Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Heparin prevents *in vitro* glycocalyx shedding induced by plasma from COVID-19 patients

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ABSTRACT

The severe forms and worsened outcomes of COVID-19 (coronavirus disease 19) are closely associated with hypertension and cardiovascular disease. Endothelial cells express Angiotensin-Converting Enzyme 2 (ACE2), which is the entrance door for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The hallmarks of severe illness caused by SARS-CoV-2 infection are increased levels of IL-6, C-reactive protein, D-dimer, ferritin, neutrophilia and lymphopenia, pulmonary intravascular coagulopathy and microthrombi of alveolar capillaries. The endothelial glycocalyx, a proteoglycan- and glycoprotein-rich layer covering the luminal side of endothelial cells, contributes to vascular homeostasis. It regulates vascular tonus and permeability, prevents thrombosis, and modulates leukocyte adhesion and inflammatory response. We hypothesized that cytokine production and reactive oxygen species (ROS) generation associated with COVID-19 leads to glycocalyx degradation. A cohort of 20 hospitalized patients with a confirmed COVID-19 diagnosis and healthy subjects were enrolled in this study. Mechanisms associated with glycocalyx degradation in COVID-19 were investigated. Increased plasma concentrations of IL-6 and IL1-β, as well as increased lipid peroxidation and glycocalyx components were detected in plasma from COVID-19 patients compared to plasma from healthy subjects. Plasma from COVID-19 patients induced glycocalyx shedding in cultured human umbilical vein endothelial cells (HUVECs) and disrupted redox balance. Treatment of HUVECs with low molecular weight heparin inhibited the glycocalyx perturbation. In conclusion, plasma from COVID-19 patients promotes glycocalyx shedding and redox imbalance in endothelial cells, and heparin treatment potentially inhibits glycocalyx disruption.

1. Introduction

The correlation between viral infection and cardiovascular disease gained strength in the last year with the emergence of the new β-coronavirus disease 2019 (COVID-19) [1]. The severe forms and worsened outcomes of COVID-19 are closely associated with hypertension and cardiovascular disease [2] and up to February 2021 the COVID-19 pandemic has affected more than one hundred and eleven million people worldwide [3].

Caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), COVID-19 may trigger intense and diffuse lung injury, that may progress to acute respiratory distress syndrome (ARDS), leading to respiratory failure and death [4,5]. SARS-CoV-2 [6] activates the immune system and increases cytokines production such as IL-1β, IL-

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6 and TNF-α [7], stimulates reactive oxygen species (ROS) generation [8] and coagulation cascade, increasing the risk for thrombosis in the macro and microvasculature [9]. Of importance, as reviewed by Libby and Lüscher [10], "*COVID-19 is, in the end, an endothelial disease*" [10].

Endothelial cells provide a crucial interface in host defenses, producing inflammatory cytokines, ROS and reactive nitrogen species (RNS), which contribute to immune responses against viral infections [11,12]. However, when inappropriately or excessively produced, ROS and RNS disrupt endothelial cell and vascular function [13,14]. The endothelial glycocalyx is one of the most redox-sensitive components that covers endothelial cells [15] and is composed of membrane-attached proteoglycans, glycosaminoglycan chains and glycoproteins [16]. The heparan sulfate proteoglycans, as syndecan and glypican, are the predominant constituent of the glycocalyx, ranging from 50 to 90% [17].

The glycocalyx structure contributes to vascular homeostasis. It modulates vascular tonus, acts as a selective permeable barrier, controlling vascular permeability, prevents microvascular thrombosis, and regulates endothelial cells interactions with immune cells [15], modulating leukocyte adhesion and inflammatory responses (see review [18]). In addition, the vascular endothelial glycocalyx responds to shear stress and is responsible for mechano-transduction signaling, being pivotal for adequate nitric oxide (NO) production [19–23], and redox control [24].

The degradation of glycocalyx is thought to contribute to vascular dysfunction in viral infections as in dengue [25,26], diseases caused by hantavirus [27], ebola virus [28] and influenza virus [29]. The vascular endothelial glycocalyx is more easily damaged in older adults than younger adults, and in common comorbidities such as chronic kidney disease [30], stroke [31,32], diabetes [33], and heart failure [34].

We hypothesized that the cytokine storm and redox imbalance present in plasma from COVID-19 patients leads to glycocalyx degradation, increasing cardiovascular risk. Therefore, this study determined the effects of plasma from SARS-CoV-2-infected patients on the glycocalyx structure of human umbilical vein endothelial cells (HUVECs).

2. Materials and methods

2.1. Human samples

The Brazilian National Committee for Ethics in Research (CONEP) approved all procedures performed in the study (CONEP CAAE: 30248420.9.0000.5440 and 30816620.0.0000.5440). In addition, written informed consent was obtained from all recruited patients.

Twenty hospitalized patients in the *Hospital das Clínicas de Ribeirão Preto* (Ribeirao Preto Medical School) with a RT-PCR of nasopharyngeal samples [35] proved SARS-CoV-2 infection as well as the detection of specific antibodies IgM and IgG against SARS-CoV-2 were confirmed in plasma samples. Blood from COVID-19 patients was collected between days 1 to 5 after admission to the emergency room in the Hospital das Clínicas de Ribeirão Preto. In addition, a control group with seven sex-matched healthy subjects was included in this study. Blood samples from all volunteers were collected in tubes containing ethylenediaminetetraacetic acid (EDTA), to obtain the plasma and in tubes without anticoagulant, to obtain the serum. Samples were stored at −80 °C for later analysis. Table 1 summarizes clinical information, biochemical parameters and therapeutic treatments for COVID-19 patients.

2.2. IgM-IgG combined antibody test

Analysis of IgG and IgM anti-SARS-CoV-2 antibodies was performed in serum from healthy subjects (control) and COVID-19 patients using the rapid test Asan Easy Test® COVID-19 IgG/IgM from Asan Pharmaceutical (Gyeonggi-do, Korea). Healthy subjects were not previously exposed to SARS-CoV-2 until to the moment of blood collection (Supplementary Fig. 1). The text is based in an immunochromatographic assay for the rapid qualitative detection of IgG and IgM antibodies through combination of particles coated with SARS-CoV-2 antigen. The *Agência Nacional de Vigilância Sanitária* (National Agency of Sanitary Vigilance, ANVISA, Brazil) licensed the Asan Easy Test COVID-19 IgG/IgM in May of 2020 (https://consultas.anvisa.gov.br/#/saude/q/?numeroRegistro=80198110005).

Table 1
Baseline characteristics of COVID-19 patients.

Characteristics	COVID-19 patients (n = 20)	Critical COVID-19 (n = 10)	Mild-to-severe COVID-19 (n = 10)	p value
Demographic				
Age (years), median IQR	57 (50–70)	57 (50–70)	59 (47–71)	0.94
Male gender (%)	48%	70%	30%	0.08
Female gender (%)	52%	30%	70%	0.08
Comorbidities				
Hypertension (%)	65%	70%	60%	0.68
Heart disease	50%	40%	30%	0.68
Diabetes (%)	45%	30%	70%	0.20
Obesity (%)	50%	30%	70%	0.09
BMI, median, IQR	28 (25–35)	27.7 (25–31)	31.3 (24–42)	0.72
Clinical characteristics				
Days of symptoms (mean, ±sd)	13 ± 5	13 ± 4	13 ± 6	0.82
Hospitalization (days), mean, ±sd	18 ± 11	26 ± 11	11 ± 4	0.0004*
Mechanical ventilation (%)	50%	100%	0	0.0001*
Acute kidney injury (%)	35%	50%	20%	0.185
Mortality rate (%)	10%	20%	0	0.0001*
Laboratory tests				
Creatinine, mg/dl, mean (IQR)	1.1 ± 0.9	1.23 ± 1.34	0.94 ± 0.47	0.85
Lymphocyte; mm^3, median (IQR)	1100 (600–1650)	650 (475–1550)	1295 (975–2450)	0.09
Platelets; $\times 10^3$/l (IQR)	246 (217–338)	251 (218–369)	243 (186–313)	0.85
RCP; mg/dl, median (IQR)	13 (9–20)	10.8 (6.9–14.1)	7.1 (1.9–13.3)	0.23
D dimer; mg/dl, median (IQR)	1.19 (0.93–1.98)	1.3 (1–3.5)	1 (0.8–1.7)	0.43
Lactate; mg/dl, median (IQR)	2.2 (1.7–2.6)	2.5 (2–2.6)	1.7 (1.5–2.5)	0.09
Lactate dehydrogenase, U/l(IQR)	361 (302–434)	340 (292–438)	383 (278–498)	0.89
Ferritin, ng/ml (IQR)	608 (345–1076)	608 (246–635)	600 (345–1409)	0.51
Fibrinogen (mg/dl)	686 (619–815)	796 (619–833)	683 (617–799)	0.55
Severity				
PaO_2/FiO_2 ratio, median (IQR)	205 (139–315)	158 (122–194)	272 (211–358)	0.01*
SOFA score	2 (1–3)	3 (2–4.5)	2 (1–2)	0.005*
SAPS-3	47 (38–50)	47 (42–51)	42 (34–47)	0.11

BMI: body mass index; IQR: interquartile range; sd: standard deviation; RCP: reactive C protein; PaO2/FiO2 ratio: the ratio of arterial oxygen partial pressure (PaO2 in mm Hg) to fractional inspired oxygen; SOFA: sequential organ failure assessment; SAPS-3: simplified acute physiology score III.

* $p < 0.05$, comparison between mild-to-severe and critical patients with COVID-19.

2.3. ELISA assay

Plasma samples were used to evaluate circulating cytokine levels by

enzyme-linked immunosorbent assay (ELISA) using Human DuoSet ELISA (R&D Systems, Minneapolis, MN, USA). The assay was performed according to the manufacturer's instructions and the detection limits were as follows: TNF-α, 15.62–2000 pg/ml; IL-6, 9.37–1200 pg/ml; and IL-1β, 3.90–500 pg/ml.

Glycocalyx components, as hyaluronan (DuoSet ELISA) and heparan sulfate proteoglycans (ELISA, Cloud-Clone Corporation, Katy, TX, USA), were determined in plasma samples and in the supernatants of HUVECs (exposed to plasma for 12 h) by ELISA assay, according to the manufacturer's protocol. The detection limit for hyaluronan was 0.37–90 ng/ml and for heparan sulfate proteoglycans was 15.6–1000 pg/ml.

Heparanase activity was measured in plasma samples using Simple Step ELISA kit (Abcam, Cambridge, United Kingdom) and the detection limit established by manufacturer's guidelines was 125–8000 pg/ml.

2.4. TBARS assay

Thiobarbituric acid reactive substance (TBARS) assay was performed to measure malondialdehyde (MDA), which is an end product of lipid peroxidation and indicative of oxidative stress [36], using plasma samples according to the manufacturer's datasheet (Cayman Chemical, Ann Arbor, MI, USA).

2.5. HUVECs cells

Human umbilical vein endothelial cells (HUVECs) were purchased from ATCC cell lines (American Type Culture Collection, Manassas, VA – USA), cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with sodium bicarbonate, HEPES, penicillin, streptomycin, amphotericin B, fetal bovine serum (FBS, 10%). Before any experiment, HUVECs were submitted to serum starvation (FBS 3%) [37,38]. Cells were cultured for at least 3 days before the experiments and used at passages 4–6.

2.6. Cytotoxicity assay

Supernatants of naive/non-treated HUVECs (Control), HUVECs treated with plasma from healthy subjects and HUVECs treated with plasma from COVID-19 patients (12 h of treatment) were used to measure lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis or damage, using the CytoTox 96® Non-Radioactive Cytotoxicity colorimetric assay according to the manufacturer's instructions (G1780, Promega Corporation, Madison, WI, USA).

2.7. Measurement of ROS and RNS in HUVECs

HUVECs were cultured at 4×10^4 cells/well in a 96-well assay plate in DMEM with fetal bovine serum (-FBS) 10% for 24 h (h). After 24 h, cells were serum deprived (3 h), washed twice with PBS (phosphate buffered saline) and incubated with 100 μL of DAF-2DA (5 μM + L-arginine 1 mM, for 30 min), or carboxy-H_2DCFA (10 μM, for 60 min), or 7-CBA (20 μM, for 30 min), or DHE (2.5 μM, for 30 min). After this period, HUVECs were treated with plasma (10%) from healthy subjects and COVID-19 patients for 30 min.

The fluorescence produced by DAF-2DA (488 nm/530 nm; excitation/emission), 7-CBA (332 nm/475 nm), carboxy-H_2DCFA (370 nm/420 nm; Excitation/Emission), DHE (379 nm/420 nm) was measured at a microplate reader (FlexStation-3, Molecular Devices, San Jose, CA, USA).

2.8. Western blot

HUVECs were cultured in DMEM and after serum deprivation for 3 h [37,38], the confluent HUVECs received no treatment (Control) or were treated with a "pool" of plasma (10%) - 2 plasma samples per pool were randomly chosen - from healthy subjects or COVID-19 patients for 30 min, 1 h, 12 h and 24 h. In a subsequent set of experiments, the time of 12 h to treat cells with plasma from COVID-19 patients in presence of low molecular weight heparin (LMWH, 10 μg/ml) was established. After treatments, cells were lysed in RIPA buffer supplemented with a protease inhibitor cocktail (PIC, P8340, Sigma-Aldrich, St Louis, MO, USA) and phosphatase inhibitors [NaF (1 mmol/l), Na_3VO_4 (1 mmol/l) and PMSF (10 mmol/l)]. Protein (30 μg) obtained from each sample was submitted to electrophoresis on polyacrylamide gel (8 to 15%) and transferred to a nitrocellulose membrane. Next, membranes were incubated with primary antibodies (overnight) against syndecan-1 (#sc-12765), syndecan-4 (#sc-12766) and glypican-1 (#sc-101827). The bands were detected by a chemiluminescent system (ImageQuant LAS 400, GE Life Science, Chicago, IL, USA). β-Actin was used to normalize the results. The bands were quantified with the ImageJ Software (NIH Image).

2.9. Immunofluorescence

HUVECs were plated in glass coverslips pretreated with 0.4% gelatin. When cells reached confluence, they were serum deprived for 3 h and treated for 12 h with plasma (10%) from control subjects, COVID-19 patients or COVID-19 patients in the presence of LMWH (10 μg/ml). Cells were fixed with 4% paraformaldehyde and permeabilized with triton 0.1% in HBSS. Cells were incubated with glypican-1 (#sc-101827) and CD34 (#IS63230-2) antibodies overnight at 4 °C. Then, cells were washed and incubated with Alexa Fluor ™ 647 and 488 antibodies for 2 h at room temperature followed up by incubation with DAPI. Slides were mounted and images obtained on a LSM 780 System on the Axio Observer microscope using Zen software. Images were quantified with ImageJ (NIH). Data are presented as relative fluorescent units (RFU, fluorescence on 647 channel normalized by the number of cells in the field assessed by DAPI staining).

2.10. Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM) and interquartile interval (IQR), depending on distribution tested by Shapiro Wilk test. In the experiments where HUVECs were used, *n* indicates the number of independent experiments. The statistical significance was determined by unpaired Student's *t*-test and one-way ANOVA with Tukey post-hoc test. The statistical analysis was performed with the Prism GraphPad 5.0 software and differences statistically significant were considered when $p < 0.05$.

3. Results

A total of 27 subjects were included in the present study, 20 patients that tested positive to SARS-CoV-2 and 7 healthy individuals in the control group. Similar numbers of men and women with COVID-19 were included (48% men and 52% women). The demographic, clinical, and biochemical characteristics for COVID-19 patients are shown in Table 1, and information from healthy subjects are shown in Supplementary Fig. 1.

In all analyses, healthy subjects *versus* COVID-19 patients as well as mild-to-severe *versus* critical individuals with COVID-19 were compared. PaO_2/FiO_2 ratio was used to identify mild-to-severe and critical patients. 70% of critical patients were men, reinforcing sex differences in the severity of disease. Patients infected with SARS-CoV-2 showed significant higher plasma levels of IL-6 (Fig. 1A) and IL-1β (Fig. 1C) compared to healthy subjects. TNF-α levels did not differ between the groups (Fig. 1E). In addition, splitting data frames in moderate-to-severe and critical cases of COVID-19, showed no differences for IL-6, IL-1β or TNF-α levels, as shown in Fig. 1B, D, and F, respectively. Moreover, MDA levels were increased in patients with SARS-CoV-2 infection, in comparison to healthy subjects (Fig. 1G). MDA levels did not differ between moderate-to-severe and critical patients

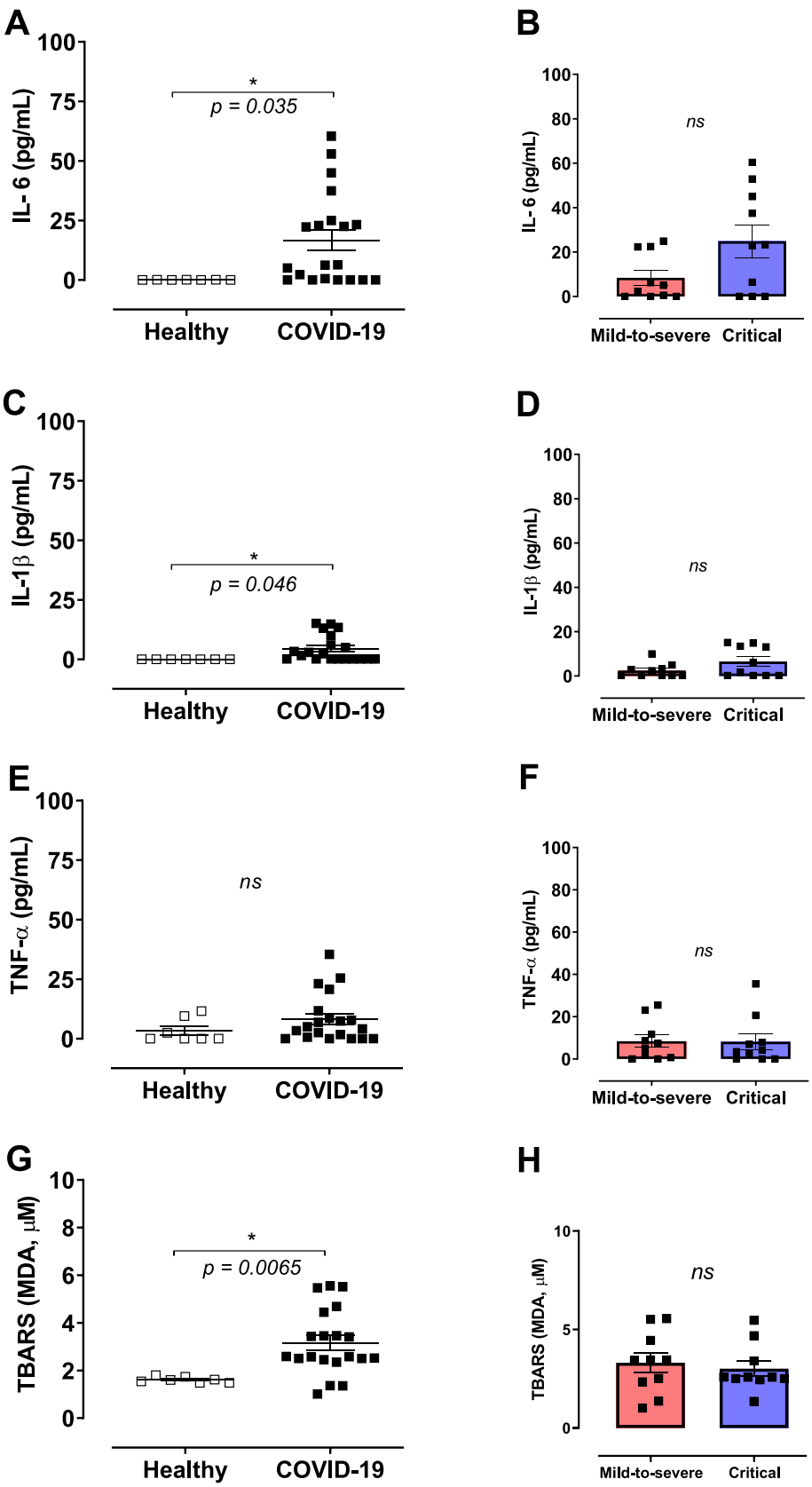

Fig. 1. COVID-19 patients exhibit high concentrations of cytokines and augmented lipid peroxidation. Plasma was obtained from healthy controls and COVID-19 patients; cytokines were measured by ELISA and lipid peroxidation was determined by malondialdehyde (MDA) levels, which is an end product of lipid peroxidation and indicative of oxidative stress. (A) IL-6, (B) IL1-β, (C) TNF-α and (D) MDA in plasma from healthy controls (n = 7) and COVID-19 patients (n = 20). Data are shown as mean ± SEM. Student's *t*-test. *p < 0.05; *ns*: non-significant.

diagnosed with COVID-19 (Fig. 1H).

Glycocalyx integrity was evaluated by heparanase activity, by heparan sulfate and hyaluronan levels. Heparanase activity was significantly higher in the plasma from COVID-19 patients compared to healthy controls (Fig. 2A). In addition, circulating levels of heparan sulfate were also increased during SARS-CoV-2 infection (Fig. 2C), possibly due to the increased heparanase activity observed in patients with COVID-19. Hyaluronan plasma levels were also increased in COVID-19 patients compared to the control group (Fig. 2E). The analyses showed no differences between mild-to-severe and critical patients (Fig. 2B, D and F).

Considering that plasma samples from COVID-19 patients displayed higher levels of cytokines, MDA and heparanase activity, the effects of plasma from COVID-19 patients on the glycocalyx of healthy endothelial cells were determined. HUVECs stimulated for 30 min with plasma from COVID-19 patients showed lower concentrations of H_2O_2 (Fig. 3A) and NO (Fig. 3C), but not changes were observed in superoxide anion or peroxynitrite levels (Fig. 3E and G, respectively). No differences were observed in H_2O_2, superoxide anion or peroxynitrite levels between HUVECs stimulated with plasma from mild-to-severe and critical patients with COVID-19 (Fig. 3B, F and H, respectively). However, NO levels were higher in HUVECs treated with plasma from severe COVID-19 patients (Fig. 3D).

Since ROS imbalance may lead to glycocalyx degradation, we questioned whether HUVECs treated with plasma from COVID-19 patients exhibit glycocalyx shedding. HUVECs treated with plasma from COVID-

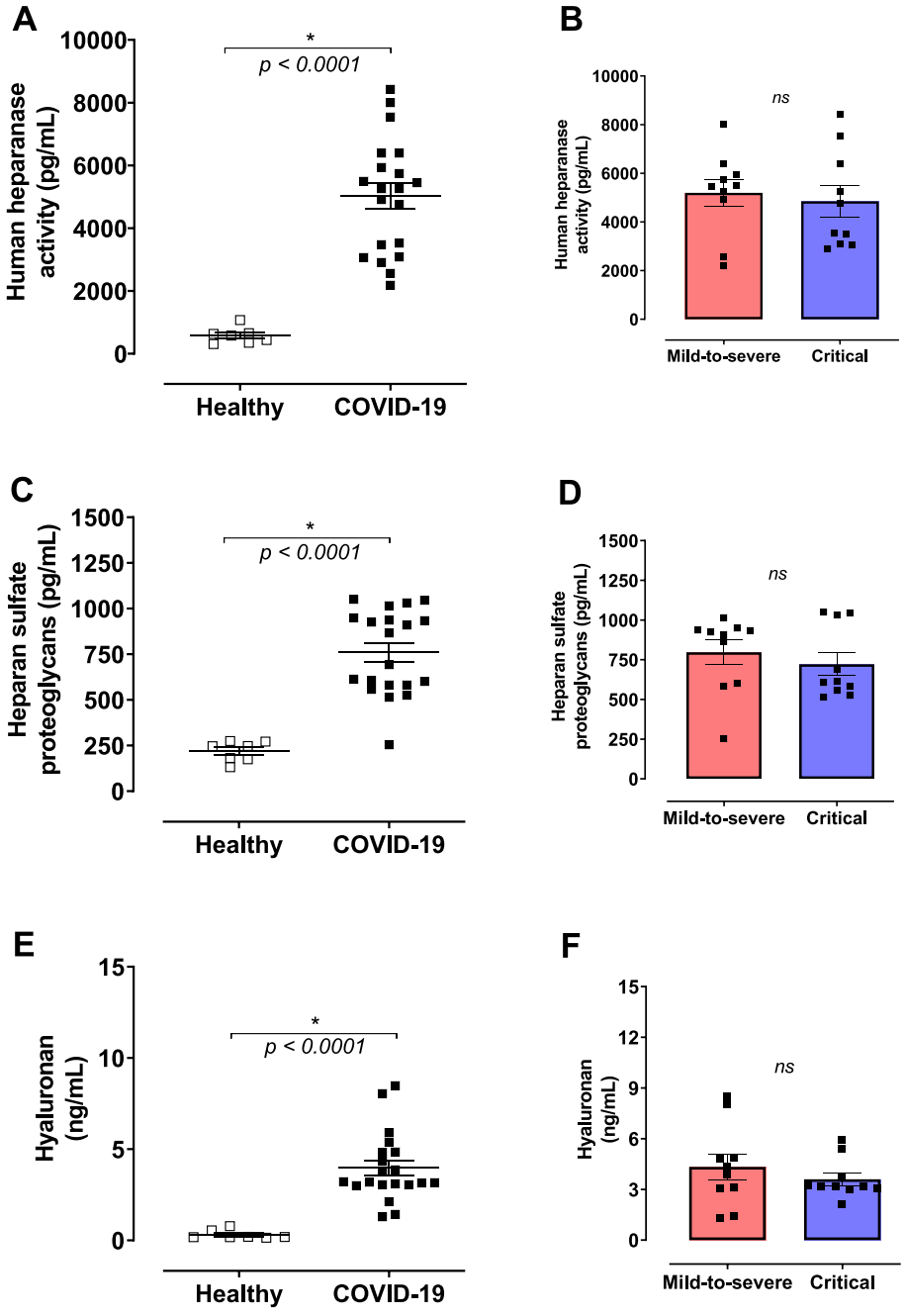

Fig. 2. COVID-19 patients exhibit high concentrations of degradation glycocalyx components. Plasma was obtained from healthy controls and COVID-19 patients and glycocalyx components measured by ELISA. (A) Human heparanase activity, (B) heparan sulfate proteoglycans, and (C) hyaluronan in plasma from healthy controls (n = 7) or COVID-19 patients (n = 20). Data are shown as mean ± SEM. Student's *t*-test. *p < 0.05; *ns*: non-significant.

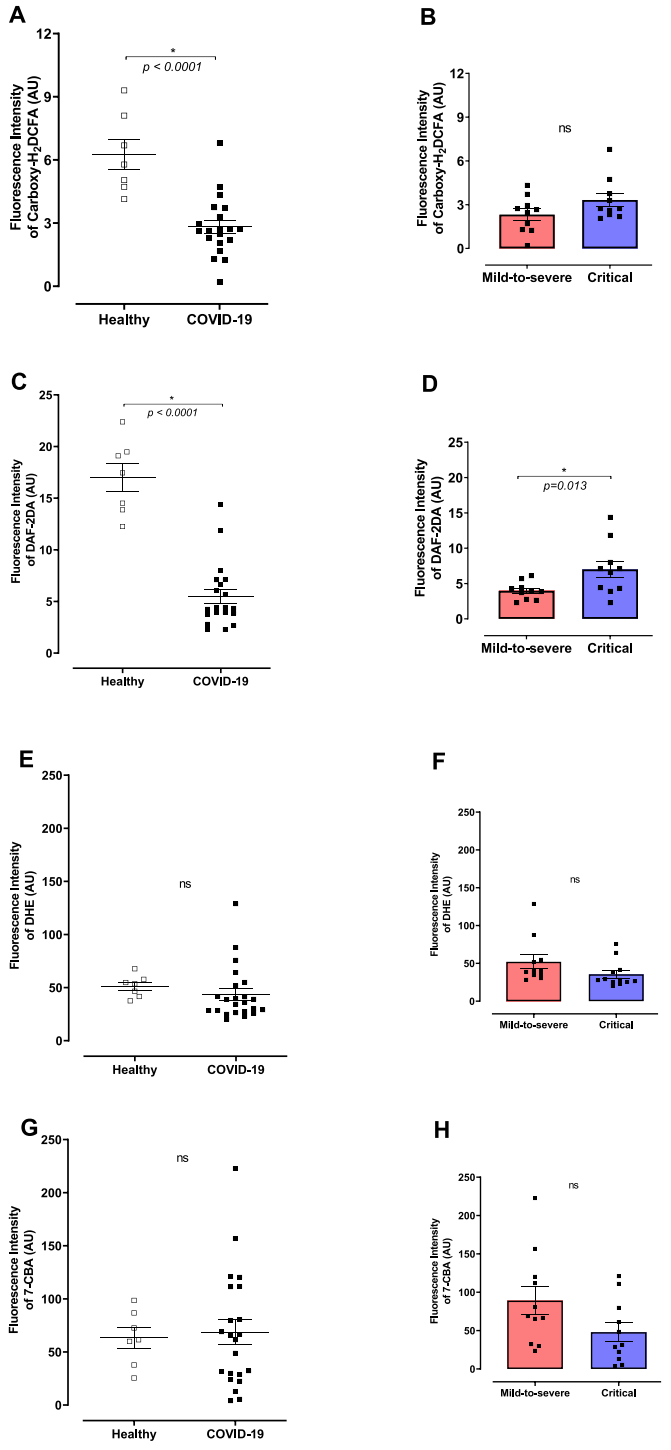

Fig. 3. Plasma from COVID-19 patients reduces H_2O_2 and NO in healthy endothelial cells. (A) Hydrogen peroxide (H_2O_2), (B) nitric oxide (NO), (C) superoxide anion ($O_2^{\bullet-}$), and peroxynitrite ($ONOO^-$) levels in HUVECs treated with plasma from healthy subjects or COVID-19 patients. H_2O_2 was measured by Carboxy-H_2DCFA (10 μM), NO by DAF-2DA (5 μM), peroxynitrite by 7-CBA (5 μM), and superoxide anion by DHE (2.5 μM) in 4×10^4 HUVECs cells. The values are expressed by as Fluorescence Intensity. Data are shown as mean ± SEM. Student's *t*-test. *$p < 0.05$; *ns*: non-significant.

19 patients or treated with plasma from healthy subjects for 12 h showed similar levels of LDH compared to Control HUVECs, indicating that plasma treatment did not damage or decrease viability of the cells (Fig. 4A). In addition, heparan sulfate levels were increased in HUVECs treated with plasma from COVID-19 patients for 12 h, demonstrating glycocalyx shedding (Fig. 4B). To confirm glycocalyx disruption, HUVECs were exposed to plasma for various time points (30 min, 1 h, 12 h, and 24 h). Treatment of HUVECs with plasma from COVID-19 patients decreased protein levels of important glycocalyx components such as syndecan-1, glypican-1, and syndecan-4 (Fig. 5). As treatment for 12 h did not decrease cells viability, but significantly decreased the expression of the analyzed proteins, this time point was used to evaluate the effects of heparin/LMWH.

To determine the role of heparanase on glycocalyx degradation, HUVECs were treated with plasma from COVID-19 patients in the presence of heparin/LMWH. Heparin/LMWH prevented the decreased expression of syndecan-1, glypican-1 and syndecan-4 (Fig. 6). These results were confirmed by fluorescence images. HUVECs exhibited decreased fluorescence intensity for glypican-1 in the presence of plasma from SARS-CoV-2-positive patients compared to plasma from healthy subjects. Furthermore, heparin/LMWH abrogated the decreased fluorescence intensity of glypican-1 observed in cells exposed to plasma from COVID-19 patients (Fig. 7).

4. Discussion

This study shows that plasma from COVID-19 patients promotes endothelial glycocalyx shedding. Glycocalyx disruption occurs in healthy endothelial cells (HUVECs) and is associated with high levels of cytokines and redox imbalance. The impairment of endothelial barrier function, represented by glycocalyx perturbation, contributes to endothelial dysfunction, procoagulant, and thrombotic events. In addition, *in vitro* treatment with heparin/LMWH prevents endothelial glycocalyx disruption in HUVECs exposed to plasma from COVID-19 patients.

Reports from China showed that about 10–15% of mild COVID-19 cases may progress to severe illness, and 15–20% of severe cases may become critical, with critical cases requiring treatment in intensive care units (ICU) [5]. It is important to mention that the criteria to define severely ill patients vary among studies. Individuals with cardiovascular and metabolic comorbidities such as hypertension, obesity, and type 2 diabetes are more likely to develop severe symptoms of COVID-19 and to be classified into as mild-to-severe and critical cases of COVID-19 [39]. In addition, mild-to-severe *versus* critical patients may show differences during the development of COVID-19, and critical cases can rapidly progress to death.

Our study used a cohort of elderly patients with comorbidities, one of the main risk groups. Although no significant differences were observed in the rate of hypertension and diabetes between mild-to-severe and critical cases of COVID-19 (Table 1), many, but not all, studies show differences in the occurrence of comorbidities between mild-to-severe *versus* critical patients. In a public hospital in New York City during the first month of the COVID-19 pandemic, similar rates of cardiovascular comorbidities and type 2 diabetes were observed between ICU *versus* non-ICU patients [40]. On the other hand, hypertension was prevalent in a critical group compared to a mild-to-severe group ($p < 0.0078$) in a cohort of forty-one patients positive to COVID-19 hospitalized at the Fifth Medical Center of PLA General Hospital in Beijing (China) [41]. Of note, a similar percentage of patients with obesity was present in these groups of patients [41].

In addition, differences in platelet activation and platelet-monocyte aggregate formation were reported between critical and mild-to-severe patients. However, the mild-to-severe patients were younger than the critical group [42]. Differences in endothelial glycocalyx were also reported in critically ill patients with COVID-19, but basically only men (95%) were enrolled in the study [43]. In the present study, however, samples from men and women were similar, with 48% of male patients, reinforcing that the cohort is representative for both aging and comorbidities.

Viral infections demand an immediate immune response of host metabolism to create an effective antiviral response [44]. SARS-CoV-2

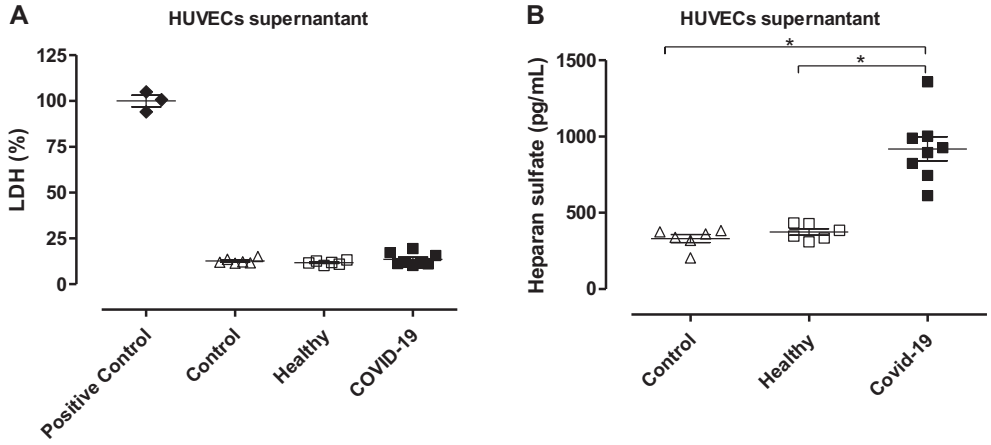

Fig. 4. Treatment of HUVECs with plasma from COVID-19 patients stimulates glycocalyx shedding but does not decrease cell viability. (A) Lactate dehydrogenase (LDH) levels and (B) heparan sulfate proteoglycans measured in supernatant from HUVECs without any treatment (Control, n = 6), HUVECs treated with plasma from healthy subjects (n = 6) or plasma from COVID-19 patients (n = 8). Data are shown as mean ± SEM. Student's *t*-test. *p < 0.05.

triggers a cascade of acute biological events, such as increased synthesis of ROS in immune and non-immune cells, promoting redox imbalance. ROS are by-products of a wide range of physiological reactions that play essential roles in living organisms. However, excessive ROS induced by infections may disrupt redox balance and lead to vascular pathology.

COVID-19 patient's exhibit increased extracellular traps (NETs), which are indicative of neutrophil activation [45,46]. Activated polymorphonuclear neutrophils migrate to target tissues of inflammation and enhance ROS generation [47]. In addition, many viruses inhibits nuclear factor erythroid 2-related factor 2 (Nrf2) activation, which is responsible for antioxidant defense [48]. Moreover, decreased expression of the antioxidant enzyme superoxide dismutase 3 (ec-SOD3) in the lungs of elderly patients infected with SARS-CoV-2 [49] has been reported. These studies reinforce that viral infection disrupts the antioxidant defense system [50], a potential mechanism associated with increased ROS generation.

Human coronaviruses activate NF-κB signaling, the major contributor to inflammation and oxidative damage during SARS-CoV infection [51]. Furthermore, the viral infection induces mitochondrial ROS production to promote efficient viral replications [52]. All these events contribute to overproduction of ROS and lead to oxidative stress and cellular damage, including lipid peroxidation and DNA oxidation [53]. MDA levels were increased in plasma from COVID-19 patients as compared to healthy individuals, which indicates a state of oxidative stress. High levels of ROS were also detected in fresh sputum of COVID-19 patients [8].

Despite the damage caused by ROS excess, ROS are essential signaling molecules for the progression of inflammatory processes and production of cytokines and chemokines [47]. Costela-Ruiz et al. [7] reviewed several studies that show hyperproduction of cytokines and interleukins in plasma and blood from COVID-19 patients. During cellular infection, RNA virus promotes NLRP3 (Nod-like receptor pyrin domain-containing 3) inflammasome activation [54,55]. NLRP3 converts inactive pro-caspase-1 to active caspase-1 [56], and then caspase-1 cleaves pro-IL-1β forming active IL-1β [57]. SARS-CoV-2 infection is associated with the activation and maturation of IL-1β, a key cytokine in the cytokine storm produced by coronavirus. IL-1β activates other proinflammatory cytokines, such as IL-6 and TNF-α [58]. In the present study, plasma from COVID-19 patients exhibited increased levels of IL-1β and IL-6, but no differences in the levels of TNF-α.

Proinflammatory cytokines, as IL-1β and IL-6, and ROS activate enzymes named sheddases, such as heparanase, metalloproteinases (MMPs) and hyaluronidase, which induce glycocalyx degradation [28,59]. Regarding the activation of MMPs, ROS decrease levels of tissue inhibitors of MMPs (TIMPs), thus increasing the activity of MMPs [60], which cleave the protein core of the syndecan proteoglycan, promoting shedding of the syndecan's family [61]. Heparanase is an endoglycosidase that cleaves the side chains of heparan sulfate present in the structure of syndecan and glypican families and, therefore, disrupts the glycocalyx [62]. Hyaluronidase degrades hyaluronan into fragments *via* hydrolysis of the disaccharides at hexosaminidic β (1 to 4) linkages [63]. A recent study demonstrated that heparanase activity was increased in both non-ICU (intensive care unit) and ICU patients with COVID-19 compared to healthy individuals [64], reinforcing data from our study. The increased heparanase activity was linked to increased heparan sulfate fragments present in the plasma from COVID-19 patients. In addition, hyaluronan fragments were also increased in COVID-19 patients, confirming that SARS-CoV-2 infection is associated with glycocalyx degradation. Although, Stahl et al. [43] did not find differences in heparanase-1 activity between COVID-19 patients and healthy subjects, using sublingual sidestream darkfield (SDF) image, they showed *in vivo* that endothelial glycocalyx thickness is decreased on the perfused boundary region in patients with COVID-19.

We then addressed whether plasma from patients with COVID-19 alters vascular homeostasis, by inducing endothelial glycocalyx degradation. HUVECs treated with plasma from COVID-19 patients showed reduced H_2O_2 and NO levels compared to endothelial cells treated with plasma from healthy individuals. Several studies have shown that the endothelial glycocalyx is the main sensor that activates mechanotransduction in vascular cells, creating immediate response to shear stress and inducing NO production [21,65]. Pharmacological tools that remove or degrade heparan sulfate and other glycocalyx constituents block NO production in endothelial cells [20–23]. These studies reinforce our results that glycocalyx is disrupted in HUVECs exposed to plasma from patients with COVID-19, since NO production was decreased. It is important to mention that plasma from critically ill patients exhibited increased NO, an event that may be linked to NO derived from iNOS, whose expression is 2–3 fold higher following infection [66]. H_2O_2 is an intracellular messenger that modulates several endothelial cell functions. Its rigidly regulated concentration modulates endothelial cell growth and proliferation, endothelium-dependent vasorelaxation and vascular remodeling, and also inflammatory responses [67]. Low levels of H_2O_2 decrease survival and proliferation of vascular smooth muscle cells [68], and the decreased H_2O_2 levels in HUVECs exposed to plasma from infected patients may impair vascular homeostasis.

HUVECs exposed to plasma from patients with COVID-19 exhibited decreased expression of specific components of the glycocalyx, including glypican-1, syndecan-1 and syndecan-4. Expression of these

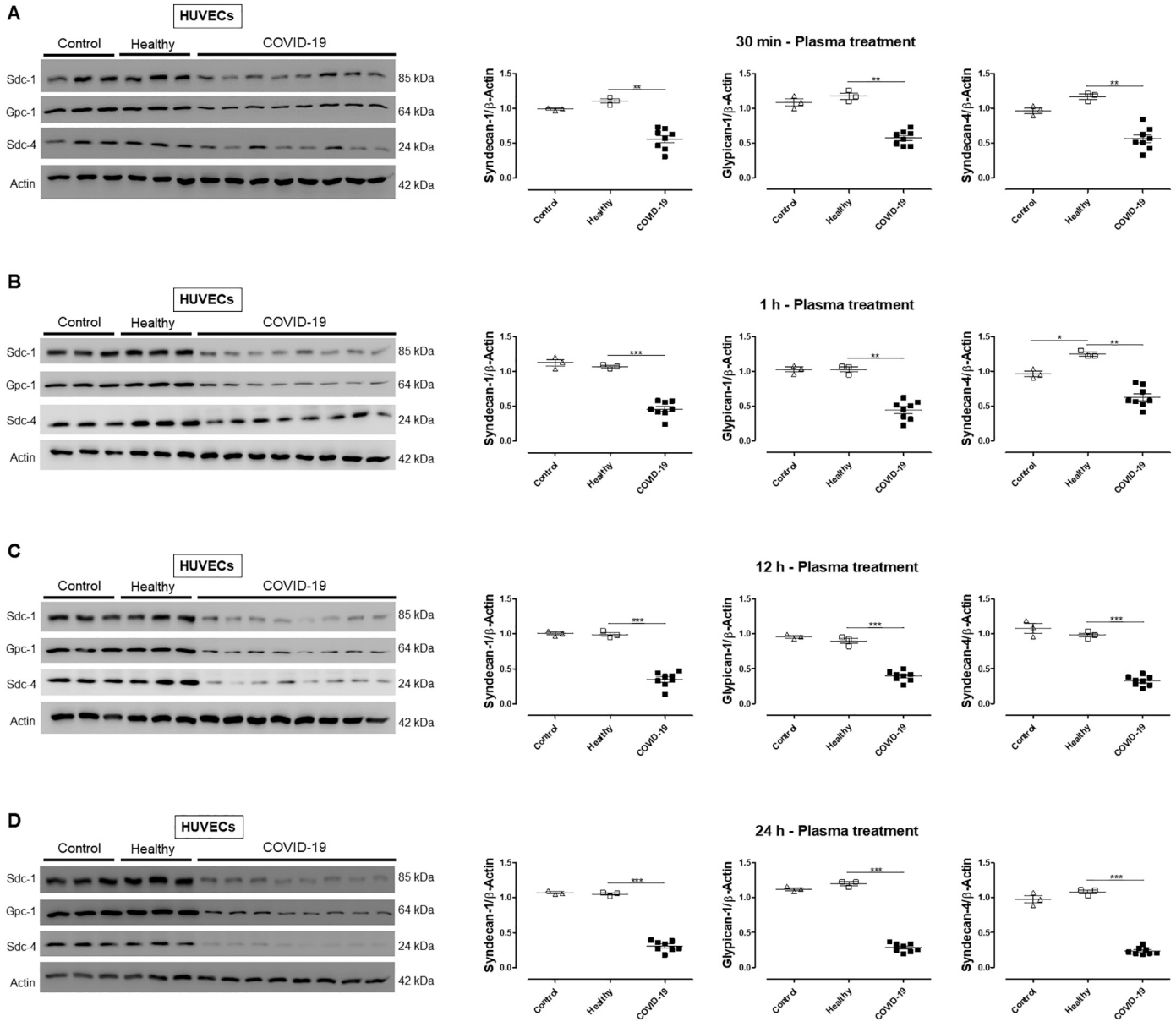

Fig. 5. Plasma from COVID-19 patients reduces the expression of glycocalyx components. Immunoblot representative images (A, B, C, and D) and densitometric analysis (right) of protein levels of syndecan-1, glypican-1, and syndecan-4 in HUVECs non-treated (Control), treated with plasma from healthy subjects and COVID-19 patients for 30 min (A), 1 h (B), 12 h (C) and 24 h (D). The values are normalized by β-actin and expressed by absolute values. Data are representative of at least 3 experiments and are shown as mean ± SEM. Student's *t*-test. *p < 0.05. We processed images of blots changing brightness and contrast and we applied equally over the entire image.

heparan sulfate proteoglycans were reduced after 30 min and 1 h of exposure to plasma from COVID-19 patients. Longer exposure times, 12 h and 24 h, promoted a drastic decrease in the expression of glypican-1, syndecan-1 and syndecan-4, confirming the degradation of glycocalyx. Syndecan-1 was increased in blood from COVID-19 patients, indicated shedding of glycocalyx and reinforce our data [43]. Intact heparan sulfate chains of the glycocalyx serve as binding sites for antithrombin III, the main anticoagulant molecule that inhibits coagulant factors [69,70]. Likewise, tissue factor pathway inhibitor (TFPI) can also bind to heparan sulfates and inhibits the early stages of procoagulant process [71]. Therefore, the endothelial glycocalyx has a role as antithrombotic and anticoagulant, and its degradation may be directly associated with thrombotic events and pro-coagulant effects in SARS-CoV2 infection. A review with 1026 patients with SARS-CoV-2 infection showed that at least 40% were considered at high risk of venous thromboembolism [72]. In addition, coagulation disorders were reported in patients infected by the new coronavirus, and more than 70% of non-survivors patients developed disseminated intravascular coagulation (DIC) [73]. Moreover, bleeding was a significant cause of death in positive cases of COVID-19, which was associated with increased plasma D-dimer levels during the admission of patients, making D-dimer an excellent predictor of coagulation disorders, bleeding complications, and thrombotic events [74].

Heparanase is a unique mammalian enzyme that degrades heparan sulfate chains [75], and its inhibition may promote beneficial effects on COVID-19 patients, such as preventing coagulation disorders and avoiding thrombotic events by blocking glycocalyx shedding and disruption. In the present study, the treatment of HUVECs exposed to plasma from COVID-19 patients with heparin/LMWH was effective in preventing glycocalyx degradation. Accordingly, heparin/LMWH has been shown to inhibit heparanase [76–80]. Corroborating our data, non-ICU patients positive for SARS-CoV-2 that received prophylactic doses of

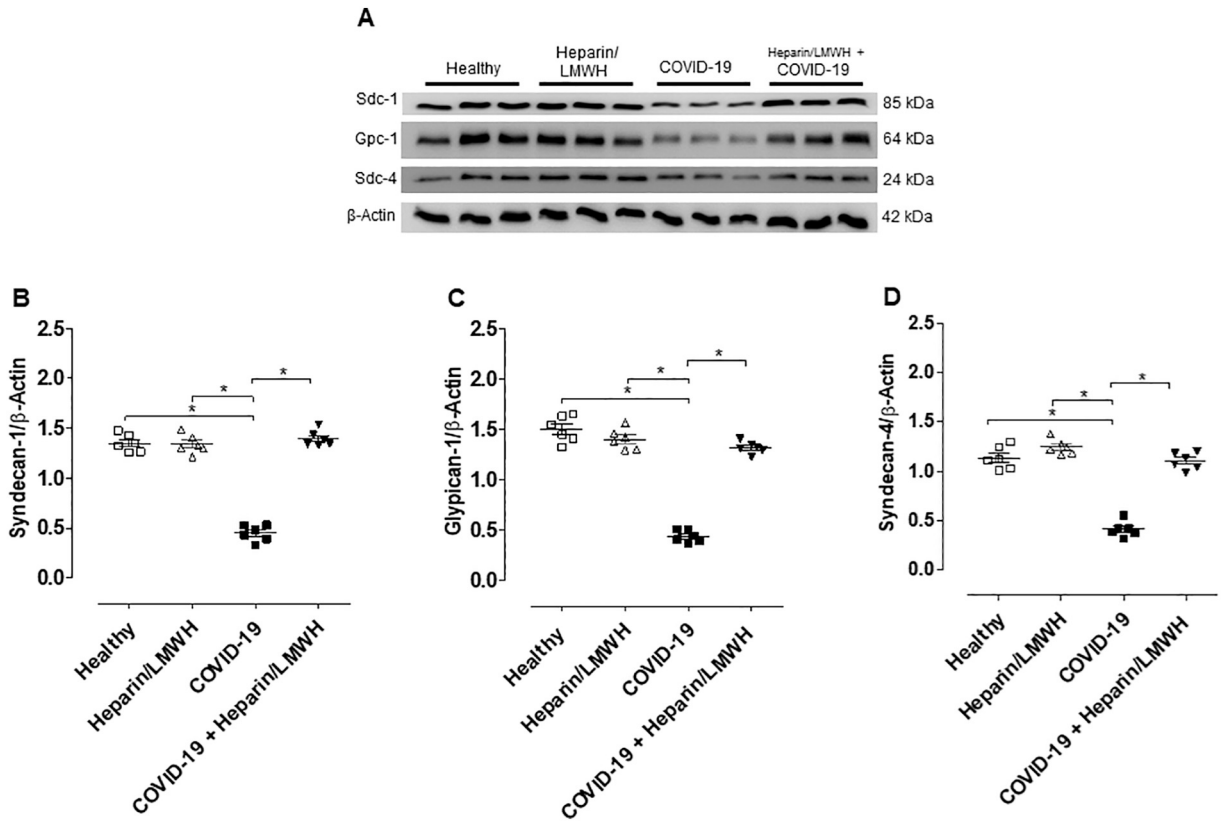

Fig. 6. Heparin/LMWH abrogates the reduced levels of glycocalyx components induced by plasma of COVID-19 patients. Immunoblot representative images (up) and densitometry analysis (A, B, and C) of protein levels of syndecan-1, glypican-1, and syndecan-4 in HUVECs treated with plasma from healthy subjects and COVID-19 patients for 12 h in presence of Heparin/LMWH (10 μg/ml, 12 h). The values are normalized by β-actin and expressed as absolute values. Data are representative of at least 6 experiments and are shown as mean ± SEM. One-way ANOVA followed by Tukey post-test. *$p < 0.05$. We processed images of blots changing brightness and contrast and we applied equally over the entire image.

dalteparin/LMWH (5000 IU, daily) exhibited reduced heparanase activity [64]. Several clinical studies demonstrated beneficial effects of heparin/LMWH during lung injury; and decreased the mortality of patients with acute respiratory distress syndrome, according review by Li et al. [81]. Moreover, heparin inhibited the infection caused by SARS-CoV strain HSR1 in Vero cells by 50% [82] and blocked of the SARS-CoV pseudovirus spike protein at the HEK293 cells, thus preventing the entry of the virus on host cells [83]. Furthermore, heparin has recently shown to induce a conformational change on the SARS-CoV-2 spike protein domain, preventing the virus attachment to host cells [84] and effectively inhibiting SARS-CoV-2 infection *in vitro* [85].

Prevention of endothelial glycocalyx degradation may effectively preserve endothelial barrier function, avoiding endothelial dysfunction as well as coagulant disorders and thrombotic events observed in critical and severe patients with COVID-19. In addition, our study reinforces the potential use of heparin and its derivatives in SARS-CoV-2 infection. More clinical trials are necessary to find the appropriate, effective dose of heparin/LMWH to improve outcomes in COVID-19 patients [86]. Therefore, knowledge on the mechanisms associated of SARS-CoV-2 infection and vascular dysfunction may certainly contribute to the development of new therapeutic strategies aiming to treat COVID-19 patients and to avoid cardiovascular complications.

Although we have used classical methods to show that plasma of patients with COVID-19 increases the enzyme responsible for the degradation of heparan sulfate proteoglycans and for the leakage of the glycocalyx components, more robust techniques, such as electron microscopy, are important to provide accurate information on the thickness of the glycocalyx. Nevertheless, our data clearly suggest that plasma from COVID-19 patients disturbs the glycocalyx of HUVEC, decreasing the content of specific components of the glycocalyx and promoting shedding of heparan sulfate, which indicates that COVID-19 induces glycocalyx degradation.

5. Conclusion

Our study shows that plasma from hospitalized COVID-19 patients contains increased levels of glycocalyx components and increased heparanase activity, indicating glycocalyx disruption. Moreover, plasma from COVID-19 patients also induces glycocalyx shedding and disturbs redox balance in healthy HUVECs. LMWH/heparin inhibits glycocalyx perturbation induced by plasma from COVID-19 patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2021.119376.

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CRediT authorship contribution statement

SRP, TJC and RT designed the research. MNB and MAM supervised the clinical study. SRP, TJC, TFCFS, RBM and KSGS performed experiments. SRP, TJC, and RT analyzed and interpreted data. SRP, TJC, MNB,

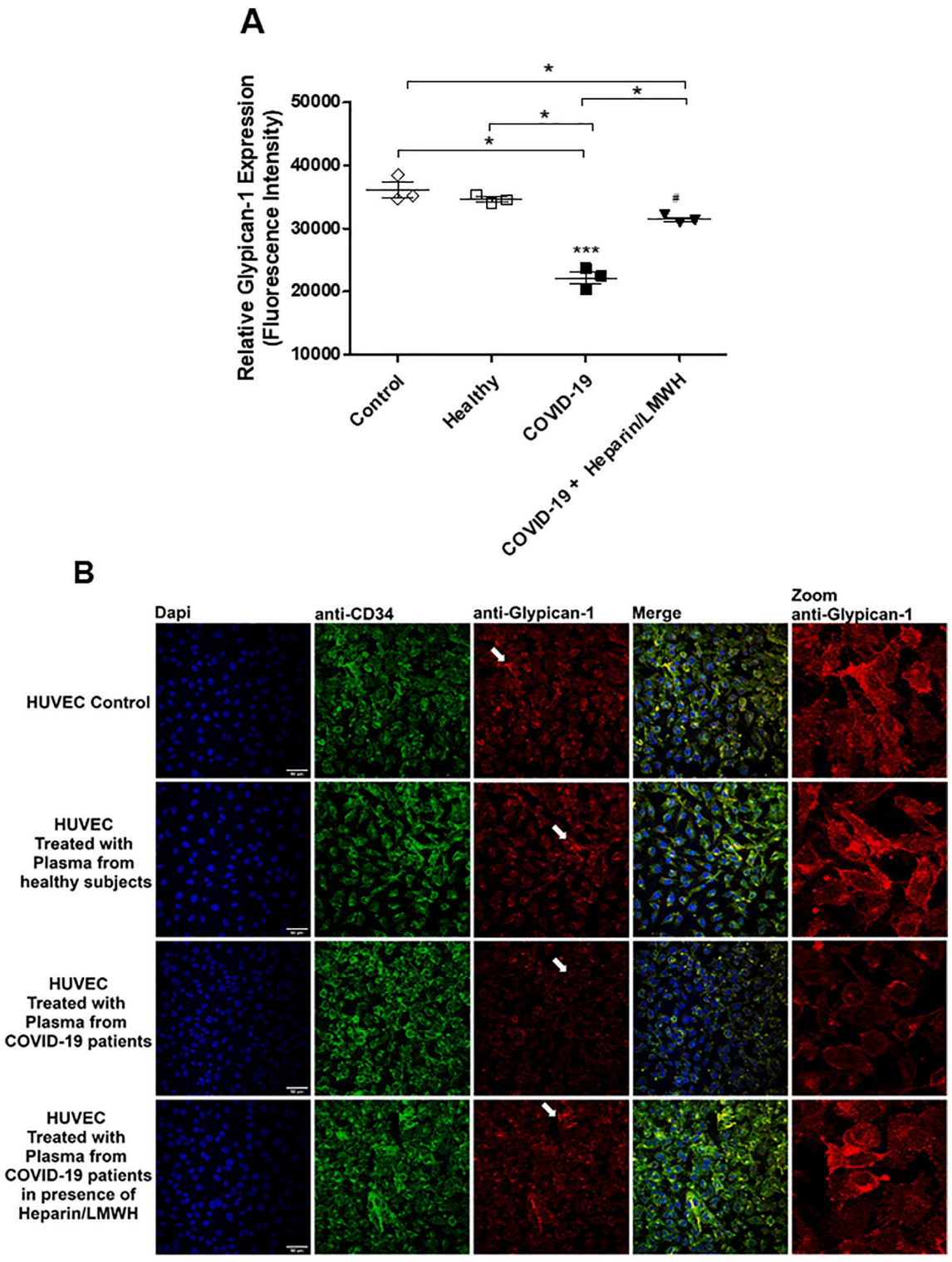

Fig. 7. Heparin/LMWH abrogates the reduced levels of glycocalyx components induced by plasma from COVID-19 patients. Immunofluorescence (40× magnification) of glypican-1 in HUVECs treated with plasma from healthy subjects and COVID-19 patients in the presence of heparin/LMWH or vehicle. Data are representative of at least 3 experiments and are shown as mean ± SEM. One-way ANOVA followed by Tukey post-test. *$p < 0.05$.

CELA, VLDB, EA, PLJ, RDRO, DSZ, CB, MAM and RT discussed data. SRP and TJC wrote the manuscript. All authors approved the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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References

[1] F. Wu, S. Zhao, B. Yu, Y.M. Chen, W. Wang, Z.G. Song, et al., A new coronavirus associated with human respiratory disease in China, Nature 579 (2020) 265–269.
[2] S. Huang, J. Wang, F. Liu, J. Liu, G. Cao, C. Yang, et al., COVID-19 patients with hypertension have more severe disease: a multicenter retrospective observational study, Hypertens. Res. 43 (2020) 824–831.
[3] (CSSE-JHU) CfSSaEaJHU, COVID-19 Dashboard. https://coronavirus.jhu.edu/map.html.
[4] W.J. Guan, Z.Y. Ni, Y. Hu, W.H. Liang, C.Q. Ou, J.X. He, et al., Clinical characteristics of coronavirus disease 2019 in China, N. Engl. J. Med. 382 (2020) 1708–1720.
[5] Z. Wu, J.M. McGoogan, Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72 314 cases from the Chinese Center for Disease Control and Prevention, JAMA 13 (2020) 1239–1242.
[6] Z. Varga, A.J. Flammer, P. Steiger, M. Haberecker, R. Andermatt, A.S. Zinkernagel, et al., Endothelial cell infection and endotheliitis in COVID-19, Lancet 395 (2020) 1417–1418.
[7] V.J. Costela-Ruiz, R. Illescas-Montes, J.M. Puerta-Puerta, C. Ruiz, L. Melguizo-Rodríguez, SARS-CoV-2 infection: the role of cytokines in COVID-19 disease, Cytokine Growth Factor Rev. 54 (2020) 62–75.
[8] Z.S. Miripour, R. Sarrami-Forooshani, H. Sanati, J. Makarem, M.S. Taheri, F. Shojaeian, et al., Real-time diagnosis of reactive oxygen species (ROS) in fresh sputum by electrochemical tracing; correlation between COVID-19 and viral-induced ROS in lung/respiratory epithelium during this pandemic, Biosens. Bioelectron. 165 (2020), 112435.
[9] R. Patell, T. Bogue, A. Koshy, P. Bindal, M. Merrill, W.C. Aird, et al., Postdischarge thrombosis and hemorrhage in patients with COVID-19, Blood 136 (2020) 1342–1346.
[10] P. Libby, T. Lüscher, COVID-19 is, in the end, an endothelial disease, Eur. Heart J. 41 (2020) 3038–3044.
[11] S. Di Meo, T.T. Reed, P. Venditti, V.M. Victor, Role of ROS and RNS sources in physiological and pathological conditions, Oxidative Med. Cell. Longev. 2016 (2016), 1245049.
[12] P.R. Kvietys, D.N. Granger, Role of reactive oxygen and nitrogen species in the vascular responses to inflammation, Free Radic. Biol. Med. 52 (2012) 556–592.
[13] T.J. Costa, P.R. Barros, C. Arce, J.D. Santos, J. da Silva-Neto, G. Egea, A.P. Dantas, R.C. Tostes, F. Jiménez-Altayó, The homeostatic role of hydrogen peroxide, superoxide anion and nitric oxide in the vasculature, Free Radic. Biol. Med. 162 (2021) 615–635.
[14] M.A. Incalza, R. D'Oria, A. Natalicchio, S. Perrini, L. Laviola, F. Giorgino, Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases, Vasc. Pharmacol. 100 (2018) 1–19.
[15] L. Möckl, The emerging role of the mammalian glycocalyx in functional membrane organization and immune system regulation, Front. Cell. Dev. Biol. 8 (2020) 253.
[16] S. Weinbaum, J.M. Tarbell, E.R. Damiano, The structure and function of the endothelial glycocalyx layer, Annu. Rev. Biomed. Eng. 9 (2007) 121–167.
[17] S. Reitsma, D.W. Slaaf, H. Vink, M.A. van Zandvoort, M.G. oude Egbrink, The endothelial glycocalyx: composition, functions, and visualization, Pflugers Arch. 454 (2007) 345–359.
[18] C.S. Alphonsus, R.N. Rodseth, The endothelial glycocalyx: a review of the vascular barrier, Anaesthesia 69 (2014) 777–784.
[19] A.M.W. Bartosch, R. Mathews, J.M. Tarbell, Endothelial glycocalyx-mediated nitric oxide production in response to selective AFM pulling, Biophys. J. 113 (2017) 101–108.
[20] M.A. Dragovich, D. Chester, B.M. Fu, C. Wu, Y. Xu, M.S. Goligorsky, et al., Mechanotransduction of the endothelial glycocalyx mediates nitric oxide production through activation of TRP channels, Am. J. Physiol. Cell. Physiol. 311 (2016) C846–C853.
[21] J.A. Florian, J.R. Kosky, K. Ainslie, Z. Pang, R.O. Dull, J.M. Tarbell, Heparan sulfate proteoglycan is a mechanosensor on endothelial cells, Circ. Res. 93 (2003) e136–e142.
[22] M.Y. Pahakis, J.R. Kosky, R.O. Dull, J.M. Tarbell, The role of endothelial glycocalyx components in mechanotransduction of fluid shear stress, Biochem. Biophys. Res. Commun. 355 (2007) 228–233.
[23] W. Yen, B. Cai, J. Yang, L. Zhang, M. Zeng, J.M. Tarbell, et al., Endothelial surface glycocalyx can regulate flow-induced nitric oxide production in microvessels in vivo, PLoS One 10 (2015), e0117133.
[24] I. Rubio-Gayosso, S.H. Platts, B.R. Duling, Reactive oxygen species mediate modification of glycocalyx during ischemia-reperfusion injury, Am. J. Physiol. Heart Circ. Physiol. 290 (2006) H2247–H2256.
[25] S. Suwarto, R.T. Sasmono, R. Sinto, E. Ibrahim, M. Suryamin, Association of endothelial glycocalyx and tight and adherens junctions with severity of plasma leakage in dengue infection, J. Infect. Dis. 215 (2017) 992–999.
[26] T.H. Tang, S. Alonso, L.F. Ng, T.L. Thein, V.J. Pang, Y.S. Leo, et al., Increased serum hyaluronic acid and heparan sulfate in dengue fever: association with plasma leakage and disease severity, Sci. Rep. 7 (2017), 46191.
[27] A.M. Connolly-Andersen, T. Thunberg, C. Ahlm, Endothelial activation and repair during hantavirus infection: association with disease outcome, Open Forum Infect. Dis. 1 (2014), ofu027.
[28] B.F. Becker, M. Jacob, S. Leipert, A.H. Salmon, D. Chappell, Degradation of the endothelial glycocalyx in clinical settings: searching for the sheddases, Br. J. Clin. Pharmacol. 80 (2015) 389–402.
[29] M.N. Benatti, A.T. Fabro, C.H. Miranda, Endothelial glycocalyx shedding in the acute respiratory distress syndrome after flu syndrome, J. Intensive Care 8 (2020), 72.
[30] J.S. Padberg, A. Wiesinger, G.S. di Marco, S. Reuter, A. Grabner, D. Kentrup, et al., Damage of the endothelial glycocalyx in chronic kidney disease, Atherosclerosis 234 (2014) 335–343.
[31] B. DellaValle, H. Hasseldam, F.F. Johansen, H.K. Iversen, J. Rungby, C. Hempel, Multiple soluble components of the glycocalyx are increased in patient plasma after ischemic stroke, Stroke 50 (2019) 2948–2951.
[32] R.J. Martens, H. Vink, R.J. van Oostenbrugge, J. Staals, Sublingual microvascular glycocalyx dimensions in lacunar stroke patients, Cerebrovasc. Dis. 35 (2013) 451–454.
[33] L.N. Broekhuizen, B.A. Lemkes, H.L. Mooij, M.C. Meuwese, H. Verberne, F. Holleman, et al., Effect of sulodexide on endothelial glycocalyx and vascular permeability in patients with type 2 diabetes mellitus, Diabetologia 53 (2010) 2646–2655.
[34] P.P. Wadowski, M. Hülsmann, C. Schörgenhofer, I.M. Lang, R. Wurm, T. Gremmel, et al., Sublingual functional capillary rarefaction in chronic heart failure, Eur. J. Clin. Investig. 48 (2018).
[35] A.K. Nalla, A.M. Casto, M.W. Huang, G.A. Perchetti, R. Sampoleo, L. Shrestha, et al., Comparative performance of SARS-CoV-2 detection assays using seven different primer-probe sets and one assay kit, J. Clin. Microbiol. (2020) 58.
[36] D. Armstrong, R. Browne, The analysis of free radicals, lipid peroxides, antioxidant enzymes and compounds related to oxidative stress as applied to the clinical chemistry laboratory, Adv. Exp. Med. Biol. 366 (1994) 43–58.
[37] Z. Chen, S.D.S. Oliveira, A.M. Zimnicka, Y. Jiang, T. Sharma, S. Chen, et al., Reciprocal regulation of eNOS and caveolin-1 functions in endothelial cells, Mol. Biol. Cell 29 (10) (2018) 1190–1202.
[38] S. Pirkmajer, A.V. Chibalin, Serum starvation: caveat emptor, Am. J. Physiol. Cell. Physiol. 301 (2) (2011) C272–C279.
[39] B. Almeida-Pititto, P.M. Dualib, L. Zajdenverg, J.R. Dantas, F.D. de Souza, M. Rodacki, M.C. Bertoluci, Brazilian Diabetes Society Study Group (SBD), Severity and mortality of COVID 19 in patients with diabetes, hypertension and cardiovascular disease: a meta-analysis, Diabetol. Metab. Syndr. 12 (2020) 75.
[40] T.D. Filardo, M.R. Khan, N. Krawczyk, H. Galitzer, S. Karmen-Tuohy, M. Coffee, V. E. Schaye, B.J. Eckhardt, G.M. Cohen, Comorbidity and clinical factors associated with COVID-19 critical illness and mortality at a large public hospital in New York City in the early phase of the pandemic (March–April 2020), PLoS One 15 (11) (2020), e0242760.
[41] J.W. Song, C. Zhang, X. Fan, F.P. Meng, Z. Xu, P. Xia, W.J. Cao, et al., Immunological and inflammatory profiles in mild and severe cases of COVID-19, Nat. Commun. 11 (1) (2020), 3410.
[42] E.D. Hottz, I.G. Azevedo-Quintanilha, L. Palhinha, L. Teixeira, E.A. Barreto, C.R. R. Pão, C. Righy, S. Franco, T.M.L. Souza, P. Kurtz, F.A. Bozza, P.T. Bozza, Platelet activation and platelet-monocyte aggregate formation trigger tissue factor expression in patients with severe COVID-19, Blood 136 (11) (2020) 1330–1341.
[43] K. Stahl, P.A. Gronski, Y. Kiyan, B. Seeliger, A. Bertram, T. Pape, et al., Injury to the endothelial glycocalyx in critically ill patients with COVID-19, Am. J. Respir. Crit. Care Med. 202 (2020) 1178–1181.
[44] E.L. Sanchez, M. Lagunoff, Viral activation of cellular metabolism, Virology 479-480 (2015) 609–618.
[45] F.P. Veras, M.C. Pontelli, C.M. Silva, J.E. Toller-Kawahisa, M. de Lima, D. C. Nascimento, et al., SARS-CoV-2-triggered neutrophil extracellular traps mediate COVID-19 pathology, J. Exp. Med. (2020) 217.
[46] Y. Zuo, S. Yalavarthi, H. Shi, K. Gockman, M. Zuo, J.A. Madison, et al., Neutrophil extracellular traps in COVID-19, JCI Insight (2020) 5.
[47] M. Mittal, M.R. Siddiqui, K. Tran, S.P. Reddy, A.B. Malik, Reactive oxygen species in inflammation and tissue injury, Antioxid. Redox Signal. 20 (2014) 1126–1167.
[48] A. Ramezani, M.P. Nahad, E. Faghihloo, The role of Nrf2 transcription factor in viral infection, J. Cell. Biochem. 119 (2018) 6366–6382.
[49] A.S. Abouhashem, K. Singh, H.M.E. Azzazy, C.K. Sen, Is low alveolar type II cell, Antioxid. Redox Signal. 33 (2020) 59–65.
[50] A. Treitinger, C. Spada, J.C. Verdi, A.F. Miranda, O.V. Oliveira, M.V. Silveira, et al., Decreased antioxidant defence in individuals infected by the human immunodeficiency virus, Eur. J. Clin. Investig. 30 (2000) 454–459.
[51] M.L. DeDiego, J.L. Nieto-Torres, J.A. Regla-Nava, J.M. Jimenez-Guardeño, R. Fernandez-Delgado, C. Fett, et al., Inhibition of NF-κB-mediated inflammation in severe acute respiratory syndrome coronavirus-infected mice increases survival, J. Virol. 88 (2014) 913–924.
[52] M.L. Cheng, S.F. Weng, C.H. Kuo, H.Y. Ho, Enterovirus 71 induces mitochondrial reactive oxygen species generation that is required for efficient replication, PLoS One e113234 (2014) 9.
[53] C.M. Sena, A. Leandro, L. Azul, R. Seiça, G. Perry, Vascular oxidative stress: impact and therapeutic approaches, Front. Physiol. 9 (2018) 1668.
[54] I.C. Allen, M.A. Scull, C.B. Moore, E.K. Holl, E. McElvania-TeKippe, D.J. Taxman, et al., The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA, Immunity 30 (2009) 556–565.
[55] L.S. da Costa, A. Outlioua, A. Anginot, K. Akarid, D. Arnoult, RNA viruses promote activation of the NLRP3 inflammasome through cytopathogenic effect-induced potassium efflux, Cell Death Dis. 10 (2019), 346.
[56] K. Schroder, J. Tschopp, The inflammasomes, Cell 140 (2010) 821–832.
[57] R. Zhou, A. Tardivel, B. Thorens, I. Choi, J. Tschopp, Thioredoxin-interacting protein links oxidative stress to inflammasome activation, Nat. Immunol. 11 (2010) 136–140.
[58] P. Conti, G. Ronconi, A. Caraffa, C.E. Gallenga, R. Ross, I. Frydas, et al., Induction of pro-inflammatory cytokines (IL-1 and IL-6) and lung inflammation by

Coronavirus-19 (COVI-19 or SARS-CoV-2): anti-inflammatory strategies, J. Biol. Regul. Homeost. Agents 34 (2020) 327–331.
[59] R. Uchimido, E.P. Schmidt, N.I. Shapiro, The glycocalyx: a novel diagnostic and therapeutic target in sepsis, Crit. Care 23 (2019), 16.
[60] D.A. Siwik, W.S. Colucci, Regulation of matrix metalloproteinases by cytokines and reactive oxygen/nitrogen species in the myocardium, Heart Fail. Rev. 9 (2004) 43–51.
[61] R. Ramnath, R.R. Foster, Y. Qiu, G. Cope, M.J. Butler, A.H. Salmon, et al., Matrix metalloproteinase 9-mediated shedding of syndecan 4 in response to tumor necrosis factor α: a contributor to endothelial cell glycocalyx dysfunction, FASEB J. 28 (2014) 4686–4699.
[62] M. Garsen, A.L. Rops, T.J. Rabelink, J.H. Berden, J. van der Vlag, The role of heparanase and the endothelial glycocalyx in the development of proteinuria, Nephrol. Dial. Transplant. 29 (2014) 49–55.
[63] T. Wang, R. Chen, C. Liu, W. Liang, W. Guan, R. Tang, et al., Attention should be paid to venous thromboembolism prophylaxis in the management of COVID-19, Lancet Haematol. 7 (2020) e362–e363.
[64] B. Buijsers, C. Yanginlar, A. de Nooijer, I. Grondman, M.L. Maciej-Hulme, I. Jonkman, et al., Increased plasma heparanase activity in COVID-19 patients, Front. Immunol. 11 (2020), 575047.
[65] J. Ando, K. Yamamoto, Flow detection and calcium signalling in vascular endothelial cells, Cardiovasc. Res. 99 (2013) 260–268.
[66] W. Fang, J. Jiang, L. Su, T. Shu, H. Liu, S. Lai, R.A. Ghiladi, J. Wang, The role of NO in COVID-19 and potential therapeutic strategies, Free Radic. Biol. Med. 163 (2021) 153–162.
[67] H. Cai, Hydrogen peroxide regulation of endothelial function: origins, mechanisms, and consequences, Cardiovasc. Res. 68 (2005) 26–36.
[68] M.R. Brown, F.J. Miller, W.G. Li, A.N. Ellingson, J.D. Mozena, P. Chatterjee, et al., Overexpression of human catalase inhibits proliferation and promotes apoptosis in vascular smooth muscle cells, Circ. Res. 85 (1999) 524–533.
[69] N.S. Quinsey, A.L. Greedy, S.P. Bottomley, J.C. Whisstock, R.N. Pike, Antithrombin: in control of coagulation, Int. J. Biochem. Cell Biol. 36 (2004) 386–389.
[70] K. Shimada, M. Kobayashi, S. Kimura, M. Nishinaga, K. Takeuchi, T. Ozawa, Anticoagulant heparin-like glycosaminoglycans on endothelial cell surface, Jpn. Circ. J. 55 (1991) 1016–1021.
[71] H. Kato, Regulation of functions of vascular wall cells by tissue factor pathway inhibitor: basic and clinical aspects, Arterioscler. Thromb. Vasc. Biol. 22 (2002) 539–548.
[72] G. Wang, S. Kostidis, G.L. Tiemeier, W.M.P.J. Sol, M.R. de Vries, M. Giera, et al., Shear stress regulation of endothelial glycocalyx structure is determined by glucobiosynthesis, Arterioscler. Thromb. Vasc. Biol. 40 (2020) 350–364.
[73] N. Tang, D. Li, X. Wang, Z. Sun, Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia, J. Thromb. Haemost. 18 (2020) 844–847.
[74] H. Al-Samkari, R.S. Karp Leaf, W.H. Dzik, J.C.T. Carlson, A.E. Fogerty, A. Waheed, et al., COVID-19 and coagulation: bleeding and thrombotic manifestations of SARS-CoV-2 infection, Blood 136 (2020) 489–500.
[75] J. Shu, G. Santulli, Heparanase in health and disease: the neglected housekeeper of the cell? Atherosclerosis 283 (2019) 124–126.
[76] M. Bar-Ner, A. Eldor, L. Wasserman, Y. Matzner, I.R. Cohen, Z. Fuks, et al., Inhibition of heparanase-mediated degradation of extracellular matrix heparan sulfate by non-anticoagulant heparin species, Blood 70 (1987) 551–557.
[77] M. Bitan, M. Mohsen, E. Levi, M.R. Wygoda, H.Q. Miao, O. Lider, et al., Structural requirements for inhibition of melanoma lung colonization by heparanase inhibiting species of heparin, Isr. J. Med. Sci. 31 (1995) 106–118.
[78] T. Irimura, M. Nakajima, G.L. Nicolson, Chemically modified heparins as inhibitors of heparan sulfate specific endo-beta-glucuronidase (heparanase) of metastatic melanoma cells, Biochemistry 25 (1986) 5322–5328.
[79] A. Naggi, B. Casu, M. Perez, G. Torri, G. Cassinelli, S. Penco, et al., Modulation of the heparanase-inhibiting activity of heparin through selective desulfation, graded N-acetylation, and glycol splitting, J. Biol. Chem. 280 (13) (2005), 12103.
[80] D. Pala, S. Rivara, M. Mor, F.M. Milazzo, G. Roscilli, E. Pavoni, et al., Kinetic analysis and molecular modeling of the inhibition mechanism of roneparstat (SST0001) on human heparanase, Glycobiology 26 (2016) 640–654.
[81] J. Li, Y. Li, B. Yang, H. Wang, L. Li, Low-molecular-weight heparin treatment for acute lung injury/acute respiratory distress syndrome: a meta-analysis of randomized controlled trials, Int J Clin Exp Med 2 (2018) 414–422.
[82] E. Vicenzi, F. Canducci, D. Pinna, N. Mancini, S. Carletti, A. Lazzarin, et al., Coronaviridae and SARS-associated coronavirus strain HSR1, Emerg. Infect. Dis. 10 (2004) 413–418.
[83] J. Lang, N. Yang, J. Deng, K. Liu, P. Yang, G. Zhang, et al., Inhibition of SARS pseudovirus cell entry by lactoferrin binding to heparan sulfate proteoglycans, PLoS One 6 (2011), e23710.
[84] C.J. Mycroft-West, D. Su, I. Pagani, T.R. Rudd, S. Elli, N.S. Gandhi, et al., Heparin inhibits cellular invasion by SARS-CoV-2: structural dependence of the interaction of the Spike S1 receptor-binding domain with heparin, Thromb. Haemost. 12 (2020) 1700–1715.
[85] P.S. Kwon, H. Oh, S.J. Kwon, W. Jin, F. Zhang, K. Fraser, et al., Sulfated polysaccharides effectively inhibit SARS-CoV-2 in vitro, Cell. Discov. 6 (2020), 50.
[86] L. Gozzo, P. Viale, L. Longo, D.C. Vitale, F. Drago, The potential role of heparin in patients with COVID-19: beyond the anticoagulant effect. A review, Front. Pharmacol. 11 (2020) 1307.