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COVID-19 generates hyaluronan fragments that directly induce endothelial barrier dysfunction

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Conflicts of Interest Statement
The authors have declared that no conflict of interest exists.

Abstract
Vascular injury has emerged as a complication contributing to morbidity in coronavirus disease 2019 (COVID-19). The glycosaminoglycan hyaluronan (HA) is a major component of the glycocalyx, a protective layer of glycoconjugates that lines the vascular lumen and regulates key endothelial cell functions. During critical illness as in the case of sepsis, enzymes degrade the glycocalyx, releasing fragments with pathologic activities into circulation and thereby exacerbate disease. Here, we analyzed levels of circulating glycosaminoglycans in 46 patients with COVID-19 ranging from moderate to severe clinical severity and measured activities of corresponding degradative enzymes. This report provides evidence that the glycocalyx becomes significantly damaged in COVID-19 patients and corresponds with severity of disease. Circulating HA fragments and hyaluronidase, two signatures of glycocalyx injury, strongly associate with sequential organ failure assessment scores and with increased inflammatory cytokine levels in COVID-19 patients. Pulmonary microvascular endothelial cells exposed to COVID-19 milieu show dysregulated HA biosynthesis and degradation leading to production of pathological HA fragments which are released into circulation. Finally, we show that HA fragments present at high levels in COVID-19 patient plasma can directly induce endothelial barrier dysfunction in ROCK- and CD44-dependent manner, indicating a role for HA in the vascular pathology of COVID-19.
Introduction

The severe acute respiratory syndrome (SARS) coronavirus disease 2019 (COVID-19) is a crippling public health crisis caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 is responsible for >49.7 million cases and 1.2 million deaths globally (1). An exacerbated immune response (2, 3) is strongly implicated as driving damage to the airspaces and extrapulmonary symptoms including acute kidney injury, (4, 5) acute cardiac injury (6), coagulopathy, (7) thrombosis (8), platelet hyperreactivity (9, 10), and circulatory shock (11). Patients with cardiovascular risk factors such as obesity, diabetes and hypertension are at increased risk of severe complications (12-14) including acute respiratory distress syndrome (ARDS), and chronic vascular endothelial injury is often present in patients with these comorbidities. Findings of endotheliopathy (15), evidence of SARS-CoV-2 infection of pulmonary (16-18) and extrapulmonary (19) endothelial cells (ECs), reports of viremia (20, 21), and multi-organ injury lead to the hypothesis that COVID-19 is in part a vascular illness.

Under healthy conditions, the endothelium possesses endogenous anti-inflammatory, anti-coagulant, and vasodilatory mechanisms which balance factors that promote inflammation, coagulation, and vasoconstriction. However, in aged individuals and those with cardiovascular disease such as diabetes and hypertension, the two most prevalent co-morbidities in COVID-19, homeostatic endothelial functions become impaired (22). Clinical studies provide evidence that endothelial dysfunction is a major determinant of severe COVID-19, and patients have markers of endothelial injury including increased levels of fibrinogen, fibrin degradation products, D-dimer, von Willebrand factor (23, 24), and soluble thrombomodulin (15) as well as signatures of impaired
endothelial function such as apoptotic ECs (17), decreased nitric oxide availability (25), and vascular leakage (26).

At the vascular surface, the glycocalyx plays an essential role regulating barrier integrity, nitric oxide production and vasorelaxation, resistance to oxidative stress, coagulation and inflammation (27, 28). The glycocalyx is a complex network composed of the glycosaminoglycans (GAGs) hyaluronan (HA), heparan sulfate (HS), chondroitin sulfate (CS) and other glycoconjugates at the interface between the cell surface and the extracellular environment. The glycocalyx undergoes constitutive remodeling and homeostasis is maintained by a balance of synthesis and degradation. However, infection, inflammation, ischemia/reperfusion, and hyperglycemia lead to destruction of the glycocalyx and release of bioactive fragments which exacerbate disease (29, 30). In bacterial sepsis, glycocalyx degradation associates with the presence and severity of disease and is a causative element in recruitment of immune cells, barrier disruption, and development of ARDS (31-34). The glycocalyx is degraded at the cell surface by the activity of endo-β-glucuronidases which regulate the availability of HA or HS chains for receptor binding by cleaving these polymers and thereby control several important ligand-binding interactions with known roles in leukocyte recruitment, neutrophil function, endothelial behavior, and cytokine and growth factor release (33, 35-39). Hyaluronidases predominantly degrade HA while heparanase predominantly degrades HS, and both exhibit limited activity toward CS which has no known specific degradation enzyme in mammals (40, 41). These enzymes are primarily regulated at the transcript level and expressed in many cell types including endothelial cells, leukocytes, mast cells, and platelets. Degradation products of HA and HS act as endogenous danger signals capable of amplifying pro-inflammatory responses both in vitro and in murine models of disease (42-45). Recent reports suggest endothelial damage and glycocalyx injury are
present in COVID-19 (46-48). However, whether direct viral infection or cytokine release are the underlying mechanisms leading to glycocalyx injury, and how glycocalyx fragments contribute to the pathology of COVID-19 are not known.

In this prospective study, we examined the concentrations of circulating GAGs and the activities of GAG-degrading enzymes in acutely ill patients with COVID-19 compared to septic patients and matched healthy donors. Analysis of plasma isolated from SARS-CoV-2 infected patients revealed significantly increased concentrations of circulating GAGs which was accompanied with elevated hyaluronidase and heparanase activities. Circulating HA and HS fragments associate with severity of disease, and HA and hyaluronidase were also associated with clinical parameters and concentrations of plasma cytokines. Stimulation of human pulmonary microvascular endothelial cells with plasma from COVID-19 patients promotes release of HA into culture media, increased hyaluronidase activity, and transcriptional activation of HA biosynthesis and degradation when compared to plasma from healthy donors. Analysis of circulating HA isolated from patients, and HA from cells exposed to COVID-19 patient plasma demonstrates that pathological HA degradation products are significantly increased during disease. Finally, treatment of endothelial cells with HA fragments purified from the plasma of infected patients, but not HA from healthy donors, promotes disruption of endothelial barrier integrity in a Rho-associated protein kinase (ROCK) and CD44-dependent manner. Our data provide new evidence that SARS-CoV-2 infection is characterized by degradation of the endothelial glycocalyx, COVID-19 cytokine milieu stimulates aberrant synthesis and degradation of HA in pulmonary endothelial cells, and HA fragments present at high concentrations in COVID-19 patient plasma are capable of directly mediating endothelial dysfunction.
Results

COVID-19 Patient Cohort

COVID-19 patients were matched by age, sex, and race to critically ill sepsis patients and to healthy donors (Table 1). SARS-CoV-2 virus was uniformly detected in patients diagnosed with SARS-CoV-2 by PCR. Approximately 36% of COVID-19 patients were in the ICU and 35% required mechanical ventilation due to respiratory failure. Hospitalized non-ICU and ICU COVID-19 patients had comorbidities of hypertension, diabetes, and obesity consistent with previous observations.(13) Mortality rate was ~17% among all COVID-19 patients, consistent with hospitalized mortality rates (13.4%) in COVID-19 patients in Utah per the Utah Department of Health as of November, 8th 2020.

Circulating glycosaminoglycans are increased in COVID-19 patients and associate with disease severity

Endothelial glycocalyx degradation has been increasingly implicated in the pathogenesis of critical illnesses including as sepsis and influenza (49). To determine the extent of injury to the endothelial glycocalyx in COVID-19, we quantified circulating HA, HS, and CS in plasma from 36 COVID-19 patients (23 non-ICU, 13 ICU) and 18 matched healthy donors recruited from the greater Salt Lake City area. As shown in Figure 1A-C, this analysis revealed significant increases in mean plasma concentrations of HA (5.8-fold), HS (4.3-fold), and CS (1.3-fold) in COVID-19 patients in comparison to healthy donors. To evaluate whether the levels of circulating glycosaminoglycans in COVID-19 were comparable with other critical illnesses, we compared our COVID-19 patient cohort with patients with clinical sepsis (n = 23, Figure 1A-C). We observed that plasma concentrations of HA and HS are significantly increased in plasma from sepsis patients (9.3-fold...
and 9.2-fold respectively) when compared to healthy donors, and also increased in septic patients as compared to measurements in patients with COVID-19, but CS concentrations were not significantly different. Because patients with COVID-19 as well as patients with sepsis in our cohort were treated with derivatives of heparin, a highly-sulfated form of HS, we evaluated whether the treatment might confound accurate detection of HS. We observed that the addition of pharmacologic concentrations of enoxaparin to patient plasma had no significant influence on detection of HS levels in patients (Figure S1). We next evaluated whether circulating glycosaminoglycan levels associate with COVID-19 disease severity and found that circulating levels of HA and HS are significantly increased in ICU-admitted patients, while CS levels are decreased compared to non-ICU admitted patients (Fig 1D-F). Taken together, these data suggest that wide-spread endothelial glycocalyx injury is a characteristic of COVID-19 and mean concentrations of HA and HS in ICU-admitted patients are comparable to circulating concentrations detected in patients with sepsis.

**Glycocalyx-degrading enzyme activities are elevated in COVID-19 patients**

The increased concentrations of circulating glycosaminoglycans observed in COVID-19 are suggestive of increased activity of enzymes known to release glycosaminoglycan fragments into circulation. We therefore measured hyaluronidase, heparanase, and chondroitinase activities in plasma of COVID-19 patients compared to either healthy controls or septic patients. Plasma activities of hyaluronidase and heparanase were significantly elevated in both COVID-19 and sepsis patients compared to healthy controls (Figure 2 A, B), and no difference in chondroitinase activity was observed between patient groups (Figure 2C). We next compared activity levels of GAG-degrading enzymes in non-ICU and ICU-admitted COVID-19 patients and observed that ICU-admitted patients show elevated circulating hyaluronidase (Figure 2D) and heparanase
(Figure 2E), and a trend toward increase in chondroitinase activity (Figure 2F). Only hyaluronidase levels were found to be significantly different between ICU- and non-ICU patients (Figure 2D). In addition to direct degradation of GAGs, the activated endothelium may also release proteases contained within Weibel-Palade bodies and secretory lysosomes that promote proteolytic shedding of proteoglycan core proteins. We therefore measured the activities of matrix metalloproteinases (MMPs 2/9), and cathepsin D, proteases with known roles in endothelial injury(50) and found that MMP (Figure S2A) and cathepsin D (Figure S2B) activities are significantly increased in COVID-19 patients compared with healthy donors.

Circulating HA and hyaluronidase activity correlates with clinical and inflammatory signatures of COVID-19

Circulating GAGs have been shown to associate with lung injury (32), clinical parameters in ARDS (51, 52) and with other forms of organ injury in sepsis (53, 54). We therefore evaluated whether circulating levels of GAGs or degrading enzyme activities associate with clinical assessments in COVID-19 patients. We found that plasma HA levels show a significant positive correlation with sequential organ failure assessment (SOFA) score, an index of illness severity in COVID-19 patients (Figure 3A) and in sepsis patients (Figure 3B), similar to previous reports.(55) Similarly, hyaluronidase activity also was found to have a significant positive correlation with SOFA score in COVID-19 patients (Figure 3C), and trended towards significance in septic patients (Figure 3D). We found no correlation with severity of illness for HS, CS, heparanase, or chondroitinase activity in COVID-19 patients (Figure S3A-C), though HS shows a strong positive association in our septic patient population (Figure S3C) similar to previous reports (32, 33). We next performed intercorrelation analysis between plasma HA levels and hyaluronidase activity
with previously published plasma cytokine levels (56) we determined to be significantly increased in our COVID-19 patients compared with healthy controls (Figure 3E, Supplemental Table 1A,1B). This analysis shows that plasma levels of HA and hyaluronidase in COVID-19 patients each are correlated with inflammatory cytokines known to have roles in either HA synthesis or degradation such as IL-6, IL-8, MCP-1, TNF-α, and IP10. Regulation of HA synthesis and degradation are typically inversely associate. Unexpectedly, of all 19 cytokines compared only IP10, a biomarker and mediator of Kawasaki disease pathology (57) shows a significant positive correlation with both HA and hyaluronidase in COVID-19 patients, but not in healthy donors (Figure S3D, Supplemental Table 2A, 2B). Interestingly, some children infected with SARS-CoV-2 develop a severe inflammatory disease with characteristics of Kawasaki disease (58).

**Treatment with COVID-19 plasma induces HA synthesis and degradation in lung microvascular ECs**

Based on our observation that cytokines present in COVID-19 patients associate with circulating HA and hyaluronidase levels, we next explored whether plasma from infected patients might promote dysregulated synthesis and degradation of HA in human lung microvascular endothelial cells (LMVECs). We found that treatment of cultured ECs with plasma from COVID-19 patients led to decreased cellular HA (Figure 4A) and increased cell-associated hyaluronidase activity (Figure 4B) when compared to cells treated with healthy donor plasma and normalized against plasma alone. Analysis of media from cells exposed to COVID-19 plasma revealed a corresponding increase in HA (Figure 4C), but not hyaluronidase activity (Figure 4D) as compared to cells treated with healthy plasma. We examined the media of treated LMVECs for sheddase activity that might be induced by plasma cytokines and observed increased MMP2/9 and cathepsin D activities in the media of cells exposed to COVID-19 plasma compared to healthy controls when
normalized against plasma alone, suggesting release by ECs themselves (Figure S2 C,D). We next asked whether plasma from COVID-19 patients could alter transcription of genes responsible for HA synthesis or degradation and found that treatment with COVID-19 patient plasma significantly increases mRNA expression of hyaluronan synthase-3 (HAS3), hyaluronidases -1 and -2 (HYAL -1, -2) compared to treatment with healthy donor plasma (Figure 4E,F). Given the reports of SARS-CoV-2 infection of endothelial cells, we next sought to determine whether the observed activation of endothelial cells by COVID-19 plasma was due to activation by humoral factors or by infection with SARS-CoV-2 that might be present in COVID-19 plasma samples. Examination of ECs cultured in the presence of COVID-19 plasma indicates that transcripts corresponding to the SARS-CoV-2 N1 gene are undetectable as compared to tracheal aspirates from COVID-19 patients (Figure S4). These findings suggest that cytokines associated with COVID-19, rather than only direct viral infection, promote dysregulated biosynthesis and degradation of endothelial HA by the pulmonary microvasculature.

Circulating HA is present as pathological low molecular weight fragments in COVID-19 patient plasma

Many of the biological responses governed by HA are dependent upon polymer size, with high-molecular weight- (HMW) HA and low-molecular weight- (LMW) HA exhibiting differing signaling properties (59). Circulating HA is reported to be in a mass range of from 1-2 × 10^5 Da in healthy individuals, and cleavage of HA from the EC surface results in fragments of varying mass ranges which stimulate angiogenic and inflammatory responses (60-62). To determine whether LMW-HA degradation products are present in COVID-19 patients, we fractionated HA from plasma of healthy donors or COVID-19 patients and determined that that the majority of HA
purified from healthy donor plasma is >100kDa, while 74.8% of plasma HA isolated from SARS-CoV-2 infected patients is present as sub-50kDa species (Figure 5A).

We next asked whether HA released into the media of treated cells might also be present as LMW degradation products and found that cells treated with plasma from COVID-19 patients release 55.7% of HA into the media as LMW fragments as compared to ~10.11% in cells treated with control plasma (Figure 5B). During inflammation, HA may become covalently modified with the heavy-chains (HCs) of inter-alpha inhibitor protein (IαI), and this unique modified form of HA (HA-HC) enhances immune cell recruitment (35, 63-65) and associates with lung injury in mice and humans (66-68). Measurement of plasma HA-HC revealed that patients with COVID-19 have significantly higher levels of HA-HC compared with control subjects (Figure 5C). These data suggest that circulating HA in COVID-19 is present in the form of degradation products with inflammatory activities.

**HA fragments present in COVID-19 plasma disrupt endothelial barrier integrity**

Circulating HA concentrations in COVID-19 patient plasma are significantly elevated compared to healthy controls (Figure 1A) and is present as LMW-HA fragments (Figure 5A) known to have barrier-disrupting effects on ECs (28). We next examined whether treatment of LMVECs with HA purified from the plasma of infected patients had a direct effect on EC barrier function. Using a transwell assay in which confluent LMVECs form a tight monolayer over a semi-permeable membrane, we observed an increase in the permeability of ECs to high-molecular weight dextran after treatment with HA purified from SARS-CoV-2 infected patients as compared to HA purified from healthy controls (Figure 6A). Importantly, the barrier-disrupting effects of HA fragments are significantly decreased by complete digestion with exogenous hyaluronidase that reduces HA to disaccharides, indicating that the increase in permeability is attributable to HA and not impurities
from purification. HA purified from biological samples is a mixture of polymer lengths, and we next examined whether biosynthetic HA fragments of defined masses could reproduce the effects we observed with HA purified from COVID-19 plasma. Our data show that LMVECs incubated with LMW-HA species <60 kDa demonstrate a size-dependent increase in barrier disruption, with HA fragments of approximately 4kDa demonstrating the largest effect compared to control-treated cells (Figure S5). We further analyzed cells treated with HA4k for changes in the mRNA levels of HA receptors, observing a significant increase in layilin (6.3-fold) and Hyal2 (8.8-fold) expression in HA4k treated cells compared with untreated cells, but found no significant changes in CD44, ICAM-1, or HYAL1 (Figure S6).

The transmembrane HA receptors CD44 and layilin both contain extracellular HA-binding domains and intracellular cytoplasmic motifs which enable association with the actin cytoskeleton and are therefore poised to recognize and transmit signals involved in maintenance, formation, or disruption of intercellular contact. Interaction of HMW-HA by CD44 can promote barrier enhancing effects in cultured ECs via AKT activation (69), while small HA fragments by recognized by CD44 or layilin are known to disrupt cell-cell junctions in a RhoA/ROCK dependent mechanism (70). Next, we tested whether RhoA/ROCK activation might mediate HA-fragment induced barrier disruption in lung endothelium by culturing LMVECs in the presence or absence of a ROCK inhibitor (Y26632) prior to treatment with either biosynthetic HA4k or COVID-19 HA-fragments. As shown in Figure 6B, ROCK inhibition led to significant reduction of HA4k-induced (85.5%) and COVID-HA induced (63.3%) permeability as compared to treatment of barrier disrupting HA-fragments alone. We then examined VE-cadherin by immunostaining of LMVECs exposed to HA purified from healthy donors, COVID-19 patients, or LMW HA4k with or without pre-treatment with ROCK inhibitor. We observed a loss of immunostaining for VE-
cadherin in cells treated with HA purified from COVID patients or biosynthetic LMW-HA fragments compared to cells treated with HA purified from healthy donors (Figure 6C, D), indicating that inhibition of ROCK signaling diminishes HA-mediated loss of VE-cadherin.

To determine the receptor that mediates barrier disruption in response to COVID-derived HA, we cultured LMVECs in the absence or presence of either scramble siRNA or siRNA targeting specific receptors with known activities regulated by LMW-HA and measured knockdown (Figure S7). We then cultured siRNA-treated cells in the presence of HA purified from COVID-19 patients and observed a significant loss in HA-induced barrier permeability in cells treated with CD44 siRNA, while knockdown of either layilin or TLR4 showed no statistically significant difference. We next pre-treated LMVEC with an anti-CD44 antibody known to antagonize HA binding prior to incubation with HA purified from COVID-19 patients and observed a similar inhibition of HA-induced barrier disruption to that in cells subject to CD44-knockdown (Figure 6E). Together, our data provides evidence that HA-fragments present in the plasma of SARS-CoV-2 infected patients induce endothelial permeability through interaction with CD44 on the surface of lung microvascular endothelial cells in a ROCK-dependent manner.
Discussion

Here, we demonstrate for the first time to the best of our knowledge that the COVID-19 cytokine storm results in aberrant degradation of endothelial glycocalyx resulting in HA-fragments capable of directly mediating endothelial dysfunction. Pathological loss of the glycocalyx during sepsis, ischemia-reperfusion injury, and other hyper-inflammatory conditions is known to induce local endothelial dysfunction and release biologically active GAG and proteoglycan fragments into circulation (31, 71). Our data shows that circulating GAGs are uniformly increased in COVID-19 along with corresponding activities of degradative enzymes and are similar to those observed in patients with sepsis. Plasma levels of HA and hyaluronidase associate with SOFA scores in COVID-19 and septic patients, and also with levels of plasma cytokines known to alter HA metabolism present at elevated levels in COVID-19 patients. Importantly, we found that treatment of pulmonary microvascular endothelial cells with plasma from COVID-19 patients promotes HA synthesis and degradation and this effect is due to transcriptional activation of HA synthesis and hyaluronidases. We show that the majority of HA present in COVID-19 patient plasma is present as low-molecular weight degradation products, and that these HA fragments are also released into the media by cells exposed to COVID-19 plasma. Circulating GAG fragments are known to function as damage associated molecular patterns and can affect endothelial cell activation and inflammation (28, 62). Finally, we provide mechanistic evidence that circulating HA fragments generated during SARS-CoV-2 infection can contribute to endothelial injury by inducing barrier dysfunction in a CD44 and ROCK-dependent manner.
Infection of cultured cells by measles virus, respiratory syncytial virus, Epstein-Barr virus, treatment of cells with viral mimetic, and infection of mice with influenza all promote synthesis of an inflammatory HA matrix (63, 65, 72, 73). In mice infected with influenza, excessive levels of luminal HA impairs lung function and is reversible by exogenous hyaluronidase administration (73). Recent reports indicate that HA is also elevated in the airway and respiratory secretions of COVID-19 patients (74, 75). While our data is suggestive that the cytokine milieu associated with COVID-19 drives dysregulated HA metabolism and subsequent endothelial dysfunction, it is also possible that SARS-CoV-2 viral components themselves could induce endothelial injury. Our data shares several intriguing parallels with a study of dengue virus, a hemorrhagic fever characterized by vascular leakage. The authors demonstrate that elevated HA correlates with disease severity, and that dengue nonstructural protein 1 directly induces endothelial injury, release of HA-fragments, and disruption of endothelial barrier function dependent on CD44 (76). Treatment of vascular endothelial cells with COVID-19 patient plasma has been demonstrated to induce endotheliopathy (77), and it is plausible that SARS-CoV-2 nonstructural proteins might also mediate these effects in a mechanism similar to those observed with dengue virus.

The precise mechanisms that initiate glycocalyx breakdown are not fully understood, and though we demonstrate a role for ECs in vitro, multiple cell types are likely involved during disease, including platelets which become hyperactivated in COVID-19 and are known to release HA fragments from the EC surface using HYAL-2 (35, 60). Interestingly, coagulopathy and platelet activation are also characteristic of Kawasaki disease (78). Increased serum levels of syndecan-1 and HA are known to be elevated in the acute phase of Kawasaki patients, and serum HA predicts future coronary artery lesion development, possibly implicating similar mechanisms could occur in COVID-19 (79).
Our data raises several interesting questions about the nature and consequence of glycocalyx degradation in critical illnesses. While cytokine release syndrome is a shared characteristic between COVID-19 and sepsis, we show that HA and HS are present at lower concentrations in COVID-19 than those observed in septic patients. Plasma GAGs are removed from circulation by clearance receptors present on liver sinusoidal endothelial cells such as stabilin-2, and injury or infection of the liver is associated with increases in circulating GAGs as in the case of hepatitis (80). Liver damage is frequently observed in septic patients, but uncommon in COVID-19 and may explain the differences in plasma GAG concentrations observed. This explanation is supported in part by elevated bilirubin in our sepsis cohort compared with COVID-19, and by similar activities of GAG-degrading enzymes in both disease groups.

It should be noted that our study has some important limitations. First, as an observational study, we cannot conclude that the associations between glycocalyx shedding, markers of endothelial activation, and clinical outcomes are causal elements of disease progression or specific for infection with SARS-CoV-2, and cannot exclude the possibility of remaining unmeasured potential confounders. Second, many of our patients were enrolled early in the pandemic and we were unable to perform longitudinal examination of whether glycocalyx components exhibit a temporal dependence of shedding and if fluid resuscitation in COVID-19 patients contributes to increased levels of released GAGs. Third, the number of patients studied represents a low sample size, especially regarding some parameters including age and race and we were only able to examine plasma samples from patients, limiting our ability to determine the cellular source of GAG degradation. Fourth, due to the fact that many of our patients were referred to our hospital for care at various stages of disease progression we were unable to obtain blood pressure measurements for many of our patients at the time of admission and before administration of

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hemodynamic support. This represents an important gap in our study as hypertension is associated with endothelial glycocalyx dysfunction (81, 82), is among the most common comorbidities found in hospitalized patients with COVID-19 and unstable blood pressure control is associated with greater risks of ICU admission and mortality in COVID-19 patients (83, 84). Nevertheless, differences in several indices of glycocalyx degradation including HA and hyaluronidase activity are very pronounced and correlate well with severity of disease. Technical limitations of our study pertain to the evaluation of heparanase, where recent reports are conflicting. A small study suggests that heparanase activity is unchanged in COVID-19 despite evidence of glycocalyx injury (85), while a recent, cross-sectional report indicates that heparanase activity strongly associates with COVID-19 severity (46). However, interpretation of these data and ours may be complicated by prophylactic use of LMW heparin. In our study, 80.4% of COVID-19 and 30.4% of septic patients received LMW heparin, and LMW heparin treatment is associated with reduced heparanase activity in COVID-19 (46).

A growing number of studies underscore the importance of proteoglycans and GAGs of the glycocalyx as endogenous regulators of thrombosis, immune cell adhesion, and maintenance of vascular integrity which become dysregulated in severe cases of influenza, endotoxemia, and systemic inflammation. We show that glycocalyx injury itself is a pathological manifestation capable of exacerbating disease and suggests that the cytokine storm associated with COVID-19 is a driver of GAG-degradation by the pulmonary microvasculature. However, the spike glycoprotein of SARS-CoV-2 (86) and related coronaviruses (87, 88) contain a GAG binding motif (89) capable of mediating attachment to the cell-surface via HS. It is therefore plausible that GAG-degrading activities could be a protective mechanism early in infection that if unchecked contributes to disease. The results of our investigation provide compelling evidence that prevention
of enzymatic degradation of HA and other GAGs may be a novel treatment modality to reduce vascular permeability observed in ARDS and critically ill COVID-19 patients.

Methods

Study Design

Patients with SARS-CoV-2 infection (n=46) requiring hospitalization due to acute illness were recruited from the University of Utah Health Sciences Center in Salt Lake City. We prospectively collected and analyzed data on patients with SARS-CoV-2 infection confirmed by RT-PCR, in accordance with current standards. ACD-anticoagulated whole blood from hospitalized patients with COVID-19 was collected from March 17 – June 5, 2020. COVID-19 patients were recruited under study protocols approved by the Institutional Review Board of the University of Utah (IRB#: 00102638, 00093575). Healthy, age-, and gender-matched donors were recruited and enrolled under a separate IRB protocol (IRB#: 0051506). Enrollment criteria included: age > 18, respiratory symptoms including cough and shortness of breath, fever, hospital admission, positive SARS-CoV-2 testing, and written informed consent. All COVID-19 patients were hospitalized and were further stratified into non-ICU and critically ill ICU COVID-19 patients. We also enrolled critically ill septic patients (n=23) for comparisons. Enrollment criteria for septic patients included age >18, SEPSIS-3 criteria, and ICU admission. All patients underwent clinical investigation to identify the pathogen causing the infection and include: Streptococcus (11%), Staphylococcus (9%), Escherichia coli (6%), and Influenza A/H1N1 (3%). The site or organ of the primary infection included pneumonia (34%), skin and soft tissue infection (26%), urosepsis (23%), intra-abdominal infection (9%), blood (6%), or unknown (2%). Patients were a subset of subjects enrolled in previous studies(56, 90). Demographic, clinical characteristics, and illness severity data including Sequential organ failure assessment (SOFA) scores are summarized in Tables 1 and 2.
Glycosaminoglycan Purification and Measurement

Plasma and media samples were proteolyzed at 55 °C with 1.8 U/mL proteinase K for 16 hours to digest proteins, including those with GAG-binding function. After proteolysis GAGs were purified stepwise by anion exchange spin columns (Thermo Scientific) using solutions of increasing concentrations of NaCl (0.15M to 0.8M), eluted, and desalted by using 2 kDa, 50kDa, or 100kDa molecular weight cut off ultrafiltration devices (Thermo Scientific).

Quantification of plasma GAGs and culture mediums were measured serially using a competitive ELISA-like assay for HA (Echelon Biosciences), HS (JM403, AMSbio), and Chondroitin Sulfate (LSbio). Plasma HA-HC modification was measured in non-proteolyzed plasma by in-house ELISA with hyaluronan binding protein (Millipore) and anti-rabbit Inter-alpha-inhibitor (DAKO) antibody as previously described (91). Spike experiments to determine HS specificity were performed with the addition of 200ng Lovenox added to patient plasma samples.

For cell treatments, HA was isolated from protease-digested plasma samples by incubation with streptavidin magnetic beads (Thermo Scientific) complexed with biotinylated versican G1 domain (Echelon Biosciences) for 24 hours at room temperature on a rocker. Beads were placed on a magnet, washed three times with PBS, and boiled at 95 °C for 15 minutes to release bound HA. All samples were assayed for residual protein contamination by Rapid Gold BCA protein assay kit (Thermo).

Enzyme activity assays

Enzyme activity was measured from equal volumes of plasma or media diluted in assay buffer. Hyaluronidase activity was measured as previously described (35). Heparanase activity measurements were performed using a FRET-based assay as described previously (92).
Chondroitinase activity was measured using 10 μM Chondroitin Sulfate-A (AMS Bio) as a substrate as described previously (93). Digestion with Streptomyces hyaluronidase (HA’se, 0.1 mU/mL), recombinant human heparanase (1μg/mL, R&D Systems), and Chondroitinase ABC (*P. vulgaris*, 100ng/mL, R&D Systems) for 1 hour at 37°C was used as a reference standard to calculate units of activity. MMP2/9 and Cathepsin D activity were measured using fluorometric assay kits containing specific inhibitors according to manufacturer’s instructions (Anaspec).

**Primary Cell Culture**

Human lung microvascular endothelial cells (LMVECs) were obtained from Lonza, cultured on fibronectin (R&D Systems) coated dishes, and maintained in EGM2-MV (Lonza) growth medium. Cells were incubated at 37°C in 5% CO₂ and 95% humidity. Plasma samples were pre-treated with 10mM CaCl₂ and defibrinated prior to addition of 1.5% plasma to culture media. Cells were incubated in the presence of plasma for 2 hours to measure changes in mRNA levels, and for 16 hours for measurement of HA, or enzyme activities.

**Transwell Permeability Assay**

LMVEC were seeded on permeable supports (3 μm pore size, Corning) placed into a 24-well plate and grown to confluence. Cells were treated with or without HA purified from patient plasma samples (750 ng HA) or 1.5% patient plasma for 16 hours at 37°C to induce endothelial barrier disruption. The upper chamber was replaced with FITC-conjugated dextran (1 mg/mL, 40kDa, MilliporeSigma) in PBS and a sample of medium from the lower chamber was measured after 1 hour.

**HA Receptor Knockdown**

Cells were grown to 70% confluency in EMG2-MV in 6-well cell culture plates and then treated with scramble, CD44, LAYN, or TLR4 siRNA (10nM, 48hr) using the RNAiMax transfection
reagent according to the manufacturer’s instructions (ThermoFisher). After 48h, cells were harvested and examined by qRT-PCR and western blot. Immunodetection (1:1000 dilution) and antibody blockade (1ug) of CD44 was performed using clone KM114 (BD Biosciences). Actin (abcam) was used as a loading control. Quantification of knock-down was measured by densitometry analysis using ImageJ. Replicate cultures were reseeded into transwell chambers for subsequent assays.

**Plasma preparation.** Whole blood was collected from patients using a 21-g needle vacutainer butterfly into acid/citrate/dextrose-anticoagulant. Whole blood was centrifuged (150xg, 20 minutes) to generate platelet-rich plasma (PRP). PRP was centrifuged at 1,500xg, 20 minutes to produce platelet-poor plasma, and was flash frozen in liquid nitrogen and stored at -80°C. Plasma from patients with COVID-19 or sepsis and healthy donors were isolated in a similar manner with respect to blood draws, centrifugations, and time from blood draw to freezer.

**Hyaluronan Size Estimation**

To estimate HA size ranges, human plasma samples were purified as described above. Following NaCl elution, and desalting, fractions were quantified measured by competitive HA-ELISA to determine the relative abundance of LMW HA (<50kDa) as a function of total HA isolated from plasma specimens and culture mediums. Purified HA was quantified using an ELISA-like assay for HA (Echelon Biosciences).

**Immunohistochemistry**

Cells were grown in 8-well chamber slides coated with fibronectin and maintained at confluency for 5 days to allow for tight junction formation. LMVECs were then treated for 16 hours in the presence or absence of purified HA or biosynthetic HA with or without addition of ROCK
inhibitor Y27632. Cells were fixed with 4% paraformaldehyde, washed 3x with PBS, and blocked for 1 hour in HBSS + 2% FBS. For VE-cadherin detection, affinity purified monoclonal antibody (1:100, Clone BV9, Biolegend) was diluted in HBSS +2% FBS and incubated at 4C overnight in a humidified chamber. After primary exposure, cells were washed with HBSS three times for 5 minutes each and incubated with donkey anti-mouse AlexaFluor 488 secondary detection antibody diluted in HBSS + 2% FBS for 1 hour. Slides were then incubated with HBSS + Hoechst 33342 (Thermo Scientific) for 10 minutes, washed with HBSS three times for 5 minutes each, and mounted under cover glass with Prolong Glass (Thermo Scientific) and sealed with nail polish. Images were obtained using an EVOS FL Auto Cell imaging system with integrated dual camera system, system specific software equipped with a 40x/1.42 NA objective was used. 16-bit monochrome images were further analyzed and changes quantified using Adobe Photoshop CS6 and ImageJ (NIH).

**Plasma Cytokine Analysis**

Plasma samples were analyzed using a Human Cytokine/Chemokine Panel I Multiplex Array (MilliporeSigma catalog# HCYTMAG60PMX41, Burlington, MA) according to manufacturer's instructions on a Luminex 200 instrument. Plasma samples from 14 healthy adults were used as controls for cross-comparison.

**Real-time Quantitative PCR**

RNA was extracted from LMVEC and tracheal aspirates using the Direct-zol RNA kit (Zymo Research) according to the manufacturer's protocol. The RNA was eluted into 30 μL of H2O. After digestion of genomic DNA by DNase, reverse transcription using the Superscript VILO kit (Thermo Fisher Scientific) was completed in accordance with the manufacturer's instructions.
The cDNA product was stored at −20°C before quantitative PCR analysis. Validated primers with conjugated 6-carboxyfluorescein (FAM) probes for HAS1-3, HYAL1&2, CD44, Layilin, ICAM1, CD44, LAYN, TLR4, SARS-CoV-2 N1, and HPRT1 rRNA were purchased from Applied Biosystems (Invitrogen, Carlsbad, CA). The real-time PCR amplifications were performed in 10-μL reaction volumes that contained TaqMan gene expression Master Mix, primers and fluorogenic probes (Invitrogen), and cDNA. All reactions were performed with four replicate reactions using a Bio-Rad C1000 Touch Thermal Cycler with attached CFX96 Real-Time System (Bio-Rad Laboratories). The real-time PCR reaction conditions were 50°C for 2 minutes and 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Changes in gene expression were calculated using the Livak (ΔΔCT) method.

Statistics

Variables from all experiments were assessed for normality with skewness and kurtosis tests using GraphPad Prism (Anderson-Darling, D’Agostino & Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov tests). For analyses comparing two groups, a parametric 2-tailed unpaired Student’s t-test was used, and differences between multiple groups were calculated using 1-way ANOVA (Kruskal-Wallis test) with Dunn’s correction for multiple comparisons. When data were not normally distributed, a Mann-Whitney U test was used when 2 groups were analyzed. For correlations, we performed simple linear regression, Pearson correlation or Spearman correlation. Summary statistics were used to describe the study cohort and clinical variables are expressed as the mean plus or minus standard deviation, or as a number and percentage where relevant. Statistical analyses were performed by using GraphPad Prism (version 8; San Diego, CA). Outliers were removed using the ROUT method with a maximum false discovery rate set to 1%. All experiments were performed
at least in triplicate, and data are represented as means ± SEM. A 2-tailed P value <0.05 was considered statistically significant.

**Study Approval**

All studies were approved by the Institutional Review Board of the University of Utah (IRB#: 00102638, 00093575, 0051506) and all participants or their legally authorized representatives completed written informed consent.
Authorship Contributions

K.Q., B.A.M., E.A.M., I.P, B.K.M, F.D., E.J.B., R.A.C., A.C.P designed and performed experiments; K.Q., E.A.M, E.J.B., M.T.R., R.A.C., analyzed results and made the figures; E.A.M., R.A.C., and A.C.P wrote the paper; and all authors reviewed and critically edited the manuscript.

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Disclosure of Conflicts of Interest

The authors declare no competing financial interests.
References


Figure 1. Circulating glycosaminoglycans are increased in COVID-19 patients and associate with disease severity. Circulating glycosaminoglycans were measured in plasma collected from COVID-19 patients (n=46) and septic patients (n=23) within 72 hours of ICU admission, or age-, race-, and gender-matched healthy donors (n=18) as described in “Methods.” Levels of HA (A), HS (B), and CS (C) in COVID-19 compared with normal and septic patients. Comparison of HA (D), HS (E), and CS (F) levels by disease status in ICU (n=20) and non-ICU (n=23) admitted COVID-19 patients. A thick dashed line indicates the median and thin dashed lines indicate either quartile. Data are reported as mean ± SEM, *p<0.05, ***p<0.001, differences between multiple groups were calculated using 1-way ANOVA (Kruskal-Wallis test) with Dunn’s correction for multiple comparisons.
Figure 2. Glycocalyx-degrading enzyme activities are elevated in COVID-19 patients. Activity of glycosaminoglycan degrading enzymes were measured in plasma collected from COVID-19 patients (n=46) and septic patients (n=23) within 72 hours of ICU admission, or age-, race-, and gender-matched healthy donors (n=18). Levels of HA (A), HS (B), and CS (C) in COVID-19 compared with normal and septic patients. Comparison of HA (D), HS (E), and CS (F) levels by disease status in ICU (n=20) and non-ICU (n=23) admitted COVID-19 patients. A thick dashed line indicates the median and thin dashed lines indicate either quartile. Data are reported as mean ± SEM, **p<0.01, ***p<0.001, differences between multiple groups were calculated using 1-way ANOVA (Kruskal-Wallis test) with Dunn’s correction for multiple comparisons.
Figure 3. Circulating levels of HA and hyaluronidase activity correlate with clinical and inflammatory signatures of COVID-19. Correlation analysis severity of sequential organ failure assessment (SOFA) scores with plasma HA concentration and hyaluronidase activity levels in COVID-19 (n=46) (A, C) and sepsis (n=23) (B, D) patients. (E) Spearman correlation matrix of plasma HA, hyaluronidase (HA’se) activity, and cytokines in COVID-19 patients (n=46) demonstrated to be statistically significant (*P <0.05) between healthy controls and COVID-19 patients. Yellow indicates a positive correlation and purple indicates a negative correlation. Pearson correlation coefficient was used to determine the $r$ value of the correlation between the 2 groups.
Figure 4. COVID-19 plasma induces HA synthesis and degradation in lung microvascular ECs.

Primary human pulmonary microvascular endothelial cells were exposed to 1.5% plasma from COVID-19 patients or healthy donors and measured for changes in HA biosynthesis and degradation. After 16 hours, levels of HA and hyaluronidase activity associated with the cell-layer (A, B) or in conditioned media (C, D) were measured and levels present in 1.5% plasma alone was subtracted as background. q-RT-PCR of HA biosynthetic (E) and HA degrading (F) enzyme transcript levels in cells treated with either COVID-19 or control plasma for 2 hours. A thick dashed line indicates the median and thin dashed lines indicate either quartile. Data are reported as mean ± SEM, **p<0.01, ***p<0.001, unpaired Student’s t-test, 2 tailed, n = at least 5 independent experiments of at least 5 patients each. Circles represent individual patient plasma samples.
Figure 5. *Circulating HA is present as pathological low molecular weight fragments.* The molecular weight profile of HA was fractionated stepwise and measured by competitive ELISA assay. HA from patient plasma (A) or pulmonary microvascular endothelial cells stimulated with either healthy donor or COVID-19 patient plasmas (B) is reported as the relative abundance of LMW-HA fragments (<50 kDa, n = 5 patients each). (C) ELISA measurement of HA-HC in plasma from COVID-19 patients (n = 46) or healthy donors (n = 18). A thick dashed line indicates the median and thin dashed lines indicate either quartile. Data are reported as mean ± SEM, ***p<0.001, unpaired Student’s t-test, 2 tailed.
Figure 6. HA fragments present in COVID-19 plasma promote endothelial dysfunction. (A) LMVECs were seeded on permeable supports (3 μm pore size) placed into a 24-well plate and grown to confluence. Cells were treated with or without HA purified from patient plasma samples (750 ng HA) or 1.5% patient plasma for 16 hours at 37°C to induce endothelial barrier disruption. The upper chamber was replaced with FITC-conjugated dextran (1 mg/mL, 40kDa) in PBS and medium from the lower chamber was measured after 1 hour. (B) LMVECs were grown to confluence in a transwell chamber and co-incubated in the presence or absence of the ROCK inhibitor Y27632 (10µM), in the presence or absence of biosynthetic 4kDa HA (1000 ng), or in the presence or absence of HA purified from patient plasma for 16 hours at 37°C to induce endothelial barrier disruption and barrier function was measured as above. (C) LMVECs were grown on coverslips until confluency in the presence or absence of Y27632 prior to treatment with either HA purified from healthy donor plasma, COVID-19 patient plasma, or LMW-HA (4kDa). Cells were fixed, permeabilized, and stained with an antibody against VE-cadherin. Scale bar indicates 20 µm. (D) VE-Cadherin immunostaining was quantified by using 3 independent experiments and normalized to the number of endothelial cells per field using ImageJ. (E) LMVECs were treated for 48 hours in the presence or absence of siRNA (10nM) or a CD44-HA blocking antibody (KM114, 1µg) prior to treatment with HA and measurement of barrier function. In some experiments, HA was digested with Streptomyces hyaluronidase (HA’aše) as a specificity control. Data is reported as mean ± SEM; n = 5 independent experiments of at least 4 patients each, 1-way ANOVA followed by Tukey’s multiple comparison tests. Different alphabetical superscripts are significantly different from each other, p < 0.05.
### Table 1. Clinical Characteristics of Healthy Donors and Hospitalized Patients with COVID-19 and Sepsis

<table>
<thead>
<tr>
<th></th>
<th>Healthy Donors (n=18)</th>
<th>COVID-19 (N=46)</th>
<th>Sepsis (N=23)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (mean, ±SD)</strong></td>
<td>53.2 (± 12.8)</td>
<td>54.4 (± 13.8)</td>
<td>57.8 (± 17.0)</td>
<td>0.644</td>
</tr>
<tr>
<td><strong>Male (%)</strong></td>
<td>61%</td>
<td>48%</td>
<td>57%</td>
<td>0.585</td>
</tr>
<tr>
<td><strong>Hispanic/Latino (%)</strong></td>
<td>5.5%</td>
<td>37%</td>
<td>30%</td>
<td>0.097</td>
</tr>
<tr>
<td><strong>BMI (mean, ±SD)</strong></td>
<td>NR</td>
<td>33.5 (±9.4)</td>
<td>34.8 (±15.2)</td>
<td>0.643</td>
</tr>
<tr>
<td><strong>Diabetes (%)</strong></td>
<td>0%</td>
<td>45.7%</td>
<td>25.0%</td>
<td>0.123</td>
</tr>
<tr>
<td><strong>Hypertension (%)</strong></td>
<td>0%</td>
<td>34.8%</td>
<td>52.3%</td>
<td>0.199</td>
</tr>
<tr>
<td><strong>Chronic Lung Disease</strong></td>
<td>0%</td>
<td>21.7%</td>
<td>26.1%</td>
<td>0.765</td>
</tr>
<tr>
<td><strong>SOFA score (mean, ±SD)</strong></td>
<td>--</td>
<td>3.2 (± 1.4)</td>
<td>6.3 (± 2.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>ARDS (%)</strong></td>
<td>--</td>
<td>41.3%</td>
<td>21.7%</td>
<td>0.179</td>
</tr>
<tr>
<td><strong>Mechanical Ventilation (%)</strong></td>
<td>--</td>
<td>17.3%</td>
<td>4.3%</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Survival (90-day follow up) (%)</strong></td>
<td>--</td>
<td>82.6%</td>
<td>95.6%</td>
<td>0.252</td>
</tr>
<tr>
<td><strong>Platelet Count (K/µL, mean, ±SD)</strong></td>
<td>--</td>
<td>242.5 (±70.8)</td>
<td>230 (± 98.1)</td>
<td>0.729</td>
</tr>
<tr>
<td><strong>White Blood Count (K/µL mean, ±SD)</strong></td>
<td>--</td>
<td>6.7 (± 2.5)</td>
<td>20.8 (± 20.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Bilirubin (mg/dL mean, ±SD)</strong></td>
<td>--</td>
<td>0.61 (± 0.3)</td>
<td>2.42 (± 3.7)</td>
<td>0.308</td>
</tr>
<tr>
<td><strong>Anticoagulation</strong></td>
<td>0%</td>
<td>89.1%</td>
<td>95.7%</td>
<td>0.661</td>
</tr>
<tr>
<td><strong>Low Molecular Weight Heparin</strong></td>
<td>0%</td>
<td>80.4%</td>
<td>34.7%</td>
<td>0.0004</td>
</tr>
<tr>
<td><strong>Heparin</strong></td>
<td>0%</td>
<td>4.3%</td>
<td>56.5%</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*Reference Range: 159-439 K/µL

*Reference Range: 4.3-11.3 K/µL

*Reference Range: 0.2-1.2 mg/dL

*Three sepsis patients were on therapeutic heparin

Study participants were a subset of subjects previously reported.

NR = not recorded
Table 2. Clinical Characteristics of COVID-19 ICU and Non-ICU patients

<table>
<thead>
<tr>
<th></th>
<th>COVID-19 ICU (N=20)</th>
<th>COVID-19 Non-ICU (N=26)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean, ±SD)</td>
<td>62.1 (± 13.7)</td>
<td>47.6 (± 16.1)</td>
<td>0.277</td>
</tr>
<tr>
<td>Male (%)</td>
<td>55%</td>
<td>42%</td>
<td>0.553</td>
</tr>
<tr>
<td>Hispanic/Latino (%)</td>
<td>45%</td>
<td>29%</td>
<td>0.537</td>
</tr>
<tr>
<td>BMI (mean, ±SD)</td>
<td>31.4 (±8.9)</td>
<td>34.1 (±9.5)</td>
<td>0.129</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>65%</td>
<td>30.8%</td>
<td>0.036</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>40%</td>
<td>30.8%</td>
<td>0.548</td>
</tr>
<tr>
<td>Chronic Lung Disease (%)</td>
<td>30%</td>
<td>15.4%</td>
<td>0.292</td>
</tr>
<tr>
<td>Smoker, Current/Former (%)</td>
<td>41%</td>
<td>36%</td>
<td>0.408</td>
</tr>
<tr>
<td>SOFA score (mean, ±SD)</td>
<td>4.9 (± 1.4)</td>
<td>1.9 (± 1.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ARDS (%)</td>
<td>85%</td>
<td>14.4%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mechanical Ventilation (%)</td>
<td>40%</td>
<td>0%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Survival (90 day follow up) (%)</td>
<td>65%</td>
<td>96.1%</td>
<td>0.008</td>
</tr>
<tr>
<td>Platelet Count (K/µL, mean, ±SD)</td>
<td>247.7 (± 71.4)</td>
<td>239.5 (± 122)</td>
<td>0.4003</td>
</tr>
<tr>
<td>White Blood Count (mean, ±SD)</td>
<td>8.1 (± 2.5)</td>
<td>5.7 (± 2.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PT (s, mean, ±SD)</td>
<td>14.4 (± 3.0)</td>
<td>13.0 (± 0.9)</td>
<td>0.964</td>
</tr>
<tr>
<td>aPTT (s, mean, ±SD)</td>
<td>42.0 (± 24.8)</td>
<td>32.5 (±3.5)</td>
<td>&gt;0.999</td>
</tr>
<tr>
<td>Anticoagulation (prophylactic)</td>
<td>100%</td>
<td>80.7%</td>
<td>0.023</td>
</tr>
<tr>
<td>Hydroxychloroquine (%)</td>
<td>25.0%</td>
<td>15.4%</td>
<td>0.473</td>
</tr>
<tr>
<td>Remdesivir (%)</td>
<td>35.0%</td>
<td>19.2%</td>
<td>0.314</td>
</tr>
</tbody>
</table>

Study participants were a subset of subjects previously reported.