/VWF:Ag ratio = 0.17 [0.12-0.26]) was present at admission suggesting an impairment in hemostasis promoting microvascular thrombosis.

Overall, when assessing the pattern of organ injury at ICU-admission, the association between immune response and biomarkers of organ dysfunction was mostly apparent for the lungs and the liver.

Occurrence of respiratory failure, liver injury, multi-organ injury and death are associated with endotheliopathy

As shown in Figure 1 and supplementary Table 2, most endothelial damage markers (VWF:Ag, syndecan-1, TFPI and PAI-1) were significantly associated with respiratory failure at admission or occurring during follow-up. After adjustment for age, sex and BMI, these associations remained significant with the strongest effect size for syndecan-1. Overall, this association of endothelial damage at admission with the occurrence of respiratory failures independently of age, sex and BMI, suggests its direct involvement in COVID-19 pneumonia pathogenesis.

We further assessed whether the degree of endothelial damage was also associated with the occurrence of specific organ dysfunctions. Occurrence of liver injury was strongly associated with baseline levels of VWF:Ag, syndecan-1, TFPI and PAI-1. After adjustment for age, sex and BMI, these associations remained significant with the highest effect sizes for TFPI and syndecan-1.

As thrombomodulin levels are dependent on renal function in patients without endothelial damage²⁵, we evaluated the association of renal failure and sThrombomodulin/creatinine ratio. Only high TFPI and sThrombomodulin/creatinine ratio baseline levels were significantly

associated with the occurrence of renal failure. This association remained significant after adjustment. No association was observed between the other endothelial damage markers at admission and the occurrence of hemodynamic failure.

Most endothelial damage markers (VWF:Ag, syndecan-1, TFPI and PAI-1) at ICU-admission were significantly associated with the occurrence of multi-organ injury. After adjustment for age, sex and BMI, this association remained significant for all parameters (see Supplementary Table 2 for unadjusted and adjusted for age, sex, and BMI relations). It was also noticed that VWF:Ag, syndecan-1, TFPI and sThrombomodulin were increased according to the number of organ-injuries (supplementary figure 2). VWF:Ag, syndecan-1 and PAI-1 levels at ICU-admission were significantly associated with all-cause-of-death but this association was no longer significant after adjustment.

Interestingly, the 10 patients who required a VV-ECMO support for acute respiratory distress syndrome (ARDS) presented at ICU-admission higher levels of endothelial damage markers (VWF, syndecan-1 and TFPI) and of IL-6 and a tendency for higher nucleosomes levels ($p=0.09$) (Supplementary Figure 3).

Overall, we demonstrated a strong association between endothelial damage markers at ICU-admission and the occurrence of liver injury and multi-organ failure in critically-ill COVID-19 patients.

Endotheliopathy is associated with dysregulated immune response

We further investigated whether cytokine levels, complement activation and NETosis were associated with the intensity of endotheliopathy (Figure 2). The relation between baseline values of endotheliopathy biomarkers and dysregulated immune response at baseline is presented in Supplementary Table 3. All cytokines correlated with VWF:Ag levels. IL-2R and TNF α also correlated with syndecan-1, PAI-1 levels and sThrombomodulin/creatinine levels. All NETs markers correlated with VWF:Ag while cell-free DNA and nucleosomes both correlated with syndecan-1 and TFPI levels. Cell-free DNA was also correlated with PAI-1 levels. When assessing complement activation, only VWF:Ag correlated with C5b-9 levels. We also observed a correlation between most of the immune markers and D-dimer levels (IL-2R: $r=0.29$, $p=0.008$; IL-6: $r=0.26$, $p=0.02$; cell-free DNA: $r=0.27$, $p=0.02$; nucleosomes: $r=0.25$, $p=0.03$; and C5b-9: $r=0.35$, $p=0.001$), a coagulopathy marker consistently associated with worse outcomes across COVID-19 studies.

Overall, dysregulated immune response including excessive NETosis was associated with endothelial damage markers highlighting an interaction between inflammatory-driven processes and endothelial damage leading to organ failure in critically-ill COVID-19 patients.

Immune response and endotheliopathy promote immunothrombosis

In inflammatory disorders, microvascular thrombosis as a consequence of endothelial damage is involved in organ failure. We aimed to investigate the main drivers of thrombogenicity in COVID-19 through the combined analysis of circulating blood biomarkers reflecting endothelial damage and by investigating the composition of *in vivo* generated thrombi collected in alive patients.

We investigated whether the composition of thrombi collected in critically-ill living COVID-19 patients reflected the involvement of both circulating markers of endothelial damage, measured by VWF staining and immune dysregulation, measured by neutrophils and NETs staining.

We performed a histological analysis of thrombi collected from VV-ECMO tubings from 8 COVID-19 patients and 3 non-COVID-19 patients presenting with ARDS (for main characteristics of patients included in this histological analysis see Supplementary Table 1).

As expected, Martius Scarlet Blue (MSB) and haematoxylin and eosin (H&E) stainings revealed a strong presence of fibrin and VWF. No prominent differences in red blood cells, fibrin, platelet or VWF content between COVID-19 and the control thrombi were observed (Figure 3). In contrast, a striking accumulation of nucleated cellular infiltration was found in

thrombi from COVID-19 patients compared to control. Neutrophil elastase staining revealed that leukocyte infiltrates in COVID-19 thrombi consisted predominantly of neutrophils, with few macrophages detected. Quantitative analysis revealed a significant higher area of H3Cit staining in COVID-19 patients compared to non-COVID-19 patients (Supplementary Figure 4).

In plasma, neutrophil count correlated with markers of NETosis (cell-free DNA: $r=0.51$, $p<0.0001$; nucleosomes: $r=0.45$, $p=0.0005$), endothelial damage (VWF: $r=0.46$, $p=0.0001$; TFPI: $r=0.55$, $p<0.0001$; syndecan-1: $r=0.43$, $p=0.0003$; PAI-1: $r=0.26$, $p=0.04$) and cytokine (IL-2R: $r=0.29$, $p=0.02$). COVID-19 patients displayed higher levels of CRP and fibrinogen than the 3 non COVID-19 patients (295 vs 50 mg/L, $p=0.01$; 8.62 vs 6.1g/L, $p=0.05$ respectively). Compared to control thrombi, COVID-19 thrombi contained significant amounts of intracellular and extracellular citrullinated histones H3 (H3Cit), suggestive of NETosis. Overall, we provide evidence for immuno-thrombosis through endothelial damage and dysregulated immune responses, in particular NETs formation, in the pathogenesis of severe COVID-19. VWF released from damaged endothelial cells interacts with NETs released from neutrophils to provide a scaffold for platelet adhesion and thrombus formation.²⁶

Discussion

Using endotheliopathy circulating biomarkers, we demonstrate that endothelial damage is associated with respiratory failure, liver injury, multi-organ failure and death in COVID-19. Most of these associations were still significant after adjustment for age, sex and BMI further underlining the direct involvement of endotheliopathy in organ failure. We also observed a relationship between endothelial damage and the extent of immune inflammatory responses highlighting that inflammatory-driven processes are likely primary drivers of endothelial damage leading to microvascular thrombosis and organ failure in COVID-19. These findings are consistent with our previous finding on the direct *in vitro* acute cytotoxic effect on vascular endothelial cells of plasma collected from critically-ill COVID-19.¹³ Finally we provide evidence for a major role of neutrophils, NETosis and VWF as a scaffold for thrombus formation and new associative data to support the concept that endothelial damage and dysregulated immune response are involved in thrombus formation and organ failure.

The pathophysiology of multi-organ dysfunction in COVID-19 patients is still poorly understood. COVID-19 patients seem to have a specific respiratory failure pattern characterized by an initial good compliance despite severe hypoxemia.²⁷ This discrepancy could be linked to ventilation-perfusion mismatch and the loss of hypoxic pulmonary vasoconstriction, because of endothelial damage with subsequent activation of coagulation and widespread microvascular thrombi formation in the lung and other organs, as well as in the same time hypoperfusion of well ventilated area (REF : Grasselli G et al. Lancet Respir Med 2020) . In the lung, the progressive injury increases vascular permeability then causes the alveolar changes, ground-glass appearance in the parenchymal lung injury, and clearly ARDS.^{28,29} Supporting this concept, we observed that endothelial damage markers are significantly associated with the development of respiratory failure in ICU upon admission to ICU.³⁰ Endothelial glycocalyx damage was evidenced by increased plasma levels of syndecan-1 and TFPI. As TFPI is mainly associated with endothelial cells through binding to heparan sulfate proteoglycans, a main component of the glycocalyx at the surface of endothelial cells, this result further strengthens the hypothesis that glycocalyx disruption is a key-feature of critically-ill COVID-19 patients.³¹ Endothelial damage was also evidenced by unusually high VWF concentrations indicative of an acute release of endothelial Weibel-Palade bodies content in COVID-19 patients, as previously reported for angiopoietin also stored in the same organelles.¹⁴ However, it should be mentioned that the presence of endothelial injury is not specific of COVID-19 since the prognostic value of endothelial biomarkers levels was already reported in patients with septic shock or acute lung injury.³² Prior reports have also extensively link aberrant NET formation to pulmonary diseases, particularly ARDS. Neutrophils from patients with pneumonia-associated ARDS appear "primed" to form NETs, and both the extent of priming and the level of NETs in blood correlate with disease severity and mortality.^{33,34} It is therefore likely that NETs, as a source of extracellular histones, contribute to ARDS and sepsis.^{35,36} There are increasing reports addressing the pathophysiology of ARDS related to COVID-19 using seasonal influenza patients as controls. To date, most of the data were derived from autopsy studies allowing comparisons with historical controls such as patients died from seasonal influenza. Ackermann et al showed distinctive vascular features between patients died from COVID-19 and influenza with lung autopsy of COVID-19 specimens showing more severe endothelial injury with disrupted endothelial cell membranes and alveolar capillary microthrombi.¹² Likewise, Nicolai et al reported a significantly higher percentage of vessels with feature of immunothrombosis in the lung vasculature in COVID-19 patients than in non-COVID pneumonia ones.^{37,38} While being pivotal in the understanding of the pathophysiology of respiratory failure in COVID-19, the major limitation of such studies is inherent to their post-mortem nature with potential confoundings related to long-term ICU complications unspecific to COVID-19. Our study was designed to overcome such limitation using a dual approach including the dosing of an extensive set of biomarkers related to endothelial damage and NETosis at ICU admission and a thrombus analysis performed on thrombi retrieved from alive COVID-19 patients at ECMO weaning. Our study gives further evidence

supporting a critical role for endotheliopathy and immunothrombosis in COVID-19 ARDS pathophysiology since we observed that these biomarkers upon admission were significantly associated with the development not only of respiratory failure but also hepatic and multi-organ failure in ICU.

Beyond respiratory failure, kidney and liver injuries were frequently observed in our COVID-19 cohort as previously reported.^{18, 39-41} The direct relation between biomarkers of endothelial damage, NETosis with different organ dysfunctions has not been established so far during COVID-19 apart from autopsy findings.^{11,12} It is noteworthy to report that the extent of endotheliopathy and microvascular damage at ICU admission in COVID-19 patients is associated not only with respiratory failure but also with the occurrence of multi-organ failure. Indeed, high baseline levels of most endothelial biomarkers were associated with the development of liver injury while this association was not found for hemodynamic failure and only observed with TFPI levels for renal failure suggesting that different mechanisms are contributing to liver and renal injuries in COVID-19 patients. Moreover, liver injury occurred at a later stage than renal dysfunction. Thrombomodulin is increased in patients with renal dysfunction, a situation frequently encountered in critically-ill COVID-19 patients.^{25,41} As such, we only found a weak association between the sThrombomodulin over creatinine ratio and the development of renal injury. A renal tropism of SARS-CoV-2 with a direct cytopathic effect on podocytes and renal tubular epithelial cells could explain our results⁴², although ultrastructure evidence of direct viral replication in the kidney is lacking.

Liver injury occurs in 14 to 53% of COVID-19 patients according to the definition criteria used across studies and patient's illness severity.^{43,44} Raised transaminase levels are also frequently reported despite the fact that ACE2 is poorly expressed on hepatocytes and that SARS-CoV-2 RNAs were not detected in hepatocytes from COVID-19 patients.^{45,46} In contrast, given that ACE2 is highly expressed in the endothelial layer of small hepatic blood vessels, hepatic dysfunction could be related to endotheliopathy rather than to a cytopathic viral effect. The significant association between the degree of endotheliopathy and the development of liver injury is in line with this hypothesis. Liver injury at admission was most frequently observed in patients with elevated cytokines, complement activation and NETosis suggesting that inflammatory-driven processes could promote hepatocyte or liver sinusoidal cell damage in COVID-19 patients. COVID-19 patients with non-alcoholic steatohepatitis (NASH) have a higher likelihood of developing a severe illness, irrespective of obesity.⁴⁷ Interestingly, altered hepatic immune function has been also recently reported to mediate the progression of fatty liver to NASH through pronounced changes in blood immune cell populations.⁴⁸ Whether NASH presence exacerbates the SARS-CoV-2-induced inflammatory process thereby contributing mechanistically to severe COVID-19 deserves further research.

We also clearly demonstrated that thrombi are infiltrated with neutrophils and contain markers suggestive of NETs, confirming the role of neutrophils and NETs in the immunothrombotic complications that characterize COVID-19 pathology.^{37,38,49,50} We also provide new evidence for a major role of VWF, a binding partner of NETs, as a scaffold for thrombus formation in critically-ill COVID-19 patients.⁵¹

Albeit being observational, our results strengthen the concept that mechanical vessel obstruction by aggregated NETs is a major pathogenic determinant of severe COVID-19 leading to vascular damage, thrombosis and organ dysfunction. *In vitro*, it has been shown that sera from COVID-19 patients trigger NET release from control neutrophils.⁵² There is also some evidence that SARS-CoV-2 infection promotes an immunological response unseen with seasonal influenza. This difference is well-illustrated in our ECMO cohort with COVID patients displaying significantly higher levels of CRP and fibrinogen than the 3 patients with non-COVID ARDS). This is in line with the recently published case series also reporting a significantly higher content of NETs in the coronary thrombi of (COVID-19 patients with STEMI compared with historical non-COVID-19 STEMI.⁵³ Whether this

increased NETosis is due to complement activation or to specific interactions between monocytes and neutrophils or to other mechanisms is currently under investigation. Our results clearly suggest a specific role of neutrophils and NETs in the thrombotic process of circulating blood. A marked imbalance between VWF and ADAMTS13 was present at admission indicative of a reduced capacity of ADAMTS13 to process ultra-large VWF multimers, a finding associated with microvascular thrombosis and organ failure in sepsis.⁵⁴ Given that the pro-thrombotic role of VWF fibers included in NETs is dependent on their multimeric size, potent inhibitors of ADAMTS13 activity such as thrombospondin-1 and α -defensins released locally by activated platelets and neutrophils, respectively could potentiate thrombus growth by VWF fibers.⁵⁵⁻⁵⁷

Targeting dysregulated immune responses including neutrophils and NETosis or endotheliopathy might be a valuable and feasible therapeutic approach to prevent thrombosis and improve clinical outcomes of COVID-19. Different targets are currently under investigation.⁵⁸ Drugs that target NETs include molecules that block neutrophils function such as anticytokine therapy (anti-interleukin 6, anti-interleukin 1), that prevent NETosis such as neutrophil elastase, peptidyl arginine deiminase type 4 (PAD4) and that dismantle NETs with DNases I.^{58, 59} Improving NETs clearance through exogenous DNase treatment with recombinant human DNase I is currently under investigation for safety and efficacy in nine clinical trials in COVID-19. Targeting the VWF-ADAMTS13 axis is also an attractive hypothesis. *N*-acetylcysteine that dissolves ultra-large VWF multimers under different conditions in both animal models and humans is also currently under investigation.⁶⁰

The main strengths of our study are: i) our outcomes were not restricted to respiratory failure or death and other organ dysfunctions were investigated, ii) we assessed the occurrence of organ injury in the first 14 days only after ICU-admission and the composition of extra-pulmonary thrombi collected at the acute phase of the disease. Thus our results are less likely to be affected by confounding related to long-term complications unspecific to COVID-19, iii) we combined a large panel of markers and adjusted our results to the main COVID-19 comorbidities to disentangle the role of SARS-CoV-2 on top of comorbidities. Assessing whether the biomarkers associated with multi-organ dysfunction in our study are specific to SARS-CoV-2 or could be translated to other viruses such as SARS, MERS or influenza is a major limitation.

In conclusion our results suggest that organ injury due to endothelial and microvascular damage is associated to immunopathology and may occur in parallel to SARS-CoV-2 intracellular infection.

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Supplemental Materials

Supplementary Table 1, 2 and 3

Supplementary Figure 1, 2, 3 and 4

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Highlights

- In severe COVID-19 patients the degree of endotheliopathy at ICU admission is associated with **different organ dysfunctions** even after adjustment for other risk factors, age, sex and body-mass-index further underlining the direct involvement of endotheliopathy in organ failure.
- There is a relationship between endothelial damage and the extent of immune inflammatory responses in severe COVID-19 patients highlighting that inflammatory-driven processes are likely primary drivers of endothelial damage leading to microvascular thrombosis and organ failure in COVID-19.
- **Histopathological comparison of COVID-19 thrombi collected from ECMO with non COVID-19 specimens shows a specific involvement of NETs in COVID-19 thrombogenesis**
- **VWF released from damaged endothelial cells interacts with NETs released from neutrophils and **could** play a major role as a scaffold for thrombus formation in COVID-19 pathophysiology.**

Table 1. Baseline patients' characteristics according to occurrence of organ injury or death in ICU

	All (n=82)	No organ Failure [†] (n=11)	Respiratory failure (n=60)	Renal injury (n=40)	Liver injury (n=31)	Hemodynamic failure (n=23)	Multi-organ injury (n=49)	Death (n=22)
Demographics								
Age (years)	60 (14)	59 (10)	59 (15)	61 (14)	56 (14)	64 (15)	60 (15)	68 (15)
Sex (male)	64 (78)	7 (70)	48 (80)	31 (77)	25 (80)	20 (87)	40 (81)	18 (82)
Body mass index (kg/m ²)	31.2 (7)	31.4 (7)	32.2 (7)	32 (8)	31.4 (7)	30.8 (6)	31.8 (7)	31.1 (7)
Comorbidities								
Diabetes	22 (27)	4 (40)	15 (25)	12 (30)	5 (15)	4 (17)	11 (22)	8 (36)
Hypertension	44 (54)	70 (70)	30 (50)	20 (50)	16 (48)	12 (52)	26 (53)	16 (73)
Chronic pulmonary disease	21 (26)	1 (10)	15 (25)	14 (35)	8 (26)	5 (22)	16 (32)	7 (32)
Chronic heart failure	6 (8)	2 (20)	5 (8)	5 (12)	1 (3)	3 (13)	5 (10)	1 (4)
Coronary/peripheral arterial disease	8 (10)	0	6 (10)	6 (15)	3 (9)	3 (13)	6 (12)	4 (18)
Stroke	4 (5)	1 (10)	1 (2)	2 (5)	0	0	1 (2)	1 (4)
Chronic liver disease	2 (2)	0	0	2 (5)	0	1 (4)	0	0
Chronic kidney disease	7 (8)	1 (10)	3 (5)	5 (12)	3 (9)	2 (8)	4 (8)	1 (4)
Time to illness onset, days	10.4 (5.5)	10.3 (4)	11.3 (5.5)	10 (6.3)	11 (4.6)	10.7 (5.5)	11.1 (5.8)	10.6 (6.3)
Organ dysfunctions								
Total SOFA*score	5.9 (4)	1.9 (1.3)	7 (4)	7 (4.4)	7.4 (4)	7.3 (4.6)	7.1 (4.2)	7 (4.1)
PaO ₂ /FiO ₂ ratio, mmHg	146 (88)	161 (76)	126 (68)	152 (103)	118 (71)	131 (74)	124 (67)	120 (74)
Need for invasive ventilation*	48 (59)	0	48 (80)	20 (50)	11 (35)	10 (43)	32 (65)	14 (63)
Renal SOFA score*	0 [0-1]	1 [0-2]	0 [0-1]	0 [0-1]	0 [0-1]	0 [0-2]	0 [0-1]	0 [0-2]
KDGO score*	1 [0-2]	0 [0-0.25]	1 [0-3]	2 [0-3]	1 [0-2]	1.5 [0-3]	1 [0-3]	1 [0-2.5]
Hepatic SOFA score*	0 [0-0]	0 [0-0]	0 [0-0]	0 [0-0]	0 [0-1]	0 [0-0]	0 [0-0]	0 [0-0]
Hepatic SOFA score ≥ 1*	10 (12)	1 (10)	8 (13)	5 (12)	8 (26)	4 (17)	7 (14)	2 (9)
ALT or AST > thrice ULN*	8 (10)	0	8 (13)	4 (10)	7 (22)	4 (17)	8 (16)	3 (14)
Hepatic SOFA score ≥ 1 or ALT/AST > thrice ULN*	14 (17)	0	12 (20)	7 (17)	15 (48)	6 (26)	11 (22)	4 (18)

Need for epinephrine or norepinephrine support at a dose > 0.1 µg/kg/min*	11 (48)	0	10 (16)	6 (15)	5 (16)	11 (48)	11 (48)	5 (23)
Median time to organ dysfunction or death, days			0 (0-4)	0 (0-7)	4 (0-14)	4 (0-14)	0 (0-7)	6 (1-38)

Laboratory markers

CRP, mg/L	169 (124)	87 (55)	201 (128)	190 (121)	199 (113)	219 (153)	208 (128)	183 (112)
PT ratio	1.28 (0.6)	1.28 (0.44)	1.22 (0.2)	1.38 (0.8)	1.20 (0.15)	1.43 (1.08)	1.33 (0.77)	1.53 (1.12)
D-dimer, µg/mL	2.4 [1.1-4.7]	1 [0.8-6.1]	3 [1.6-4.7]	2.7 [1.3-4.6]	3 [1.4-5.7]	3.3 [2.1-6.6]	2.9 [1.5-4.6]	3.1 [1.8-4.7]
Platelets, G/L	271 (114)	261 (82)	287 (122)	258 (108)	289 (132)	276 (121)	278 (126)	276 (133)
Neutrophils, G/L	9 (5.3)	5.8 (2.4)	10.2 (5.5)	11.7 (6.3)	11.2 (6.2)	10.6 (4.9)	11.1 (5.7)	10.2 (4.5)
ALT, IU/L	54 (41)	40 (10)	58 (45)	47 (33)	77 (52)	57 (44)	59 (47)	52 (39)
AST, IU/L	73 (58)	39 (13)	81 (64)	74 (53)	107 (68)	89 (73)	89 (69)	87 (74)
Bilirubin, mg/L	6.8 (4.3)	6 (2.9)	6.9 (4.5)	6.7 (4.2)	9.2 (5.5)	8.2 (5.8)	7.2 (4.8)	7.1 (5.1)
Creatinine, mg/L	9 [7-14]	13 [9-23]	9 [7-13]	9 [7-14]	9 [7-12]	9 [8-19]	9 [7-12]	9.5 [7.7-20]

Values are number (%) otherwise stated: mean (standard deviation) or median [interquartile]. Secondary respiratory failure was defined by the need for mechanical ventilation. Kidney, liver and shock components of the SOFA score were used to assess the presence of renal, liver or hemodynamic dysfunctions. Renal failure was defined as presence or any aggravation of renal SOFA and/or KDIGO score from ICU admission. Liver injury was defined as presence or any aggravation of hepatic SOFA score from ICU admission or ALT or AST values more than thrice the upper limit of normal in accordance with the modified version of the US National Cancer Institute's (NCI's) Common Terminology Criteria for Adverse Events (CTCAE) (2017). Hemodynamic dysfunction was defined as a need for epinephrine or norepinephrine support at a dose > 0.1 µg/kg/min. SOFA scores were evaluated for all ICU-patients at ICU-admission and on ICU days 4, 7 and 14. If the patient was discharged from ICU before day 14, the last SOFA score recorded during ICU stay was used. Multi-organ dysfunction was defined by the presence of more than one dysfunction.

Abbreviations: ALT: Alanine Transaminase, AST: Aspartate Transaminase, CRP: C-reactive protein, FiO₂: fraction of inspired oxygen, ICU: Intensive Care Unit, KDIGO: Kidney Disease: Improving Global Outcomes, PaO₂: arterial oxygen partial pressure, PT: prothrombin time, SOFA: Sequential Organ Failure Assessment

* baseline values

† Indication for ICU admission (n=1 unless otherwise stated): pneumonia (n=9): 8 of the 9 patients required high-flow oxygen therapy during ICU stay but none required mechanical ventilation, myocardial infarction, ischemic stroke.

Table 2: Correlations between baseline values of all biomarkers and respiratory and liver function criteria

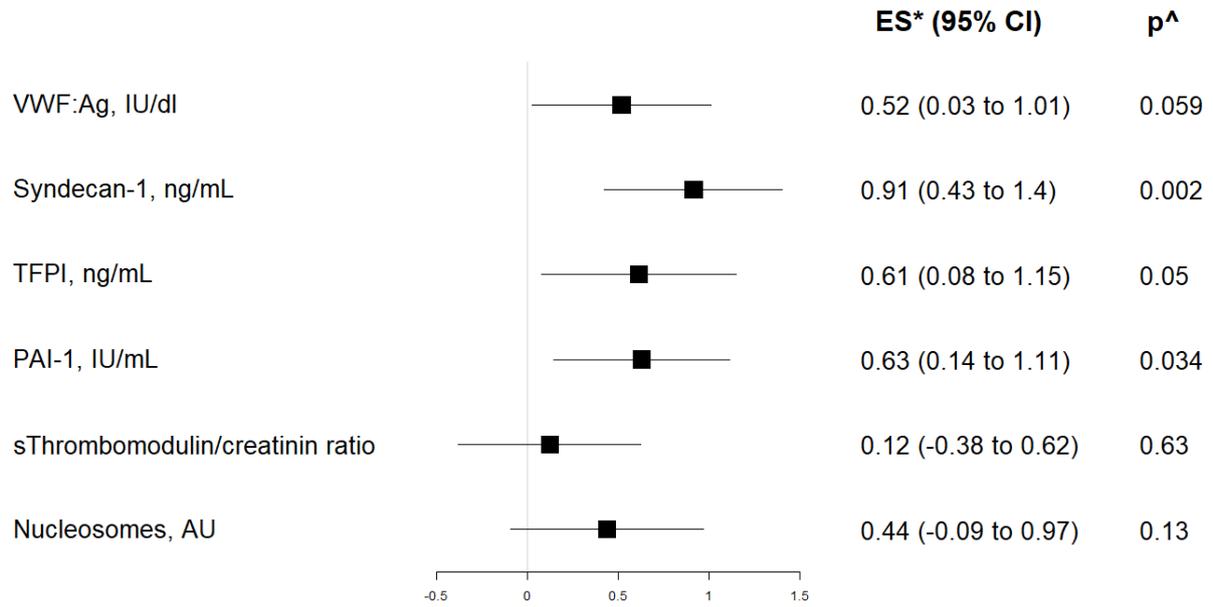
		VWF:Ag	Syndecan-1	TFPI	PAI-1	sTM	C5a	C5b-9	TNF α	IL-6	IL-2R	Neutrophils	Cell free DNA	MPO-DNA	Nucleosomes
PaO ₂ /FiO ₂	R*	-0.28	-0.43	-0.33	-0.13	-0.10	-0.25	-0.032	0.006	-0.14	-0.14	-0.36	-0.20	0.039	-0.31
	p	0.01	<0.0001	0.005	0.26	0.38	0.02	0.77	0.96	0.21	0.21	0.004	0.08	0.73	0.008
ALT	R*	0.35	0.34	0.28	0.09	0.046	0.19	0.25	-0.02	-0.009	0.03	0.26	0.17	0.088	0.25
	p	0.001	0.002	0.02	0.4	0.69	0.09	0.02	0.83	0.93	0.76	0.04	0.15	0.44	0.03
AST	R*	0.41	0.38	0.37	0.35	0.28	0.24	0.04	0.31	0.21	0.31	0.27	0.38	-0.014	0.27
	p	0.0001	0.0004	0.001	0.001	0.014	0.03	0.7	0.005	0.05	0.004	0.03	0.001	0.9	0.02
Bilirubine	R*	0.24	0.24	0.27	0.26	0.09	0.37	0.40	0.12	0.30	0.14	0.32	0.44	0.31	0.32
	p	0.03	0.03	0.02	0.017	0.44	0.001	0.0002	0.26	0.006	0.21	0.009	0.0001	0.006	0.005

Abbreviations: ALT: alanine aminotransferase, AST: aspartate aminotransferase, FiO₂: fractional inspired oxygen, IL: interleukin, PAI-1: plasminogen activator inhibitor-1, PaO₂: arterial oxygen partial pressure, sTM: soluble thrombomodulin, TFPI: tissue factor pathway inhibitor, TNF: tumor necrosis factor, VWF:Ag: von Willebrand factor antigen, p: p-value. * Squared Spearman correlation coefficient

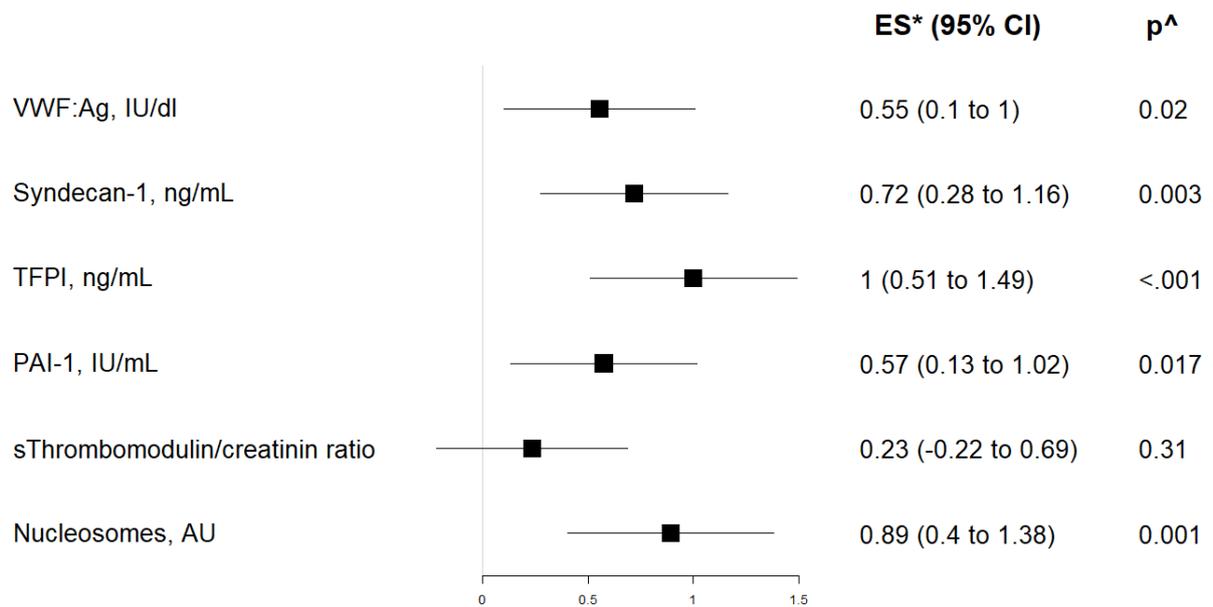
Figures

Figure 1

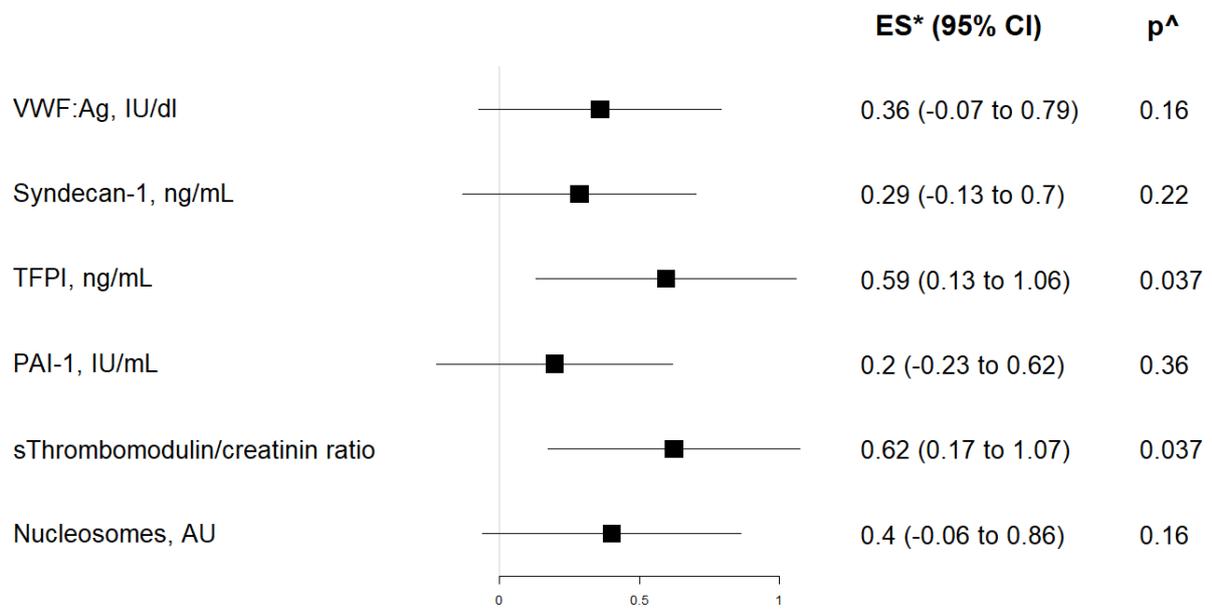
Respiratory failure



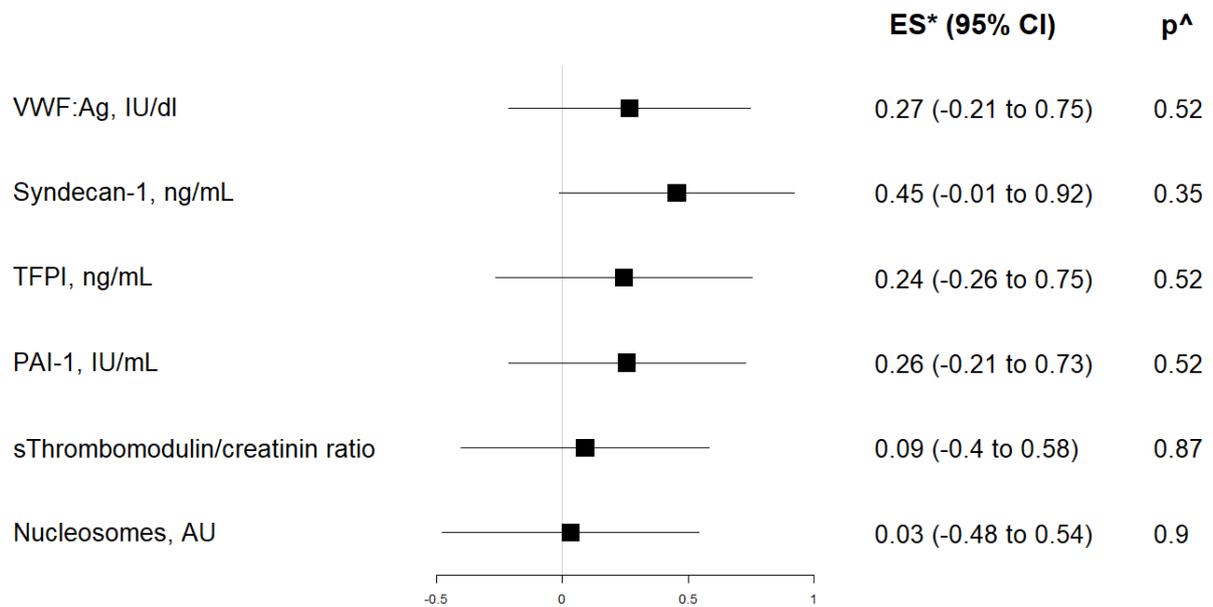
Liver injury



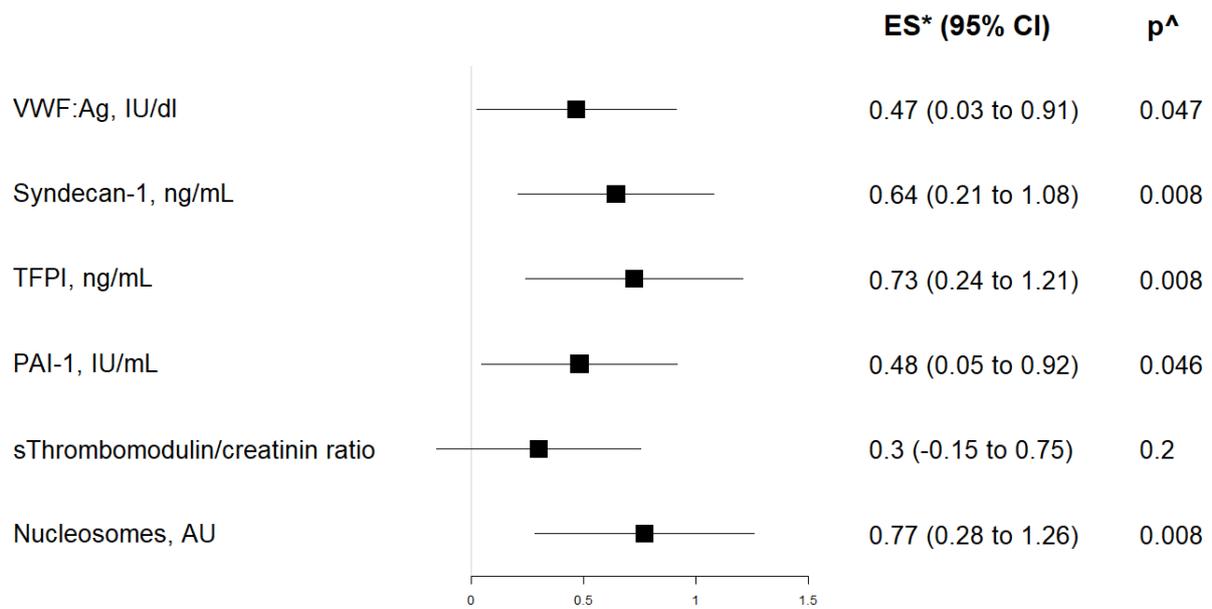
Renal failure



Hemodynamic failure



Multi-organ failure



Death

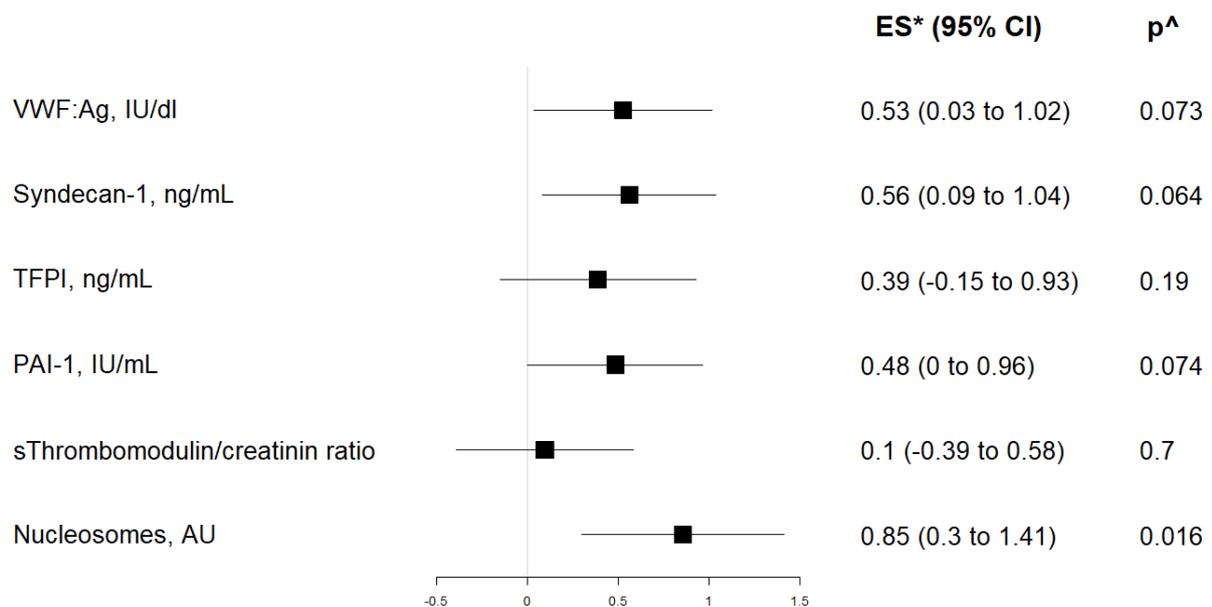


Figure1: Association of biomarkers related to endothelial damage and nucleosomes measured at admission in ICU with occurrence of organ failures within 14 days of ICU admission or all-cause 28-day mortality

Forest plots of adjusted effect sizes; 1A: respiratory failure; 1B: liver injury; 1C: renal failure 1D: hemodynamic failure; 1E: multi-organ failure; 1F: death. Associations were tested using analysis of covariance (ANCOVA) after log transformation of original data.

*: effect size (Cohen's d for ANCOVA) adjusted on pre-specified covariates (age, sex and BMI)

^: False Discovery Rate adjusted p-value; correction for multiple comparisons was performed using the Benjamini–Hochberg procedure

Abbreviations: CI: confidence interval; ES: effect size; ICU: intensive care unit; PAI-1: plasminogen activator inhibitor-1; s: soluble; TFPI: tissue factor pathway inhibitor, VWF:Ag: von Willebrand factor antigen.

Figure 2

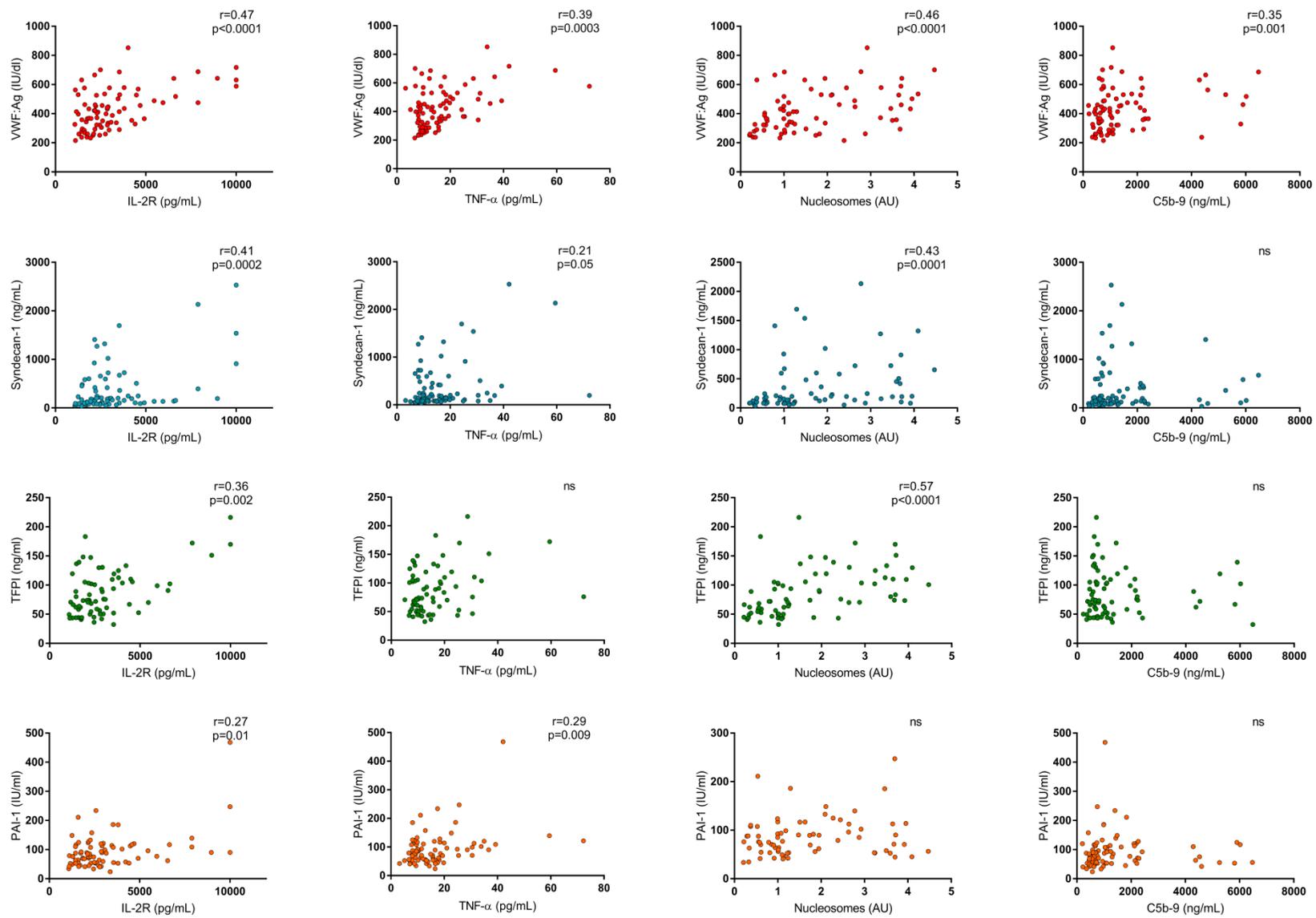


Figure 2: Association of VWF:Ag, syndecan-1, TFPI and PAI-1 levels with plasmatic levels of cytokines, NETosis and complement markers

Abbreviations: AU: arbitrary units, C5b-9: complement membrane attack complex, IL-2R: soluble interleukin-2 receptor, TFPI: tissue factor pathway inhibitor, TNF α : Tumor Necrosis Factor alpha, VWF:Ag: von Willebrand factor antigen. Correlations were evaluated with Spearman's rank-correlation statistical tool.

Figure 3

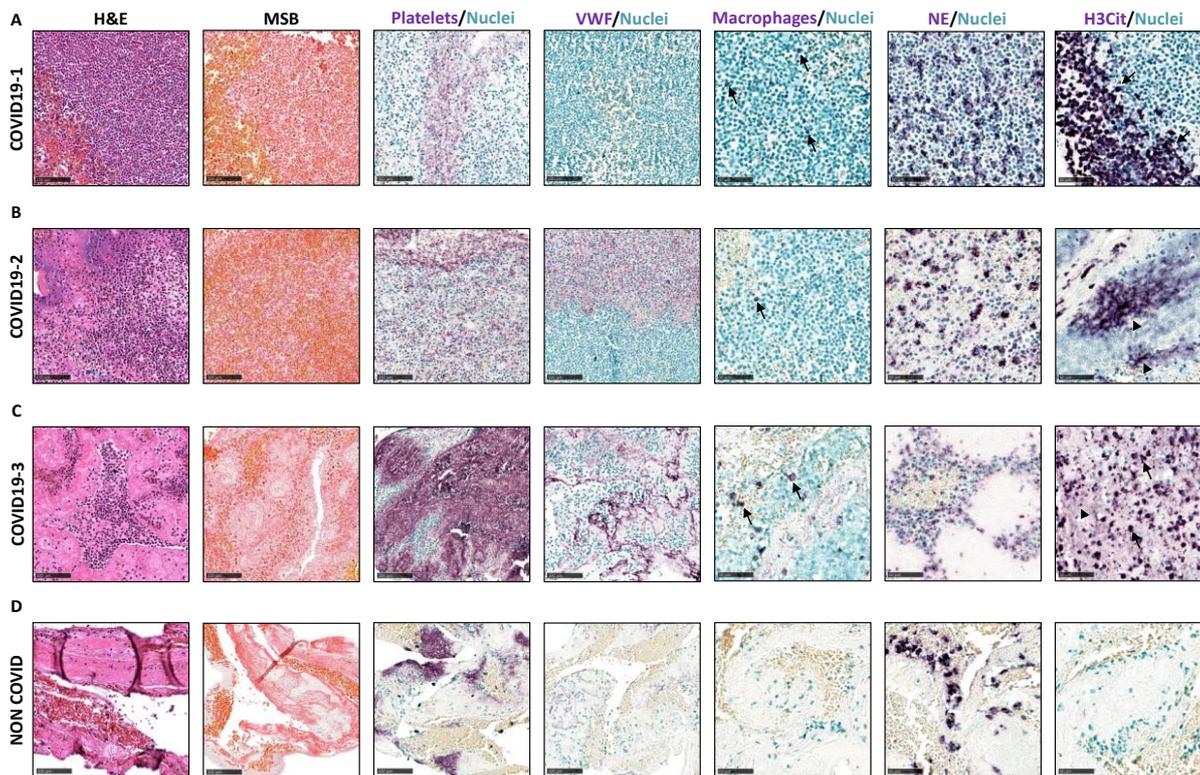


Figure 3: Histological analysis of clots retrieved from ECMO circuits after venovenous ECMO removal in recovered patients

ECMO thrombi were collected from 3 living COVID-19 patients on (A) 2 days, (B) 8 days and (C) 13 days of ECMO support and (D) from a non-COVID-19 patient suffering from acute respiratory distress syndrome on 3 days of ECMO support. Consecutive thrombus sections were stained with H&E to have a general overview, MSB to identify fibrin (red) and RBCs (yellow) and immunohistochemical agents (purple) for platelets, VWF, macrophages, NE and H3Cit. Nuclei are indicated in light green in the immunohistochemical stainings. (A-C) H&E, MSB and the nuclear counterstain on immunostainings reveal a massive amount of cellular infiltration in COVID-19 thrombi compared to (D) control. (A-C) No substantial differences in fibrin, platelet and VWF content is observed compared to (D) control. (A-C) NE staining indicates that the large proportion of leukocytes in COVID-19 patients are neutrophils whereas only few macrophages (arrows) are found. (A-C) COVID-19 thrombi contain both intracellular (arrows) and extracellular H3Cit (arrowheads), suggestive of NETosis. Scale bar = 100 μ m for H&E, MSB, platelet and VWF staining. Scale bar = 50 μ m for macrophages, NE and H3Cit staining. Abbreviations: ECMO: extracorporeal membrane oxygenation; H&E: hematoxylin and eosin; H3Cit: citrullinated histone H3, MSB: Martius Scarlet Blue stain; NE: neutrophil elastase; VWF: von Willebrand factor.