Between inflammation and thrombosis: endothelial cells in COVID-19

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To the Editor:

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is causing the current coronavirus disease (COVID-19) pandemic [1]. Over recent months, a plethora of novel research articles has been published, dealing with multiple aspects and manifestations of the disease. Increasing evidence points to a central role of endothelial cells in SARS-CoV-2 infection [2–5]. Early studies have already indicated increased expression of vascular and inflammatory factors (such as vascular cell adhesion molecule (VCAM)-1, interleukin (IL)-8 or monocyte-chemoattractant protein (MCP)-1) in COVID-19 lung tissue [2]. Such markers of endothelial dysfunction and altered endothelial cell integrity are important predictors of a poor outcome in SARS-CoV-2 infections [6], and they are associated with pulmonary oedema, intravascular thrombosis and acute respiratory distress syndrome (ARDS). The pulmonary endothelium is crucial for regulation of vascular tone, inflammatory responses, coagulation/fibrinolysis and maintenance of vascular homeostasis and permeability. Disturbances of these tightly regulated processes may directly contribute to morbidity and mortality. However, the exact mechanisms leading to pulmonary vasculopathy in COVID-19 are still unclear. Here, we provide an analysis of several important vascular markers implicated in the inflammatory response (E-selectin, intercellular cell adhesion molecule (ICAM)-1, VCAM-1), maintenance of microvascular integrity (CD31, vascular endothelial growth factor receptor (VEGFR)-2), platelet activation and coagulation (P-selectin, von Willebrand factor (vWF)) in lung tissue and plasma samples of COVID-19 patients.

Lung tissue samples were collected *post mortem* from 19 critically ill COVID-19 patients and 11 age and sex-matched autopsy controls without underlying lung pathology. The median age was 79 years for COVID-19 and 75 years for the respective autopsy controls (figure 1a). The study protocol and tissue usage were approved by the ethics commission of the Medical University of Graz (EK-number: 32–362 ex 19/20). RNA was isolated from homogenised lung tissue and cDNA synthesis and quantitative real-time PCR were performed as described previously [7]. Immunofluorescence staining was performed on formalin fixed paraffin embedded lung tissue from corresponding COVID-19 patients and autopsy controls using vWF (1:1000, #M0616, Agilent, Santa Clara, CA, USA) and CD45 (1:250, #10558, Abcam, Cambridge, UK) and secondary antibodies labelled with Alexa Fluor 555 or Alexa Fluor 488 (1:500, Thermofisher Scientific, Waltham, MA, USA) respectively. Plasma samples were collected from 21 critically ill COVID-19 patients and 22 healthy controls at Hannover Medical School, Hannover, Germany and from the Dept of Clinical Immunology and Transfusion Medicine at the Justus-Liebig University of Giessen, Giessen, Germany. The median age was 59 years for COVID-19 patients and 61 years for the respective controls. 90% of patients and 89% of the controls were male. All COVID-19 patients were severely ill and required use of vasopressors and mechanical ventilation (figure 1b). Investigations were approved by the local ethics committee (Hannover samples: SEPSIS/ARDS Registry 8146_BO_K_2018; Giessen samples: 05/00) and written informed consent was obtained from all participants or their next-of-kin. Plasma levels of E-selectin, P-selectin, ICAM-1, VCAM-1 and CD31 were determined in a LEGENDplex assay. Sandwich High Sensitivity ELISA kits were used to quantify vWF (#EHVWF, Thermofisher Scientific) and IL-6, IL-8, tumour necrosis factor (TNF)-α and MCP-1 (Biolegend, San Diego, CA, USA) plasma levels. Statistical analysis was performed in R (version number 4, www.r-project.org). Data are expressed as single data points with boxplot overlay indicating median and interquartile range. Different groups were compared using Wilcoxon Mann-Whitney U-test. Correlations were performed using Spearman's rank correlation.

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Elevated levels of several endothelial markers, including CD31, VEGFR-2, ICAM-1, VCAM-1, E-selectin, P-selectin and vWF, in lung tissue and circulation support an important role of the pulmonary endothelium in local and systemic COVID-19 pathology https://bit.ly/3eQObIR

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a) Autopsy lung tissue samples

	COVID-19	Control
Subjects (n)	19	11
Age (years)	79 (54–94)	75 (59–84)
Males (%)	65	55
CRP (mg·L^{-1})	114 (31–358)	55 (3–340)
D-dimer (mg·L^{-1})	3 (0.6–10)	
Fibrinogen (mg·mL^{-1})	5 (3–6)	3 (2–5)
Thrombocytes (nL^{-1})	201 (8–324)	214 (113–297)

b) Plasma samples

	COVID-19	Control
Subjects (n)	21	22
Age (years)	59 (19–82)	61 (19–78)
Males (%)	90	89
BMI (kg·m^{-2})	29 (15–62)	
CRP (mg·L^{-1})	151 (68–292)	
PCT (µg·L^{-1})	1 (0.1–66)	
D-dimer (mg·L^{-1})	4 (1–35)	
Fibrinogen (mg·mL^{-1})	7 (4–9)	
PTT (s)	36 (26–55)	
SOFA score	13 (9–17)	
Thrombocytes (nL^{-1})	247 (99–581)	
Mechanical ventilation (%)	100	
Received vasopressors (%)	100	

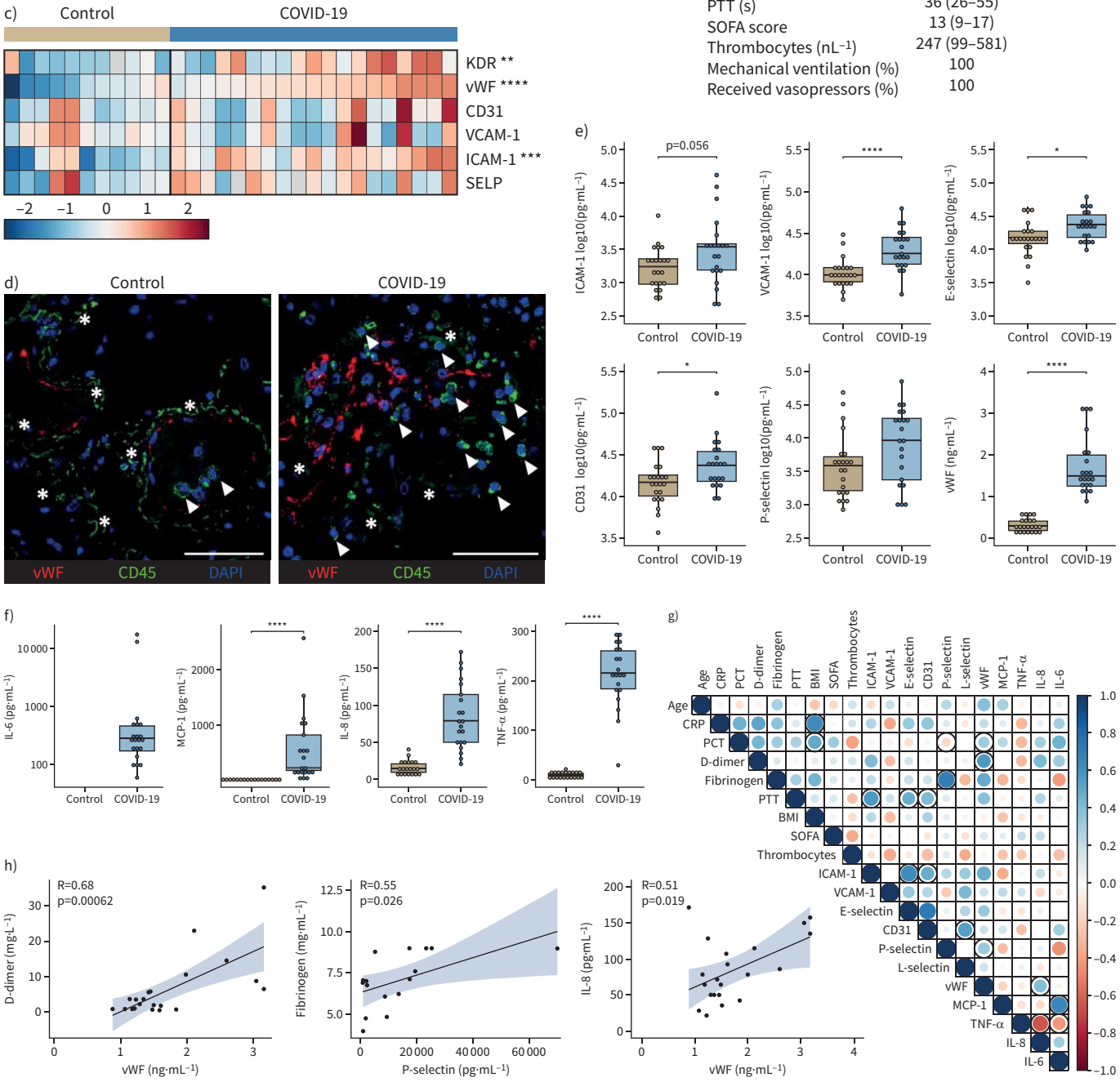

FIGURE 1 a) Patient characteristics of coronavirus disease 2019 (COVID-19) and control lung autopsy samples. Median (range) values are shown. b) Patient characteristics and clinical parameters of the COVID-19 cohort used to assess plasma levels of endothelial cell and inflammatory markers. Median (range) values are shown. c) Quantitative real-time PCR analysis of important endothelial markers in COVID-19 (n=19) and control (n=11) lung tissue homogenates. Heatmap representation of relative expression changes. z-scores are shown. d) Immunofluorescence staining of von Willebrand factor (vWF) (red) and inflammatory marker CD45 (green) in control and COVID-19 lung tissue. White arrowheads highlight

inflammatory cells. White asterisks indicate auto-fluorescent extracellular matrix. Scale bar: 50 µm. Circulating levels of e) endothelial markers intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, E-selectin, CD31, P-selectin and vWF, and f) inflammatory mediators interleukin (IL)-6, monocyte-chemoattractant protein (MCP)-1, IL-8 and tumour necrosis factor (TNF)-α in the plasma of COVID-19 patients (n=21) and healthy controls (n=22). g) Correlation matrix of clinical parameters and ICAM-1, VCAM-1, E-selectin, CD31, P-selectin, vWF, IL-6, IL-8 and MCP-1 circulating levels in COVID-19 patients. Dot size and colour correspond to the correlation coefficient, circled dots indicate significance using a threshold of p<0.05. h) Selected correlations as calculated in (d). *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001. KDR/VEGFR2: vascular endothelial growth factor receptor 2; CD31/PECAM1: platelet and endothelial cell adhesion molecule 1; SELP: P-selectin; CRP: C-reactive protein; PCT: procalcitonin; PTT: partial thromboplastin time; BMI: body mass index; SOFA: sequential organ failure assessment score.

Gene expression analysis revealed an upregulation of ICAM-1, vWF and VEGFR2 (KDR) in COVID-19 lung tissue compared to control tissue (figure 1c). Immunofluorescence staining for inflammatory cell marker CD45 and vWF revealed increased extravasation of inflammatory cells and a prominent, more diffuse vWF signal (figure 1d) in COVID-19 compared to control lungs. The latter may reflect increased vWF shedding, an assumption supported by elevated levels of circulating vWF (figure 1e). Next to vWF, we found significantly increased levels of VCAM-1, E-selectin and CD31 in COVID-19 plasma (figure 1e), indicating endothelial cell activation/dysregulation in COVID-19. In addition, levels of inflammatory cytokines IL-6, IL-8, MCP-1 and TNF-α were significantly increased in COVID-19 plasma (figure 1f). Detailed correlation analysis revealed strong associations between some of the endothelial derived markers but only weak associations with age or general inflammatory parameters such as C-reactive protein (figure 1g). D-dimer levels strongly correlate with soluble vWF, while increased partial thromboplastin time correlates with shedding of surface markers ICAM-1, E-selectin and CD31 (figure 1g and h). Fibrinogen levels correlate with increased P-selectin (figure 1h). In addition, plasma levels of IL-8 were strongly associated with circulating vWF levels (figure 1h). In summary, local, as well as systemic, circulating inflammatory markers coincide with various markers revealing endothelial activation and damage in COVID-19 patients.

The pulmonary endothelium is a tightly regulated organ, comprising a great variety of functions, both under physiological and pathological conditions. Inflammatory cell recruitment to the lung is mediated by selectins (E-selectin, P-selectin) and cell adhesion molecules (ICAM-1, VCAM-1) expressed on the surface of endothelial cells. These molecules are either stored intracellularly and released (P-selectin) or arise from *de novo* synthesis (ICAM-1, VCAM-1, E-selectin) in response to a specific cytokine milieu [8]. An excessive production of pro-inflammatory cytokines, a so-called “cytokine storm”, has been previously reported in severe COVID-19 [9–11] and confirmed here. The cytokine storm contributes to a switch from a protective to an inflammatory endothelial cell phenotype, associated with vascular leakage, tissue injury and immuno-thrombosis. Shedding of adhesion molecules indicates a highly activated endothelium, while elevated levels of plasma CD31 are additionally linked to endothelial cell apoptosis [8]. Given the increased circulating levels of adhesion molecules (E-selectin, VCAM-1, ICAM-1) and CD31, our data suggest that the endothelium is highly inflammatory and partly damaged in severe COVID-19. This notion is corroborated by local alterations in vWF mRNA and protein levels, as well as its patchy and diffuse distribution in COVID-19 lungs. The correlation of vWF and IL-8, as seen in our data, implies a concomitant release of these proteins from Weibel–Palade bodies, which ensures rapid reaction to damage and recruitment of inflammatory cells, further highlighting endothelial stress and activation in critically ill COVID-19 patients. Simultaneous upregulation of VEGFR-2 expression might display compensatory angiogenesis, which has been observed in COVID-19 patients of various disease severity [2]. Of note, increased expression of VCAM-1, IL-8 and MCP-1 in the lung tissue has been shown to be specific for COVID-19 compared to ARDS due to influenza [2]. Overall, our data showing local *de novo* synthesis of endothelial activation and damage markers suggest that the lung contributes to the pool of these proteins in plasma. However, the magnitude and the rate of lung contribution in relation to other organs has to be addressed in future studies.

In general, the resting pulmonary endothelium supports maintenance of fibrinolysis [9]. However, the presence of proinflammatory mediators drives a pro-coagulant state that finally leads to over-activation of coagulation and thrombosis, as seen in patients with COVID-19. We found that not only circulating levels of vWF but also its expression in the lung is strongly elevated in severe COVID-19 patients. vWF is stored in and rapidly released from Weibel–Palade bodies upon endothelial cell injury. It locally promotes platelet adhesion and thrombus formation [8]. Several recent publications suggested an important role of vWF in COVID-19 vasculopathy [12, 13] and for residual pulmonary obstruction after acute pulmonary embolism [14]. Importantly, elevated vWF levels were reported to be associated with severity of illness and mortality in patients infected with SARS-CoV2 [12]. A strong positive correlation between circulating vWF and D-dimer, a marker of coagulation activation and poor prognosis in severe COVID-19, underpins the

imbalance between pro-and anti-coagulatory processes directly related to endothelial dysfunction in critically ill COVID-19 patients. A recent study also highlighted the use of IL-8 to discriminate COVID-19 patients with high mortality [11]. The recent findings suggest lower circulating cytokine levels in COVID-19 ARDS compared to non-COVID-19 ARDS, while an increased local prothrombotic state, accompanied by hypofibrinolysis in severe COVID-19 patients has been described [15]. Together, cumulative assessment of several markers of endothelial cell activation/damage and inflammation might be a valuable tool to assess the immuno-thrombosis status and thus risk estimation of embolic vessel occlusion in hospitalised patients with COVID-19.

This study is limited by the lack of non-COVID-19 disease controls; however, our data argue in favour of an important role of the pulmonary endothelium in the systemic immunopathology underlying COVID-19. In line with other reports, our work provides a link between endothelial dysfunction and pulmonary coagulopathy seen in severe COVID-19 patients. Although further studies are required to fully understand endothelial changes in the onset, progression and resolution of the disease, our data unravel endothelial cell markers that participate in inflammatory cell recruitment, maintenance of endothelial cell integrity and coagulation in critically ill COVID-19 patients. While integrating such markers into clinical practice may allow for a better prediction of COVID-19 outcome, further prospective studies using larger cohorts are warranted.

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