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Endotheliopathy is induced by plasma from critically-ill patients and associated with organ failure in severe COVID-19

Running title: *Rauch et al.; Plasma cytotoxicity and endothelium in COVID-19*

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Data Sharing: The data that support the findings of this study are available from the corresponding author upon request.

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Lung histological analyses revealed the presence of vascular inflammation and severe endothelial injury as a direct consequence of intracellular SARS-CoV-2 infection and ensuing host inflammatory response in COVID-19[1]. Endothelial cells promote coagulation following injury, leading to widespread formation of microthrombi, provoking microcirculatory failure or large-vessel thrombosis[2]. Growing evidence suggests that microvascular thrombosis is a major pathophysiological event in COVID-19 pathogenesis. Damaged endothelial cells could be closely implicated in the pro-thrombotic state commonly reported in severe intensive care unit (ICU)-patients. How SARS-CoV-2 exerts its cytopathic effects is still a matter of debate and ultrastructural evidence of direct viral replication in endothelial cells remains to be demonstrated. Although direct viral tissue damage is a plausible mechanism of injury[3], endothelial damage and thrombo-inflammation associated with dysregulated immune responses, inducing microvascular thrombosis, represents an attractive alternative hypothesis[2]. Using cultured human pulmonary microvascular endothelial cells (HPMVEC), we assessed whether plasma collected from COVID-19 patients at different disease stages could trigger endothelial damage *in vitro*. The cytotoxicity of plasma samples on HPMVEC was evaluated by assessing mitochondrial activity (WST-1 test) 1 hour after incubation of cells with plasma as previously described[4]. We further investigated the association of plasma-induced cytotoxicity with levels of circulating biomarkers related to organ dysfunction (PaO2/FiO2, widely used as an indicator of oxygenation requirements, LDH, creatinine and aspartate transaminase(AST)), endothelial damage (VWF:Ag; ADAMTS13; PAI-1; Syndecan-1), tissue injury (cell-free DNA, a damage-associated molecular patterns-marker) and levels of circulating cytokines related to the activation of innate (IL-6 and TNF-α) and adaptative immune cell responses (IL-2R). Inclusion criteria were: individuals aged 18 years or older with a positive SARS-CoV-2 real-time-reverse transcriptase-polymerase chain

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reaction on nasal or tracheal samples admitted at the Lille University Hospital. Patients on treatment with direct oral anticoagulant or vitamin K-antagonists were switched to therapeutic heparin therapy upon admission. Non-ICU patients received once daily thromboprophylaxis with enoxaparin according to their body weight. ICU-patients received enoxaparin or unfractionated heparin according to their renal status, their body weight and the need for invasive procedures. This study was approved by the French institutional authority for personal data protection (CNIL-number DEC20-086), and ethics committee (IRB 2020-A00763-36), and informed consent was obtained from all participants.

HPMVEC viability was assessed after co-incubation with plasma sampled upon admission from 28 consecutive patients (non-ICU (n=16), ICU (n=12)) hospitalized for COVID19 at the Lille University Hospital between March 30th and April 8th 2020, in convalescent COVID-19 patients (n=6 from the 12 ICU-patients) sampled after ICU discharge (21±7 days) and in control healthy donors (n=8). Compared to healthy donor plasma, COVID-19 patient plasma significantly decreased HPMVEC viability, with plasma from ICU-patients inducing the greatest cytotoxicity (Figure 1A). Interestingly, HPMVEC viability was partially restored to control when plasma from convalescent patients after ICU discharge was tested and compared to plasma of the same patients at the time of ICU admission. Moreover, markers of organ dysfunction were correlated with plasma-induced cytotoxicity (Figure 1B). HPMVEC viability also correlated with most plasma markers related to endothelial damage or tissue injury (Figure 1C). Finally, IL-2R and TNF-α levels negatively correlated with HPMVEC viability (Figure 1D). Overall, the degree of vascular endothelial cell injury induced by plasma sampled from COVID-19 patients correlated to both clinical illness severity at admission and to the levels of biomarkers related to endothelial, tissue injury and proinflammatory cytokines.

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Our data shed new light on the pathophysiology of COVID-19 by demonstrating the direct and rapid cytotoxic effect of plasma collected from critically-ill patients on vascular endothelial cells. This rapid effect (1h after plasma exposure) excludes a direct cytopathic effect of SARS-CoV-2 infection as the progression of viral infection and visible cytopathogenic effects are in general only apparent 12h to 24 h after infection[5]. A higher cytotoxic effect of plasma on endothelial cells was associated with a more pronounced hypoxemia and organ-dysfunction as reflected by the correlation with PaO2/FiO2, LDH, creatinine and AST. This cytotoxic effect also correlated with circulating markers of endothelial damage indicating that this *in vitro* functional assay reflects microvascular endothelial damage *in vivo*. Different pathways could be involved in endothelial cell injury during the course of COVID-19, i.e. complement activation, cellular hypoxia, platelets and direct cytotoxicity of cytokines such as IL-6, IL-1-β and TNF-α. We observed a relationship between this cytotoxic effect and the level of pro-inflammatory cytokines suggesting that cytotoxicity could be related to overproduction of proinflammatory cytokines. However, this paper does not provide the supportive evidence of convalescent plasma for treating severe COVID-19 patients.

In conclusion, we provide for the first time the results of a functional assay demonstrating a direct effect of dysregulation of immune response on endothelial damage in COVID-19. Endotheliopathy is an essential part of the pathological response upon severe COVID-19, leading to respiratory failure, multi-organ dysfunction and thrombosis. Endothelial and microvascular damage are associated to immunopathology and may occur in parallel to intracellular SARS-CoV-2 infection.

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Disclosures

None.

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Authorship Contributions

AR, AD collected clinical data, analyzed the data, and wrote the manuscript**.** JG, MC, SSt, MDM, EJ, DC, GL, FL, KF, ML, DG, SDM and JP, collected data. JG, MC, SSt, EJ and SDM analyzed the data. JL and AD performed the statistical analysis. BS, EV, FV, JP, EK and PL provided critical input in the interpretation of data and critically reviewed the manuscript. SSu designed the study, analyzed the data, wrote and critically reviewed the manuscript. All authors provided editorial review and assisted in writing the manuscript.

Appendix

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Figure Legend

Figure 1: Endothelial cell cytotoxicity induced by plasma sampled from critically-ill and convalescent COVID-19 patients

The cytotoxicity of platelet-poor plasma samples (obtained after a double centrifugation of citrate tubes at 2500g for 15 min at room temperature) from COVID-19 patients and controls on HPMVEC was evaluated with a colorimetric assay using 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.3-benzene disulfonate (WST-1), which in viable cells is cleaved by mitochondrial dehydrogenases. After incubation, the cells were washed with PBS and incubated with WST-1 (Roche, Basel, Switzerland) at a dilution of 1:10 (10 µL) for 2 h at 37°C. Absorbance was measured using a multi-well plate reader (Synergy HTX multi-mode plate reader, BioTek Instruments, Highland Park, VT, USA) at 450 nm with a reference wavelength of 620 nm. As a positive control for endothelial cell injury, Shiga toxin 145 (Sigma-Aldrich, Saint Quentin Fallavier, France) was spiked in plasma from healthy adults (10 µg/mL final concentration) and incubated at 37°C for 15 min, before addition to HPMVECs. Experiments were performed in triplicates for each patient sample.

(A) HPMVEC viability after exposure to plasma sampled in healthy subjects (n=8), in non-ICU (n=16), ICU (n=12) upon admission and in 6 convalescent COVID-19 patients sampled after ICU discharge (21±7 days). Datapoints represent individual sample measurements, whereas horizontal bars show the mean (+/-SD). Comparisons between groups were done using the Mann-Whitney U test, except for comparison between ICU and convalescent patients where we used Wilcoxon signed-rank test on matched pairs (n=6). Correlations between HPMVEC viability and **(B)** markers of organ dysfunction: the PaO_2/FiO_2 ratio, widely used as an indicator

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of oxygenation requirements, LDH, Creatinine and AST; **(C)** parameters related to endothelial dysfunction and tissue injury: VWF:Ag, ADAMTS13, ADAMTS13:VWF ratio, PAI-1, syndecan-1 and cell-free DNA and **(D)** plasma cytokine concentrations: IL-2R, IL-6 and TNF-α. Correlations were evaluated with the Spearman's rank-correlation statistical test. No adjustment for multiple comparisons was done and the result should be interpreted as hypothesis generating. AST: Aspartate Transaminase, HPMVEC: human pulmonary microvascular endothelial cells, ICU: intensive care unit, IL-2R: soluble interleukin-2 receptor, LDH: lactate dehydrogenase, PAI-1: plasminogen activator inhibitor-1, VWF: Ag: von Willebrand factor antigen.

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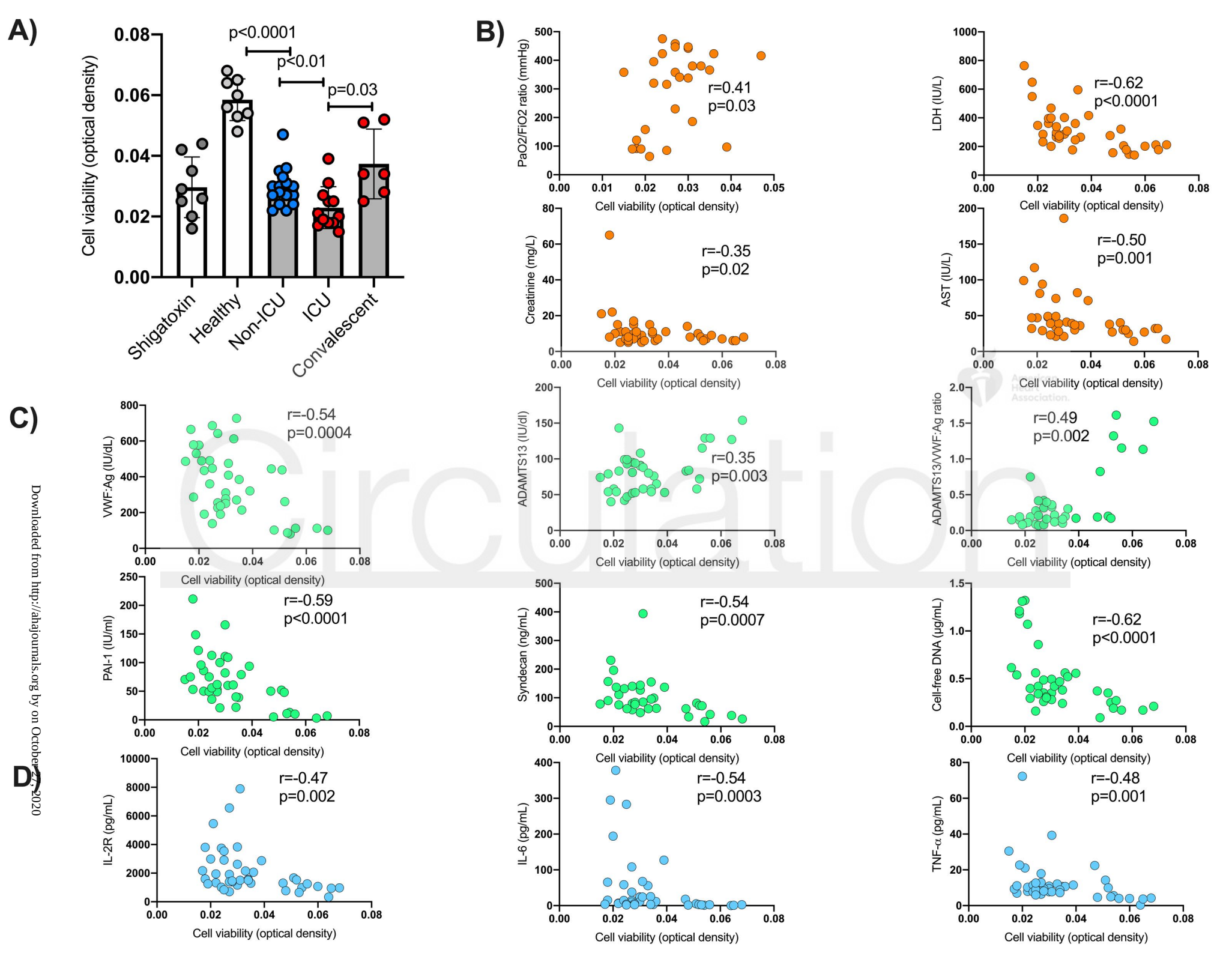

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