Injury to the Endothelial Glycocalyx in Critically Ill COVID-19 Patients

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KS, PAG, BS, TW, HH, MMH and SD obtained retrospective data. KS, PAG and SD performed SDF measurements. PAG performed ELISA and JK flow measurements. KS, PAG, HH, TW, MMH and SD analysed and discussed the data and generated figures and tables. KS, PAG and SD wrote the manuscript; all authors proof-read the manuscript.

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To the Editor,

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes the so-called coronavirus disease 2019 (COVID-19) that is characterized by a broad spectrum of clinical presentations ranging from asymptomatic patients to critically ill individuals with a high case fatality rate (1). The critical care community has increasingly recognized that cardiovascular and thrombotic complications are relatively common in COVID-19 (2). Indeed, direct involvement of the vascular endothelium was recently reported in a series of patients suffering from severe COVID-19 (3). The endothelial glycocalyx (eGC), covering the luminal surface of endothelial cells, contributes to the maintenance of vascular homeostasis while disruption of the eGC is observed early in critical ill patients and is associated with inferior outcomes (4, 5).

Here, we investigated in translational human and cellular studies if injury to the eGC can be found in critically ill COVID-19 patients early after admission to the intensive care unit (ICU). We collected plasma and serum from 19 adult individuals within 24 hours after invasive ventilation for acute respiratory distress syndrome (ARDS) and 10 healthy human donors following written informed consent of patients or their legal representative. The first patient was admitted on 03-19-2020 and the observation period was until 05-17-2020. The median (IQR) observation duration was 47 (40-54) days.

Baseline patient characteristics at study inclusion as well as a description of the further clinical course are summarized in **Table 1**. Organ failure was not restricted to the lungs and multi-organ dysfunction was common both at inclusion and during the further clinical course. Surprisingly, global markers of endothelial injury such as Angiopoietin (Angpt)-1 (Median (IQR) control: 29 (26.2-30.9) ng/ml vs. COVID-19: 27.8 (23.4-36.2) ng/ml, p=0.79) and Angpt-2 (control: 0.655 (0.336-1.113) ng/ml vs. COVID-19: 0.434

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(0.035-1.338) ng/ml, p=0.6) were unchanged in COVID-19 patients. In contrast, a marked increase in the soluble form of the Tie2 receptor (sTie2) (**Fig. 1A**) and in Syndecan-1 (**Fig. 1B**) - indicating pathological shedding of transmembrane proteins involved in glycocalyx structure and processing - was observed. The key eGC sheddase Heparanase-1 (Hpa-1) and its enzymatic activity were both not significantly increased (data not shown). To the contrary, the Hpa-1 counterpart, i.e. the protective heparanase-2 (Hpa-2) was pertinently reduced in all COVID-19 patients (**Fig. 1C**). Driven by this acquired Hpa-2 deficiency the Hpa-1 to Hpa-2 ratio was higher in COVID-19 patients ($p = 0.012$, data not shown). Together this indicates that critically ill COVID-19 patients suffer from an acquired Hpa-2 deficiency that can contribute to degradation of the eGC maybe even before classical endothelial activation and injury. Next, eGC structure was analyzed *in humans* employing sublingual side-stream darkfield imaging (SDF). We quantified the size of the individual patients' eGC using an indirect surrogate termed *perfused boundary region* (PBR) and found a decrease of PBR indicating reduced eGC thickness in COVID-19 patients. To demonstrate that the deficiency of Hpa-2 is mechanistically involved in the degradation of the eGC we used a microfluidic chamber with cultured endothelial cells (EC) under flow that synthesize an intact and stable eGC under *in vitro* conditions. After stimulation with COVID-19 or control serum, the eGC was visualized by confocal microscopy followed by computerized 3D reconstruction. Its thickness is then quantified by analyzing the heparan sulfate (HS) positive area. We found that stimulation with COVID-19 was sufficient to severely damage the eGC (**Fig. 1E**). The HS positive area was reduced by 34% (control: 6.1 ± 0.9% vs. COVID-19: 4 ± 0.4%**,** $p<0.001$). Consistent with our observation in patients, we found that the transcription of Hpa-2 in COVID-19 stimulated endothelial cells was significantly reduced after 6 hrs (0.63 ± 0.02 relative expression to control, p=0.003). Of note, transgenic overexpression of Hpa-2 in a lentivirus-transduced EC line was sufficient to reverse this phenotype as HS area in COVID-

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19 serum treated lenti-control cells was 1.9 ± 0.6% but 4.2 ± 1.2% in lenti-Hpa-2 overexpressing cells ($p<0.001$). In other words, if ECs overexpress Hpa-2 the serum of COVID-19 patients cannot degrade the eGC anymore.

This exploratory study has obvious limitations most importantly its small sample size and hypothesis generating nature. Injury to the eGC is not a finding specific for COVID-19 but can be found in a wide range of critical ill patients (5). As only a small selection of molecules that may participate in endothelial injury have been investigated in this study, we cannot exclude that further mediators may play a critical role in endothelial and eGC injury in COVID-19 patients. Additionally, due to concerns of viral transmission, SDF imaging values could not be obtained from the same control patients from which blood analysis were performed, but from a separate historic in center control cohort. Both control cohorts were not matched to the individual patients in terms of age, but the control group blood, was collected from, was matched in terms of male predominance.

In summary, we found injury of the eGC and speculate that this might represent a potentially critical hallmark of later widespread endothelial injury in severe COVID-19. Reduced eGC thickness was visualized *in vivo* employing sublingual SDF imaging in patients. At the same time, increased syndecan-1 and sTie-2 concentrations in the blood of these patients indicated shedding of important endothelial trans-membrane proteins responsible for both building (5) and maintaining (6) the structure of the eGC, respectively. Interestingly, eGC shedding could be reproduced when patient blood was transferred *ex vivo* into an endothelial micro-capillary chip model. While Hpa-1 and its enzymatic activity, primarily responsible for HS degradation (7), was found to be normal, Hpa-2, a protein that has been described as a protective antagonist of Hpa-1 (8, 9), was severely depleted in COVID-19. Importantly, degradation of the eGC following perfusion with COVID-19 serum

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could be attenuated in ECs overexpressing Hpa-2. We therefore postulate that acquired Hpa-2 deficiency, might represent a potential mechanism of injury to the eGC, which could later progress to widespread endothelial dysfunction in COVID-19.

In conclusion, our data suggest that in critically ill patients with COVID-19, endothelial injury involves glycocalyx integrity and acquired Hpa-2 deficiency might be a potential causative factor.

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FIGURE AND TABLE LEGENDS

TABLE 1 - Patient characteristics at study inclusion and further clinical course.

Given are demographic and clinical characteristics at the time of study inclusion as well as a description of the further clinical outcome within the observation period. Values are presented as median (25% to 75% interquartile range) or if categorical as numbers and percentages.

Demographic characteristics for the control patients were: Median (IQR) age 32 (28-33) years and 9/10 males for laboratory investigation controls; 39 (36-44) years and 5/10 males for SDF measurement controls. All individuals of the control cohorts had no relevant medical preconditions.

FIGURE 1 – Injury to the endothelial glycocalyx in severe COVID-19.

Scatter dot blots (Median and Interquartile Range) showing syndecan-1 **(A)** and sTie-2 **(B)** concentrations for control and COVID-19 patients. Median (IQR) concentrations for both Syndecan-1 (control: 41.5 (32.6-105.4) ng/ml vs. COVID-19: 336.5 (196.7-377.1) ng/ml) and sTie-2 (control: 18.4 (16-21.3) ng/ml vs. COVID-19: 22.3 (19.7-27.3) ng/ml) were increased in COVID-19 patients. While both Hpa-1 concentration and activity were not significantly altered in comparison to controls, COVID-19 patients showed an acquired deficiency in Hpa-2 (control: 18.7 (10.6-31.1) U/ml vs. COVID-19: 4.7 (2.6-5.1) U/ml) **(C)**. Consequentially the Hpa-1 to Hpa-2 ratio was higher in COVID-19 patients (control: 0.08 (0.05-0.17) ng/U vs. COVID-19: 0.35 (0.27-0.66) ng/U). SDF imaging in patients allows quantification of both eGC thickness as indicated by increased PBR was increased in COVID-19 patients (control: 1.9 (1.8-1.9) μm vs. COVID-19: 2.1 (1.8-2.2) μm) indicating reduced eGC thickness **(D)**. For comparison of groups, first normal distribution (D'Agostino-Pearson omnibus - and Shapiro-

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Wilk normality test) of variables was tested and then two-sided unpaired t-tests (A, D) and Mann–Whitney tests (B, C) were used accordingly. Exemplary 3D reconstruction of the heparan sulfate (HS) layer images of naive endothelial cells in a microfluidic chip (HS in red, DAPI nuclei staining in blue) after perfusion with serum of a COVID-19 patient (middle row) compared to a healthy control (upper row) in both front and isometric view angles demonstrates diffuse loss of HS rich glycocalyx layer in cells treated with COVID-19 serum. In Hpa-2 overexpressing endothelial cells (lower row) the HS surface layer is protected from injurious effects by perfusion with COVID-19 serum **(E)**.

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LIST OF ABBREVIATIONS

ACE-2 = Angiotensin converting enzyme 2 receptor

Angpt-1 = Angiopoietin-1

Angpt-2 = Angiopoietin-2

ARDS = Acute respiratory distress syndrome

CKD = Chronic kidney disease

COVID-19 = Corona virus disease 2019

ECMO = Extracorporeal membrane oxygenation

eGC = endothelial Glycocalyx

GAG = Glycosaminoglycan

Hpa-1 = Heparanase-1

Hpa-2 = Heparanase-2

HS = Heparan Sulfate

IQR = Interquartile Range

NIV = Non-Invasive Ventilation

PBR = Perfused Boundary Region

PG = Proteoglycan

Sars-CoV-2 = Severe acute respiratory syndrome – Corona Virus - 2

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SOFA = Sequential Organ Failure Assessment score

(s)Tie2 = (soluble) Tyrosine kinase with Ig and EGF homology domains-2

vv = Veno-venous

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Figure 1

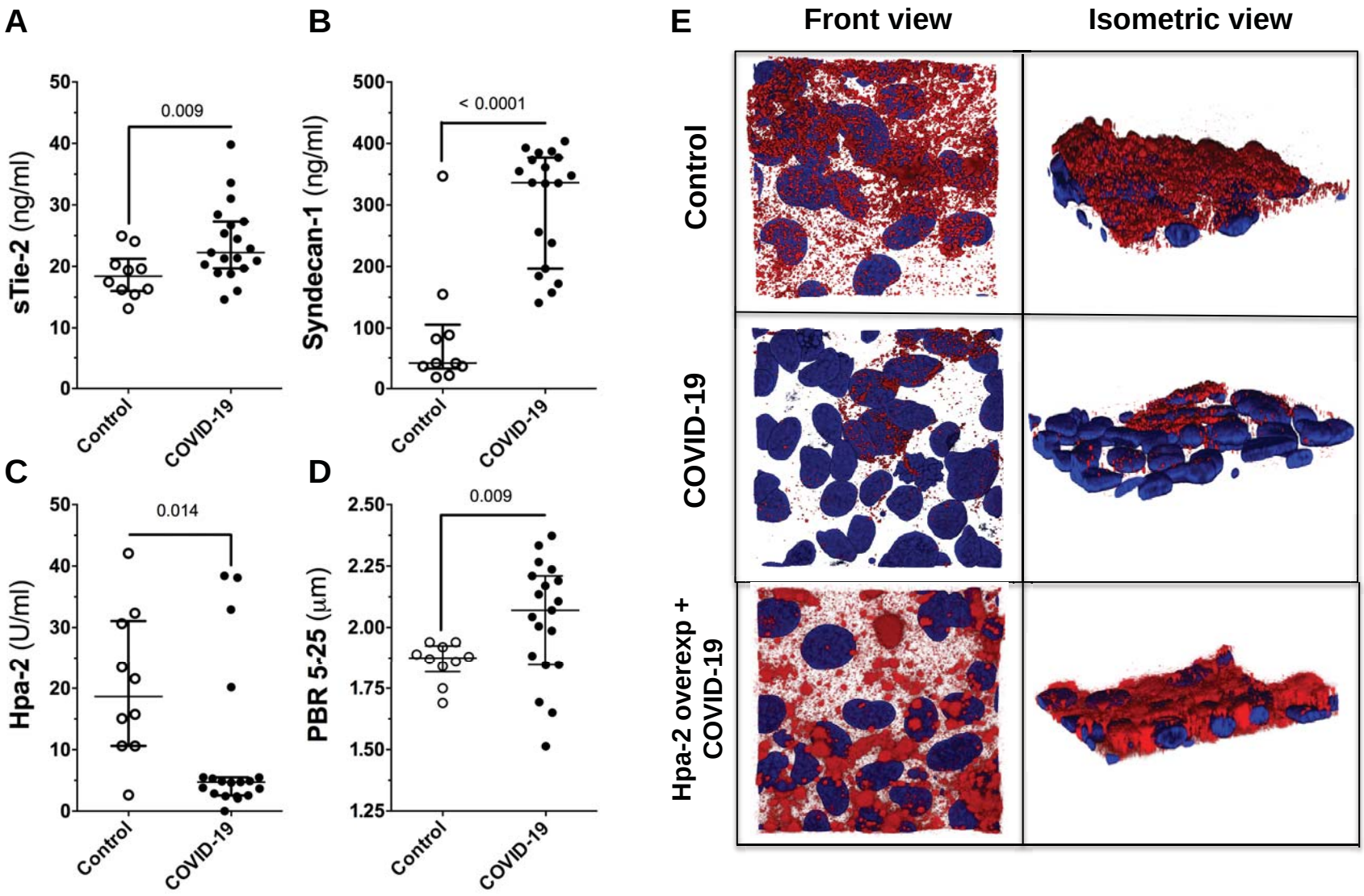

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Table 1: Patient characteristics at study inclusion and further clinical course

Characteristic	Median (Interquartile range) / No (%)
Number of patients	19
Age - years	57 (45-69)
Sex - no (%)	
male	18 (95)
female	1 (5)
BMI - kg/m^2	27.8 (26.2-33.5)
Comorbidities - no (%)	
Asthma	1 (5)
HTN	10 (53)
CHF	2 (11)
CAD	1 (5)
Diabetes	5 (26)
CKD	1 (5)
Obesity	7 (37)
Malignancy	2 (11)
Immunosuppression	2 (11)
Invasive Ventilation - no (%)	19 (100)
Time from hospital to ICU admission - days	0 (0-2)
Time from ICU admission to invasive ventilation - days	0 (0-0)
Respirator parameters	
FiO_2 - %	50 (30-60)
PEEP - mbar	12 (12-15)
Pplat - mbar	27 (23-30)
Oxygenation index (PaO_2/FiO_2) (mmHg)	173 (152-260)
$paCO_2$ - mmHg	46 (40-51)
pH	7.36 (7.32-7.41)
Lactate - mmol/l	1.6 (1.5-2.9)
Vasopressor - no (%)	16 (84.2)
Norepinephrine dose (µg/kg/min)	0.083 (0.019-0.182)
Renal replacement therapy - no (%)	3 (16)
vv-ECMO support - no (%)	2 (11)
SOFA-Score - points	11 (9-14)
Organ specific failures - no (%)	
respiratory (PaO2/FiO2<300)	19 (100)
coagulation (Thrombocytes<100)	1 (5)
liver (Bilirubin>33µmol/l)	1 (5)
cardiovascular (dobutamine or norepinephrine)	16 (84)
neurological (GCS<13)	19 (100)
renal (Creatinine>170µmol/l)	4 (21)
Lab	

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CRP - mg/l (normal: < 5)	174 (121-203)
PCT - µg/l (normal: < 0.5)	3.8 (0.5-10.9)
IL-6 - ng/l (normal: < 7)	272 (142-541)
LDH - U/l (normal: < 248)	548 (384-657)
D-Dimer - mg/l (normal: < 0.5)	3.56 (0.84-8.88)
Troponin T - ng/l (normal: < 14)	17 (11-23)
NT-proBNP - ng/l (normal: < 86)	260 (82-1108)
Further clinical course	
prone position	19 (100)
inhaled NO	4 (21)
vv-ECMO	7 (37)
Renal replacement therapy	8 (42)
septic shock	10 (53)
died until end of observation period	3 (16)
still dependeant on critical care	6 (32)
still dependeant on mechanical ventilation	2 (11)

ABBREVIATIONS:
ARDS – Acute respiratory distress syndrome, BMI – body mass index, CAD – Coronary artery disease, CHF – Congestive heart failure, CKD – Chronic kidney disease, CRP – C-reactive protein, ECMO – Extracorporeal membrane oxygenation, FiO_2 - Fraction of inspired oxygen, HTN – Hypertension, ICU – Intensive care unit, HTN – Hypertension, LDH – Lactate dehydrogenase, MV – Minute ventilation, PCT – Procalcitonin, PEEP – Positive end-exspiratory pressure, Pplat – Plateau pressure, RR – Respiratory rate, SOFA - Sequential Organ Failure Assessment

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