

Cell-Free Hemoglobin Upregulates Pulmonary Endothelial Heparanase

Expression to Drive Glycocalyx Destruction and Inflammation in Sepsis

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Abstract

Both elevated plasma cell-free hemoglobin (CFH) and heparanase-driven endothelial glycocalyx shedding are contributors to microvascular dysfunction and organ injury in sepsis. However, the mechanisms governing heparanase activation, and the potential role of CFH in this process, are not understood. Utilizing patient samples, mice with cecal slurry-induced (CS) peritonitis and elevated CFH, and human lung microvascular endothelial cells (HLMVECs), we tested the hypothesis that CFH upregulates heparanase production to drive endothelial glycocalyx degradation. In human sepsis, elevated circulating CFH was associated with higher heparanase and heparan sulfate levels, which in turn correlated with adverse clinical outcomes. CS+CFH-treated mice had increased plasma heparanase, glycocalyx degradation, and pulmonary and systemic inflammation; endothelial heparanase deletion abrogated these effects. Additionally, in both pulmonary endothelial cells isolated from CS+CFH-treated mice and HLMVECs exposed to CFH and $\text{TNF}\alpha$, heparanase transcription and active enzyme production were increased. The deleterious effects of CFH were attenuated by acetaminophen, a hemoprotein reductant. In summary, we demonstrate that CFH oxidation stimulates endothelial heparanase expression and activation during sepsis, leading to endothelial glycocalyx degradation, which may disrupt the endothelial barrier and result in organ injury. Our findings highlight the CFH-heparanase axis as a potential therapeutic target for endothelial glycocalyx preservation in sepsis.

Introduction

Sepsis, the state of life-threatening organ dysfunction due to a dysregulated host response to infection, is a critical public health issue with high morbidity and mortality (1). A primary reason for poor outcomes from sepsis is an incomplete understanding of its complex pathophysiology, creating challenges with developing pharmacologic interventions. Sepsis is characterized by damage to the microvascular endothelium due to widespread inflammation (2). Sepsis patients with more severe endothelial injury have higher sepsis severity, organ failure, and mortality (3). A hallmark of endothelial injury in sepsis is loss of endothelial barrier integrity, which is normally tightly regulated to maintain fluid homeostasis and organ function (2, 4). Loss of barrier integrity results in increased permeability, fluid extravasation and edema formation, and end-organ dysfunction including acute respiratory distress syndrome (ARDS) and acute kidney injury (2, 4, 5). As active contributors to sepsis-associated organ dysfunction, the molecular processes that underlie microvascular endothelial barrier disruption represent an important area of investigation for identification of potential therapeutic targets.

A fundamental regulator of endothelial permeability is the endothelial glycocalyx, a mesh-like network of proteoglycans, which are comprised of core proteins and glycosaminoglycans, along with glycoproteins (6, 7). The gel-like matrix formed by these components acts as a semi-permeable barrier that controls fluid movement across the vascular wall (8). Damage to the glycocalyx is an integral step in endothelial injury during sepsis (9, 10). We previously showed that glycocalyx disruption is associated with higher severity of ARDS and mortality in patients with sepsis (11). Loss of the endothelial glycocalyx occurs in part through activation of heparanase, a glycocalyx-

degrading enzyme (12, 13). Upregulation of heparanase activity leads to increased glycocalyx cleavage and microvascular permeability (13), however the molecular underpinnings of sepsis-associated heparanase upregulation have not been elucidated.

Red blood cells are damaged in sepsis due to increased fragility and, upon lysis, release hemoglobin into the intravascular space (i.e., CFH) (14). Hemoglobin is composed of a tetramer of globin molecules (two α - and two β -subunits), each containing a heme group bound to iron, which can redox cycle from its 2+ oxidation state, the oxygen-carrying form, to the 3+ state in the oxidative environment of sepsis (15, 16). Through its oxidation, hemoglobin generates reactive oxygen species (ROS), which cause cellular and tissue injury (15, 17). CFH is normally quickly removed from the circulation by the endogenous scavenger haptoglobin; however, in situations where CFH is elevated, such as sepsis, haptoglobin is rapidly depleted and CFH freely circulates (15, 16). Plasma CFH is elevated in ~80% of sepsis patients and higher levels are independently associated with risk of development of ARDS and mortality (18). Our prior studies demonstrated that CFH increases barrier permeability in primary human lung microvascular endothelial cells, murine polymicrobial peritoneal sepsis, and the isolated perfused human lung, and that this effect is not mediated by common cell death mechanisms (19–21).

While it is evident that CFH is an important driver of endothelial barrier dysfunction in sepsis, the precise mechanisms of CFH-mediated endothelial injury remain unknown. Defining the molecular mechanisms of CFH-induced endothelial hyperpermeability may identify potential treatment targets in sepsis and its associated organ dysfunction. Thus, in this study, we investigated the endothelial glycocalyx, a

central effector of endothelial integrity, as a possible site of CFH-mediated endothelial injury during sepsis.

Results

Higher plasma CFH is associated with increased circulating heparanase and heparan sulfate levels in critically ill adults

Plasma CFH is elevated in sepsis and is associated with worse outcomes, including increased development of ARDS and lower survival (18, 22). CFH oxidation yields high levels of ROS, and ROS are known to stimulate heparanase synthesis (17, 23, 24). We therefore postulated that CFH is a driver of heparanase production and consequent endothelial glycocalyx degradation. To test this, circulating CFH concentrations from critically ill adults were compared to plasma levels of heparanase and the major glycocalyx glycosaminoglycans (GAGs).

Demographic and clinical features of the patients included in this analysis are summarized in Table 1. Of the 67 patients, 15 (22.4%) died during hospitalization. Survivors and non-survivors did not differ by age, sex, or race. There were similar rates of ARDS and need for mechanical ventilation between survivors and non-survivors. Individuals who died had significantly fewer days alive and free from the ICU ($P < 0.0001$, Table 1). Sequential organ failure assessment (SOFA) scores on study day 0 were numerically higher among non-survivors versus survivors (12.5 vs. 9.0, $P = 0.055$, Table 1).

In patients with higher plasma CFH, levels of circulating heparanase were increased ($P = 0.022$) (Figure 1A). Consistent with this finding, plasma CFH levels were positively correlated with circulating heparanase ($\rho = 0.28$, $P = 0.023$). Additionally, total plasma glycosaminoglycans ($P = 0.024$), heparan sulfate ($P = 0.0045$), and heparan sulfate fragments that specifically indicate cleavage by heparanase were higher in the

plasma of patients in higher quartiles circulating CFH (tri-sulfated: $P = 0.033$, N -sulfated: $P = 0.032$) (Figure 1B-E). Positive correlations were also observed between plasma CFH and total glycosaminoglycans ($\rho = 0.28$, $P = 0.023$), total heparan sulfate ($\rho = 0.29$, $P = 0.015$), tri-sulfated heparan sulfate ($\rho = 0.29$, $P = 0.018$), and N -sulfated heparan sulfate ($\rho = 0.33$, $P = 0.0066$). These findings are consistent with previously published data demonstrating increased circulating heparan sulfate and heparan sulfate fragments in patients with indirect lung injury compared with healthy individuals (25). However, there was no association between CFH levels by quartile and chondroitin sulfate ($P = 0.14$) or hyaluronic acid ($P = 0.20$) (Supplemental Figure 1A, B), glyocalyx components that are not subject to cleavage by heparanase. Similarly, no significant correlations were identified between circulating CFH and chondroitin sulfate ($\rho = -0.007$, $P = 0.95$) or hyaluronic acid ($\rho = 0.22$, $P = 0.079$).

To demonstrate that plasma heparanase levels are reflective of heparanase enzymatic activity, we tested the association between plasma heparanase and total heparan sulfate levels, as well as tri- and N -sulfated heparan sulfate fragments; heparanase is the only sheddase in humans capable of cleaving heparan sulfate and has a particular affinity for these highly sulfated sites (12, 13). There was a significant relationship between plasma heparanase concentrations and total heparan sulfate ($\rho = 0.53$, $P < 0.001$), tri-sulfated heparan sulfate ($\rho = 0.53$, $P < 0.001$), and N -sulfated heparan sulfate concentrations ($\rho = 0.58$, $P < 0.001$), suggesting that higher circulating heparanase levels reflect increased heparanase activity (**Figure 1F-H**).

Plasma heparanase and heparan sulfate levels are associated with worse illness

severity and outcomes in sepsis

To determine whether the higher heparanase levels that were associated with CFH levels also correlated with illness severity and clinical outcomes in sepsis, we compared circulating heparanase concentrations to SOFA scores, severity of hypoxemia, rates of ARDS, and in-hospital mortality. Plasma heparanase levels were significantly associated with higher SOFA scores ($\rho = 0.32$, $P = 0.010$) (Figure 2A). Additionally, patients with ARDS had almost two-fold higher heparanase concentrations than patients without ARDS (0.41 [0.08-0.51] vs. 0.22 [0.24-1.1] pg/mL, $P = 0.015$) (Figure 2B). Severity of hypoxemia also significantly increased with plasma heparanase levels ($P = 0.0014$) (Figure 2C). Further, patients who died during admission had significantly higher circulating heparanase than those who survived (0.50 [0.35-1.3] vs. 0.24 [0.10-0.55] pg/mL, $P = 0.0027$) (Figure 2D). Plasma total heparan sulfate levels and specific heparan sulfate fragments were also tested for association with SOFA scores, rates of ARDS, severity of hypoxemia, and death. Higher circulating total heparan sulfate levels (Figure 2E-H), and specific highly sulfated fragments yielded by heparanase (Figure 2I-P), corresponded to higher SOFA scores, increased rates of ARDS, worse hypoxemia, and greater mortality.

CFH increases heparanase transcription and active protein production in the inflamed endothelium

We next sought to test whether CFH impacts regulatory pathways controlling heparanase expression in lung endothelial cells. Given that CFH is a known source of ROS (15), and that heparanase expression is in part regulated by redox-sensitive

transcription factors, we hypothesized that CFH redox cycling increases heparanase production. To test the impact of CFH on heparanase transcription and protein synthesis, we treated HLMVECs with CFH for 24 hours and measured heparanase mRNA and protein levels by qPCR and ELISA, respectively. Contrary to our initial hypothesis, CFH did not affect heparanase transcription or protein production compared to vehicle control (Supplemental Figure 2A, B).

Because associations between higher CFH and higher heparanase levels and activity were observed in sepsis patients, we next tested whether endothelial cells require priming with an inflammatory stimulus to respond to CFH. To investigate this, HLMVECs were pre-treated for 1 hour with the proinflammatory cytokine TNF- α , followed by CFH for 24 hours. HLMVECs exposed to TNF- α and CFH had higher heparanase transcript abundance (Relative expression (RE): 2.45 [2.27-2.73]) versus vehicle (RE: 1.01 [0.75-1.27], $P = 0.029$) and TNF- α (RE: 0.59 [0.52-1.68], $P = 0.0060$) (Figure 3A). HLMVECs exposed to TNF- α /CFH also had higher active heparanase protein levels, with increased ratio of the active 50-kDa subunit to the inactive 65-kDa proenzyme (median ratio: 2.21 [0.72-5.39]) compared to vehicle (0.12 [0.069-0.28], $P = 0.039$) and TNF- α (0.075 [0.036-0.25], $P = 0.0081$) (Figure 3B). These results reveal that CFH upregulates heparanase transcription and activation only in the setting of a proinflammatory stimulus.

Acetaminophen inhibits CFH-mediated heparanase induction in the inflamed endothelium

We next sought to resolve the mechanism by which CFH induces heparanase

gene expression. Redox cycling of CFH generates ROS, which can activate redox-sensitive transcription networks (16, 17, 23, 24, 26–28). To test whether CFH redox cycling triggers heparanase expression, HLMVECs were treated with TNF- α for 1 hour, followed by CFH with or without 24-hour treatment with acetaminophen (24 μ g/mL), a hemoprotein reductant that inhibits redox cycling by CFH (21, 29, 30). Exposure of cells to acetaminophen in addition to TNF- α and CFH restored heparanase mRNA to baseline levels (Figure 3C). Additionally, HLMVECs that received acetaminophen, TNF- α , and CFH had significantly lower active heparanase levels (median active-to-inactive ratio: 0.22 [0.066-0.39]) compared to those treated with TNF- α /CFH (1.87 [1.71-3.64], P = 0.046) (Figure 3D). These findings imply that the oxidant effects of CFH mediate heparanase upregulation.

Exogenous intravenous CFH enhances heparanase-mediated endothelial glycocalyx degradation in murine sepsis

To determine if CFH also promotes heparanase transcription and enzymatic activation in a physiologic model of sepsis, we utilized our established murine model of CS-induced intraabdominal polymicrobial sepsis with elevated CFH (20). This model recapitulates levels of circulating CFH that have been reported in patients with sepsis. We first confirmed that CFH exacerbates illness severity, as we previously observed (Figure 4A) (20). Renal damage was also worse in CS/CFH treated mice, demonstrated by higher expression of the kidney injury biomarkers NGAL and KIM-1 (Figure 4B, C). Spleen bacterial counts, BALF protein concentrations, and lung wet-to-dry weight ratios did not differ between the groups (Supplemental Figure 3A-D).

To test the influence of circulating CFH on endothelial glycocalyx shedding in vivo, we quantified plasma levels of syndecan-1, a glycocalyx core protein, and GAGs. Mice given CS/CFH had significantly increased plasma syndecan-1 concentrations (12.70 [7.23-20.42] ng/mL) compared to Sham/Vehicle (0.34 [0.22-0.67] ng/mL, $P < 0.0001$), Sham/CFH (0.15 [0.13-0.64] ng/mL, $P < 0.0001$) and CS/Vehicle animals (2.56 [1.50-3.78] ng/mL, $P < 0.0001$) (Figure 4D). Circulating GAGs were also significantly elevated in CS/CFH treated mice (9.48 [7.89-18.15] μ g/mL) versus all other groups (Figure 4E). These results show that CFH is an upstream mediator of endothelial glycocalyx cleavage during sepsis.

To further investigate the processes by which CFH stimulates damage to the endothelial glycocalyx, we measured circulating heparanase levels in septic mice. Those that received CFH had increased plasma concentrations of heparanase (215.1 [185.5-686.5] pg/mL) in comparison to vehicle exposed animals with sepsis (57.7 [38.8-80.4] pg/mL, $P < 0.0001$) (Figure 4F). We also quantified heparanase mRNA and active protein levels in pulmonary endothelial cells isolated from septic mice at 24 hours. Treatment with CS/CFH resulted in higher heparanase transcript abundance (RE: 3.38 [3.01-3.96]) than CS alone (RE: 1.01 [0.97-1.02], $P = 0.024$) (Figure 4G). A similar effect was observed on heparanase activation, where CS/CFH exposed mice had elevated pulmonary endothelial levels of active heparanase (Active-to-inactive ratio: 1.61 [1.36-1.70]) versus CS/Vehicle (0.71 [0.54-0.86], $P = 0.024$) (Figure 4H). These findings indicate that, in sepsis, CFH drives the induction of active heparanase and subsequent breakdown of the endothelial glycocalyx.

Lung and systemic inflammation are increased in CFH-treated septic mice

Our lab previously demonstrated that CFH exacerbates pulmonary and systemic inflammation in sepsis (20); to confirm these findings, we also examined the impact of CFH on pulmonary and systemic cytokine expression in mice with sepsis. BALF total immune cell counts were similar between groups (Supplemental Figure 3E). Whole lung transcription of the proinflammatory mediators TNF- α , IFN- γ , IL-6, and CXCL1 were significantly increased in CS/CFH treated animals relative to CS/Vehicle (Figure 4I-L). Plasma cytokine concentrations were also elevated in the septic mice exposed to CFH mice versus vehicle (Figure 4M-P).

The adverse effects of CFH in murine sepsis are mediated by heparanase

To ascertain whether the effects of CFH on illness severity, endothelial glycocalyx destruction, and pulmonary and systemic inflammation were specifically mediated via endothelial heparanase activity, we utilized tamoxifen-inducible endothelial-specific heparanase KO (hpse ^{Δ Endo}; VE-Cadherin-CreERT2^{+/-} Hpse^{fl/fl}) mice (10, 31, 32). Heparanase deletion was confirmed by a reduction in active heparanase expression in isolated pulmonary endothelial cells from healthy mice (Figure 5A) and significantly decreased circulating heparanase in CS/CFH-treated hpse ^{Δ Endo} animals (Figure 5B). CS/CFH-treated hpse ^{Δ Endo} mice also had significantly lower plasma syndecan-1 (6.89 [4.13-7.73] vs. 11.29 [9.06-12.36] ng/mL, $P < 0.0001$) and GAGs (10.41 [8.59-14.57] vs. 24.70 [21.62-27.13] μ g/mL, $P < 0.0001$) (Figure 5C, D). Compared to their littermate controls (WT; VE-Cadherin-CreERT2^{-/-}Hpse^{fl/fl}), CS/CFH-treated hpse ^{Δ Endo} animals also had less severe illness at both 12 ($P = 0.0008$) and 24 ($P = 0.038$) hours (Figure 5E).

Kidney injury (NGAL: $P < 0.0001$, KIM-1: $P = 0.025$) was reduced in CS/CFH-treated $hpse^{\Delta Endo}$ mice as well (Figure 5F, G), despite having the same weight change and bacterial burden (Supplemental Figure 4A, B). Consistent with the results of our initial experiments, BALF protein and lung wet-to-dry weight ratios did not differ between genotypes (Supplemental Figure 4C, D). However, transcript abundance of the proinflammatory cytokines TNF- α , IFN- γ , CXCL1, and IL-6 was significantly decreased in the whole lungs of CS/CFH-treated $hpse^{\Delta Endo}$ animals (Figure 5H-K). Similar results were observed for circulating levels of these cytokines (Figure 5L-O). These findings confirm that the detrimental effects of CFH are directly linked to the upregulation of heparanase.

CFH redox cycling promotes endothelial glycocalyx shedding in sepsis

Finally, to begin elucidating the molecular pathways through which CFH upregulates heparanase during sepsis, we used acetaminophen to test the contribution of CFH redox cycling to endothelial glycocalyx cleavage and its consequences. Though weight change and bacterial burden were similar, CS/CFH-exposed mice given clinically relevant doses of acetaminophen had lower severity of illness compared to placebo controls at both 12 ($P = 0.0004$) and 24 hours ($P = 0.028$) (Supplemental Figure 5A, B; Figure 6A). Kidney injury markers were also lower in the acetaminophen-treated CS/CFH animals (Figure 6B, C). BALF protein and lung wet-to-dry weight ratios were similar between treatment groups, in line with earlier data (Supplemental Figure 5C, D). Acetaminophen exposure reduced glycocalyx shedding, as these mice had significantly lower plasma concentrations of syndecan-1 (5.99 [2.48-7.86] vs. 17.61 [16.46-19.60])

ng/mL, $P < 0.0001$) and GAGs (9.06 [5.55-14.01] vs. 27.07 [21.55-30.87] $\mu\text{g/mL}$, $P < 0.0001$) (Figure 6D, E). Plasma heparanase levels were significantly lower in CS/CFH animals given acetaminophen as well (54.86 [48.05-73.24] vs. 181.9 [134.60-248.00] pg/mL) (Figure 6F). Additionally, whole lung mRNA levels and systemic concentrations of proinflammatory cytokines were significantly decreased in acetaminophen-treated CS/CFH mice (Figure 6G-N). Altogether, these results demonstrate the pathologic role of CFH redox cycling in promoting endothelial glycocalyx shedding during sepsis, likely via stimulation of heparanase transcription and activation in pulmonary and systemic endothelial cells, leading to lung and systemic inflammation and more severe illness.

Discussion

In this study, we sought to determine the mechanism by which CFH induces endothelial dysfunction in sepsis, with a focus on the endothelial glycocalyx. In patients with sepsis, we discovered that there is a specific and direct correlation between circulating CFH, heparanase, and heparan sulfate degradation products. Our findings further revealed that higher concentrations of circulating heparanase correlated with worse illness severity and higher rates of ARDS and in-hospital mortality. Elevated heparanase also corresponded to greater degradation of heparan sulfate, which in turn translated to poorer prognosis. To investigate the molecular processes underlying these observations, we determined that CFH stimulated both heparanase transcription and activation in human and murine pulmonary endothelial cells. This effect was inhibited by acetaminophen, a potent hemoprotein reductant, in cultured human lung endothelial cells and in mice with sepsis. Moreover, in septic mice, treatment with CFH enhanced endothelial glycocalyx shedding, lung and systemic inflammation, and illness severity. Since inducible endothelial-specific heparanase KO animals had improvements in these endpoints, we concluded that heparanase activation contributes to the deleterious effects of excess CFH. Taken together, these data support a working model where release of CFH in the circulation during sepsis upregulates active heparanase to augment breakdown of the endothelial glycocalyx, leading to more severe illness and adverse clinical outcomes (Figure 7).

Past research has consistently highlighted endothelial glycocalyx degradation as a central pathophysiologic feature of sepsis (11, 12, 33). We and others have previously reported that both plasma syndecan-1 and heparan sulfate are increased in septic

patients, and that this is associated with worse illness severity and outcomes (11, 12, 33). Additionally, increased heparanase transcription and active enzyme synthesis have been observed in platelets from septic patients, with platelet levels of active heparanase correlating with mortality (34). However, to our knowledge, no prior studies have quantified plasma heparanase levels or correlated circulating heparanase with clinical outcomes, nor have there been measurements of heparan sulfate fragments that reflect selective heparanase activity in the context of glycocalyx shedding during human sepsis. Here, we demonstrate that heparanase and heparan sulfate fragments produced by heparanase are higher in individuals with more severe sepsis and poor outcomes. By resolving the circulating abundance of heparanase and the tri- and *N*-sulfated heparan sulfate moieties, our investigation provides a granular assessment of sepsis-induced glycocalyx cleavage and offers mechanistic insight into the molecular pathways underlying this process. These findings also provide evidence that glycocalyx fragments may be useful biomarkers in clinical trials targeting sepsis-induced endothelial injury.

Another key observation is the direct relationship between circulating CFH and heparanase expression and activity. While it is appreciated that CFH is elevated in septic individuals, particularly those who develop ARDS or succumb to the condition (15, 18, 22), and that CFH is implicated in microvascular barrier dysfunction (19–21), we have yet to establish the molecular pathways connecting CFH with these adverse effects. The present findings suggest a role for CFH as a potential upstream driver of heparanase activation and subsequent endothelial glycocalyx degradation in sepsis. Loss of the endothelial glycocalyx contributes to increased vascular permeability,

inflammation, and organ failure, all characteristics of sepsis (13, 23, 35). The interplay between CFH and heparanase thus may represent a critical axis in the pathogenesis of endothelial dysfunction during sepsis and could inform the design of therapeutic strategies for sepsis, aimed at preserving the endothelial glycocalyx.

One promising target is CFH redox cycling. Our findings suggest that redox cycling by CFH is an upstream driver of heparanase activation which can be inhibited by acetaminophen. A recently published clinical trial investigating whether acetaminophen improves outcomes in critically ill patients with sepsis and pulmonary or cardiovascular dysfunction showed that, although there was no difference in the number of days alive and free of organ support between the acetaminophen and placebo arms, participants that received acetaminophen had significantly lower total, respiratory, and coagulation SOFA scores and were less likely to develop ARDS (36). Additionally, among patients with higher plasma CFH levels (>10 mg/dl), mortality and rates of mechanical ventilation were lower in acetaminophen-treated patients compared to their placebo counterparts (36). The current study suggests that preservation of endothelial glycocalyx integrity may be a key potential mechanism of benefit of acetaminophen in this trial.

Our findings demonstrate a mechanistic link between CFH oxidation, heparanase activity, and endothelial damage in sepsis. However, the specific molecular pathways by which oxidation of CFH promotes heparanase transcription and active enzyme production remain undetermined. Nevertheless, our investigation provides a strong basis for future studies that seek to define the biologic processes underlying CFH-mediated upregulation of heparanase. Several redox-sensitive transcription factors, including NF- κ B, Egr-1, and HIF-1, are known to modulate heparanase expression and

thus represent plausible candidate transcriptional networks that merit further exploration (23).

There are several limitations to this study. First, the relatively small sample size, single-center enrollment, and single timepoint of the human studies may restrict the generalizability of these results, as variability in sepsis across time and location were not captured. Future studies investigating if and how endothelial glycocalyx shedding changes throughout sepsis progression and resolution may provide valuable insights into glycocalyx dynamics in sepsis. Another consideration is that CFH may impact organ dysfunction in sepsis through additional mechanisms such as activation of TLR4, and release of the free heme moiety, neither of which were addressed by this study (16, 37–42). However, the finding that acetaminophen treatment almost entirely abolished CFH-induced endothelial glycocalyx shedding and pulmonary and systemic inflammation compared to CS control animals suggests that the upregulation of heparanase synthesis is primarily driven by the oxidant effects of CFH. Oxidized CFH also readily dissociates from its heme group, thus the inhibition of CFH oxidation by acetaminophen indirectly addresses this mechanism (41, 42). Moreover, although our data strongly suggest that CFH redox cycling drives heparanase induction, neither the oxidation state of CFH nor ROS production were explicitly measured, and given that acetaminophen is a non-specific inhibitor of redox cycling by CFH, contributions from off-target effects cannot be excluded. Additionally, because we exclusively utilized TNF- α in our in vitro experiments, subsequent investigations are needed to ascertain whether CFH impacts heparanase expression in the context of other proinflammatory cytokines. We also did not identify differences in measures of pulmonary edema formation in our in vivo

studies, although our data were collected at a single timepoint and lung injury in murine models of non-pulmonary sepsis is generally mild (43). Future time course experiments and investigations utilizing complementary sepsis models and more robust models of pulmonary injury in sepsis will be important to determine if and how CFH-induced endothelial glycocalyx shedding impacts pulmonary microvascular barrier function (43).

In summary, our findings provide new insight into how release of CFH into the circulation in sepsis injures the lung and systemic endothelium by triggering degradation of the endothelial glycocalyx. These observations are highly relevant to our understanding of human sepsis pathogenesis and advance our understanding of the potential mechanisms of benefit of acetaminophen in sepsis patients with elevated CFH.

Methods

Sex as a Biological Variable

For all studies, both sexes were involved. Results are reported for both sexes combined, as the effects were similar between males and females. For all Figures, males are represented as circular symbols and females are depicted as squares.

Human studies

Study Population

Our study population comprised 67 critically ill adults from the Validating Acute Lung Injury Markers for Diagnosis (VALID) study (44). All patients had a diagnosis of sepsis on enrollment to this prospective observational cohort. Details regarding inclusion and exclusion criteria, as well as informed consent, have been previously described (44). Briefly, patients admitted to one of four intensive care units (ICUs; medical, surgical, cardiovascular, or trauma) for at least two days at Vanderbilt from October 2007 to February 2010 were enrolled in VALID. Plasma was collected at enrollment on the morning of ICU day two. When possible, blood was drawn from a central venous catheter rather than using peripheral access to minimize hemolysis. Samples were centrifuged for ten minutes at 3000 rpm to remove cells and plasma was stored at -80°C. Clinical information including demographics, severity of illness (SOFA) scores, lowest PaO₂/FiO₂ ratio, and in-hospital mortality were gathered from the electronic health record by trained personnel. ARDS classification was based on the Berlin Criteria and was completed by two physician investigator review of the participants' medical records and chest radiographs (45).

Plasma Measurements

Plasma CFH levels were previously quantified by commercially available ELISA (Abcam, ab157707) (18). Total plasma heparanase concentrations (i.e., both the active and inactive forms) were also determined by ELISA (CUSABIO, CSB-E09899h). Mass spectrometric analysis was used to measure the quantity, composition, and sulfation pattern of shed GAGs (25). Plasma samples were lyophilized, defatted, and proteolyzed to yield purified GAGs, which were then depolymerized using polysaccharide lyases and AMAC-labeled at their hemiacetal reducing ends for detection by HPLC-tandem mass spectrometry with multiple-reaction monitoring (MS-MRM) (25).

Outcomes

The primary outcome assessed in this cohort was in-hospital mortality. Secondary outcomes included rates of ARDS, severity of hypoxia by Berlin category of lowest $\text{PaO}_2/\text{FiO}_2$ ratio during admission, and severity of illness based on SOFA scores.

Statistical Analysis

Descriptive statistics were used to summarize the demographic and clinical characteristics of survivors and non-survivors. Due to the non-normal distribution of the majority of the data, medians with interquartile ranges were reported for continuous variables and frequencies were stated for categorical variables. Differences in patient features based on mortality were assessed by Chi-square test and t-test (or Mann-Whitney for nonparametric data) for categorical and continuous data, respectively. A

Mann-Whitney test was applied to determine whether plasma heparanase and heparan sulfate levels differed by hospital survivorship and rates of ARDS. The Jonckheere-Terpstra trend test was utilized to analyze hypoxia severity based on the circulating concentration of heparanase and heparan sulfate. Spearman's rank correlation was employed to assess the relationship between SOFA scores and plasma heparanase and heparan sulfate levels, as well as CFH levels and circulating heparanase and heparan sulfate fragments. A Jonckheere-Terpstra test for trend was also implemented to evaluate if there was an association between circulating CFH, heparanase, and glycocalyx GAGs. The ROUT method was used to detect outliers. A two-sided P value of < 0.05 was deemed statistically significant. Statistical analyses were completed with GraphPad Prism software version 10.4.1 for macOS. Because this was a convenience sample of available biospecimens, no prior sample size calculations were done. A post-hoc power analysis indicated our study had 75% power to detect the observed effect size at an alpha level of 0.05.

Study Approval

This study was approved by the Vanderbilt Institutional Review Board (IRB; No. 051065; Nashville, TN).

In vitro and in vivo studies

Cells and Culture Conditions

HLMVECs (C-12281, PromoCell) were cultured with Endothelial Cell Growth Media MV2 (C-22121, PromoCell) plus penicillin-streptomycin (100 U/mL; 15140-122,

Gibco) in a humidified 5% CO₂ incubator at 37°C. Cells were grown to confluence on tissue culture plates coated with Attachment Factor (S006100, Gibco). Experiments were performed 2-3 days post-confluence between passages 5-8. Each experiment was completed with at least one male and one female donor and a minimum of three unique donors per group. Results are reported for both sexes combined. HLMVECs were treated with purified human CFH diluted in phosphate buffered saline (PBS) to 1.0mg/mL or PBS vehicle control for 24 hours (19). In a subset of experiments, cells were pre-treated with TNF- α (50ng/mL, Thermo Fisher Scientific) for 1 hour to simulate the inflammatory conditions seen in sepsis (13). In another group of experiments, HLMVECs were exposed to acetaminophen (24 μ g/mL), a hemoprotein reductant, for 24 hours, in addition to TNF- α and CFH (18, 21). This concentration of acetaminophen was selected based on our prior studies (21).

Purification of CFH

CFH was purified from whole blood collected from healthy human donors by trained research nurses. Written informed consent was obtained in accordance with Vanderbilt IRB No. 061088. CFH was purified using anion exchange high-performance liquid chromatography (HPLC) as described previously (46).

Heparanase mRNA and Protein Expression

Total mRNA was isolated from treated HLMVECs and the pulmonary endothelial cells of septic mice using the Qiagen RNeasy Mini Kit (74104) according to the manufacturer's instructions. 200ng of mRNA were used per 20 μ L of iScript cDNA

Synthesis reaction mix (1708891, Bio-Rad). TaqMan quantitative polymerase chain reaction (qPCR) primers specific to human (Hs00935036) and mouse (Mm00461768) heparanase were used to measure gene expression (4331182, Thermo Fisher Scientific). Quantitative PCR amplifications were completed in a QuantStudio 3 thermocycler (A28567, Applied Biosystems) with TaqMan Fast Advanced Master Mix (4444557, Applied Biosystems). The PCR cycling parameters were: 2 minutes at 50°C, followed by 10 minutes at 95°C, then 40 cycles each consisting of 15 seconds at 95°C and 1 minute at 60°C. Fold change in transcript abundance compared to the housekeeping gene GAPDH was calculated by $2^{-\Delta\Delta C_t}$ (relative quantitative value, RQV) (47).

Total protein was harvested from treated HLMVECs and isolated pulmonary endothelial cells of septic mice using a radioimmunoprecipitation assay (RIPA) lysis and extraction buffer (89900, Thermo Fisher Scientific). Cells were washed with cold PBS, incubated in RIPA buffer for 20 minutes at 4°C, then centrifuged at 12,000rpm at 4°C for 20 minutes. The total protein concentration in the supernatant was quantified by bicinchoninic acid (BCA) assay (23227, Thermo Fisher Scientific) and 30µg of protein was added to Bolt LDS Sample Buffer (B0007, Thermo Fisher Scientific) with Bolt Sample Reducing Agent (B0004). Samples were vortexed, boiled for 5 minutes, and separated by SDS-PAGE using Bolt Bis-Tris 4-12% polyacrylamide gels (NW04125BOX, Thermo Fisher Scientific). Protein was then electro blotted onto a PVDF membrane (IB401001, Thermo Fisher Scientific) and blocked with Intercept TBS Blocking Buffer for 1 hour at 25°C (927-60001, LI-COR). The membrane was incubated with anti-heparanase primary antibody (human: 1:800, polyclonal rabbit, ANT-155,

Prospec; mouse: 1 µg/mL, polyclonal rabbit, ABC1687, Sigma-Aldrich) at 4°C overnight. Blots were washed five times in 0.1% TBS-T and incubated with goat anti-rabbit secondary (1:15,000; 926-32211, LI-COR) for 1 hour at 25°C. Visualization was completed with the Odyssey infrared imaging system (LI-COR). Densitometric measurements were collected. Total heparanase levels (i.e., both the active and inactive forms) from HLMVEC supernatants were determined by ELISA (ab256401, Abcam) per the manufacturer's protocol. Samples were run undiluted. Active heparanase levels were quantified by comparing the ratio of the inactive 65-kDa proenzyme to the 50-kDa subunit of the active form. Murine total plasma heparanase levels (i.e., both the active and inactive forms) were measured by ELISA (LS-F6621, LSBio) following the manufacturer's assay procedure.

Animals

Unless otherwise stated, murine sepsis experiments were performed with 8- to 12-week-old wildtype (WT) C57Bl/6 male and female mice purchased from The Jackson Laboratory. For all animal experiments, results are reported for both sexes combined, as the effects were similar between male and female mice. Tamoxifen-inducible endothelial-specific deletion of heparanase (Hpse^{ΔEndo}) was accomplished using the Cre/Lox system (31). Homozygous floxed heparanase mice on a C57Bl/6 background were crossed with heterozygous VE-Cadherin-CreERT2 mice; both strains were provided as a generous gift from Dr. Eric Schmidt (Mass General Brigham, Boston, MA) (10, 32). Animals were acclimatized for at least 1 week in a pathogen-free animal facility and housed in a climate-controlled room with 12-hour light/dark cycles and ad libitum

access to water and standard chow diet prior to sepsis initiation.

Experimental Murine Sepsis Procedure and Sample Processing

The cecal slurry (CS) peritonitis model with elevated CFH has been described previously (20, 48, 49). CS was prepared using 6-week-old wildtype female C57Bl/6 donor mice purchased from the Jackson Laboratory. Briefly, donor mice were euthanized immediately upon arrival to VUMC, then cecal contents were collected, resuspended in 5% dextrose, and filtered through a 70 μ m cell strainer. To minimize inter-experiment variability, CS was pooled, aliquoted, and stored at -80°C.

Polymicrobial intraabdominal sepsis was induced in recipient mice by intraperitoneal injection of CS (2.0mg/g body weight) or control (5% dextrose). Human CFH was isolated as described above, diluted to 20mg/mL in PBS, and sterile filtered prior to use. At the time of sepsis initiation, recipient mice also received a retro-orbital injection of CFH (0.15mg/g body weight) or PBS control. This dose of CFH approximates the levels seen in clinical sepsis (18, 50). The set of mice utilized for the endothelial-specific heparanase KO experiments (VE-Cadherin-CreERT2 and their littermate VE-Cadherin-CreERT2^{-/-}Hpse^{fl/fl} controls) received tamoxifen (75mg/kg body weight) for 5 days prior to sepsis induction (T5648, Sigma-Aldrich). Another group of mice were pre-treated with acetaminophen in their drinking water (1.25 g/L) for 1 week to inhibit the oxidative effects of CFH. This dose was based on a standard acetaminophen (Tylenol®) dose in humans to reach circulating therapeutic levels of 10-20 μ g/mL. Water was prepared based on the assumption that a 25g mouse would drink a minimum of 4mL of water per day. Because water consumption drops after induction of sepsis, an intraperitoneal

injection of acetaminophen (200mg/kg body weight) was given at 12 hours following sepsis initiation. Also at 12 hours post-sepsis induction, animals were weighed, administered intraperitoneal antibiotics (imipenem-cilastatin) and subcutaneous fluids (0.9% NaCl), and evaluated for illness severity by sepsis scoring (20). In this assessment, animals are scored from 0 (deceased) to 12 (normal) based on responsiveness, signs of encephalopathy, and physical appearance (20). Mice were immediately euthanized if a score of less than 5 was observed. Animals that did not develop signs of sepsis (i.e., the sepsis score was never <12) were excluded from all analyses.

Mice were euthanized at 24 hours via intraperitoneal injection of sodium pentobarbital (Sigma-Aldrich). Whole blood was collected by blind cardiac puncture with a 23G needle, transferred to EDTA-containing tubes (Becton Dickinson), and spun at 1,000 x g for 10 minutes at 4°C for plasma isolation. The left lung was then harvested, weighed, and dried at 70°C for 72 hours to determine the wet-to-dry weight ratio. The right lung was used to collect bronchoalveolar lavage fluid (BALF) with 0.6mL of sterile saline. Total immune cell counts were completed on a hemocytometer from 10 µL of BALF and then the remaining sample was centrifuged for 10 minutes at 4°C.

Supernatants were frozen at -80°C. BALF protein concentrations were quantified by BCA assay (23227, Thermo Fisher Scientific). For spleen bacterial counts, organs were dipped in 100% ethanol to eradicate adherent bacteria, homogenized, serially diluted in PBS, streaked onto Lysogeny broth agar plates, and incubated at 37°C. After 24 hours, colony-forming units (CFUs) were counted. All other organs were harvested and immediately flash frozen in liquid nitrogen. All samples were stored at -80°C until further

analysis.

In a subset of murine sepsis experiments, pulmonary endothelial cells were isolated from the bilateral lungs. Mouse lungs were perfused with PBS to remove blood and then placed in MACS® Tissue Storage Solution (130-100-008, Miltenyi Biotec). Lung tissue was dissociated using the Miltenyi Lung Dissociation Kit (130-095-927, Miltenyi Biotec) and gentleMACS™ tissue dissociator (130-093-235, Miltenyi Biotec) to create a single cell suspension, which was passed through a 70µm filter. Leukocytes were first depleted by incubation with anti-CD45 (130-052-301, Miltenyi Biotec) magnetic beads, then endothelial cells were selected for using anti-CD31 magnetic beads (130-097-418, Miltenyi Biotec), Miltenyi LS columns (130-042-401, Miltenyi Biotec), and MACS manual cell separator (130-042-302, Miltenyi Biotec). Cells were immediately processed for RNA and protein isolation.

Endothelial Glycocalyx Degradation

Murine plasma was analyzed to quantify endothelial glycocalyx degradation. Syndecan-1 levels were measured by ELISA (ab273165, Abcam). Total circulating GAGs were determined by dimethylmethylene blue (DMMB) assay (51). Samples were run undiluted for both assays.

Lung Inflammation

Pulmonary inflammation was assessed by transcript abundance of the proinflammatory cytokines TNF- α , IFN- γ , IL-6, and chemokine (C-X-C motif) ligand 1 (CXCL1) in the whole lung homogenate. RNA extraction from whole lungs, cDNA

synthesis, and qPCR were completed as above. Primer information is listed in **Supplemental Table 1**.

Kidney Injury

Kidney injury was evaluated by gene expression of the established biomarkers neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) (52). RNA extraction from whole kidneys, cDNA synthesis, and qPCR were completed as above. Primer information is listed in **Supplemental Table 2**.

Systemic Inflammation

The concentration of plasma TNF- α , IFN- γ , IL-6, and CXCL1 were quantified in duplicate with the V-PLEX Proinflammatory Panel 1 (K15048D, Meso Scale Discovery).

Statistical Analysis

Statistical analyses were performed in GraphPad Prism (10.4.1). The statistical test for each Figure is indicated in the corresponding Figure Legend.

Study Approval

The Vanderbilt University Medical Center (VUMC) Institutional Animal Care and Use Committee reviewed and approved all animal experiments (Protocol Number: M1600006-02; Nashville, TN).

Data Availability

Underlying data can be found in the associated supporting data values XLS file.

Author Contributions

AMB: Conceptualization, validation, formal analysis, investigation, writing – original draft, visualization, project administration, and funding acquisition. **AH, JL, NP, HNRL, DA, SKG, NW, KJR, JEM, and KO:** Investigation, writing – review and editing. **CMS, JAB, EPS, and LBW:** Conceptualization, resources, writing – review and editing, supervision, project administration, and funding acquisition.

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Figures

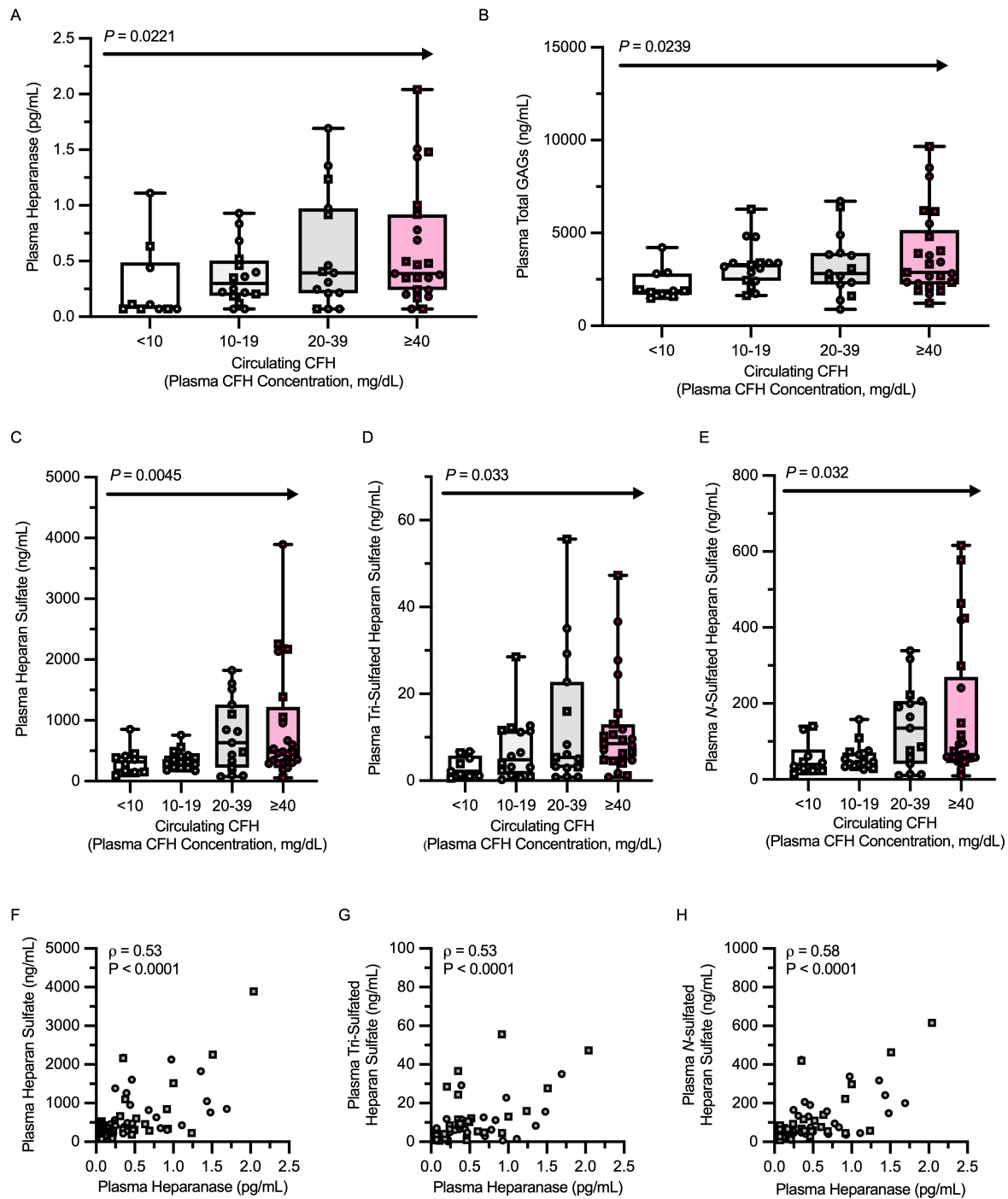


Figure 1. Elevated cell-free hemoglobin is associated with increased plasma heparanase levels and activity. (A) Patients with higher circulating CFH had

significantly greater plasma heparanase levels. **(B)** Increasing CFH concentrations corresponded to significantly more total circulating GAGs. **(C)** Elevated CFH levels correlated with significantly increased plasma heparan sulfate and **(D, E)** tri-sulfated and *N*-sulfated heparan sulfate fragments. *N* = 10 for CFH < 10 mg/dL, *N* = 16 for CFH 10-19 mg/dL, *N* = 15 for CFH 20-39 mg/dL, *N* = 23 for CFH ≥ 40 mg/dL. **(F-H)** There is a direct relationship between plasma heparanase levels and circulating heparan sulfate, tri-sulfated heparan sulfate, and *N*-sulfated heparan sulfate, all specifically produced by heparanase. Data in A-E are presented as box-and-whisker plots, with boxes representing the interquartile range and the horizontal line representing the median, whiskers extending from minimum to maximum values, and all individual data points shown. Males are represented as circular symbols and females are depicted as squares. Comparisons were made by Jonckheere-Terpstra test for trend (A-E) or Spearman's correlation (F-H). CFH, cell-free hemoglobin; GAGs, glycosaminoglycans.

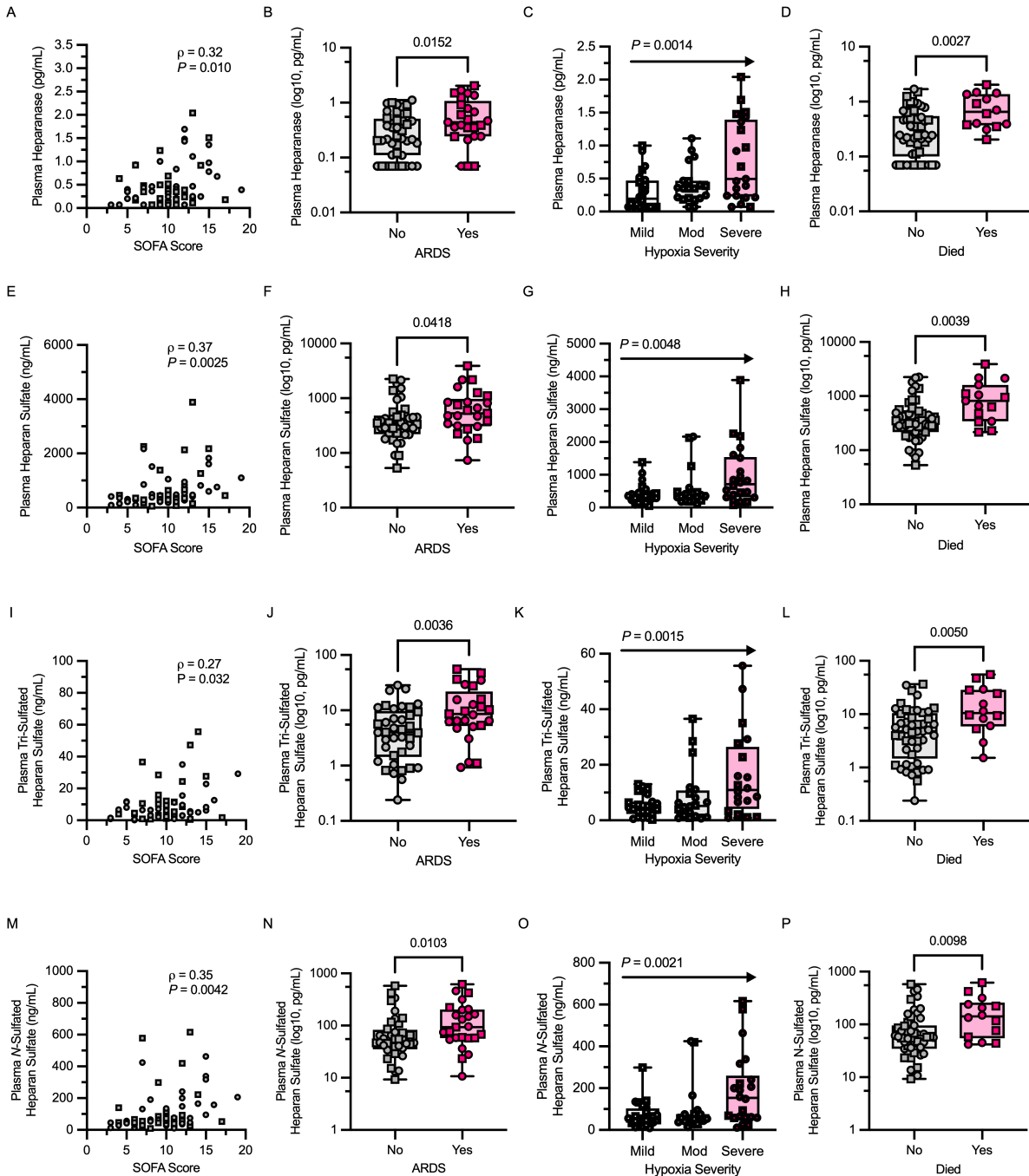
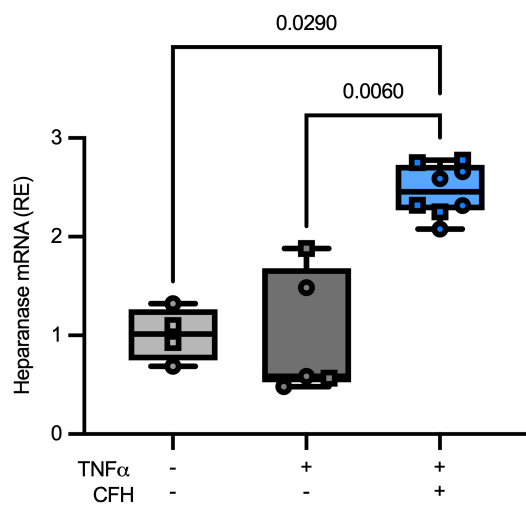


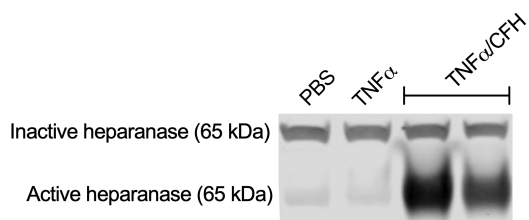
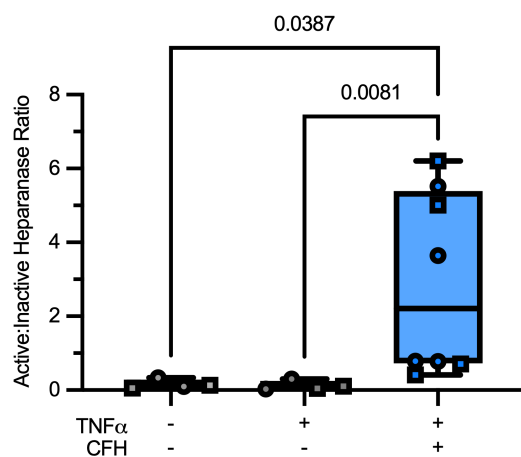
Figure 2. Heparanase-mediated endothelial glycocalyx degradation is associated with more severe illness and worse clinical outcomes in sepsis. SOFA scores significantly increased with higher plasma heparanase levels (A) and activity (E, I, M). $N = 65$. Circulating heparanase and heparanase-generated glycocalyx fragments are

significantly elevated in patients with ARDS (**B, F, J, N**). $N = 41$ without ARDS, $N = 26$ with ARDS. Hypoxia severity significantly increased in patients with greater plasma heparanase levels (**C**) and glycocalyx cleavage (**G, K, O**). $N = 24$ with mild hypoxia, $N = 20$ with moderate hypoxia, $N = 21$ with severe hypoxia. Among patients who died, heparanase levels and shed glycocalyx components were significantly increased (**D, H, L, P**). $N = 52$ survived, $N = 15$ died. Data in B-D, F-H, J-L, and N-P are presented as box-and-whisker plots, with boxes representing the interquartile range and the horizontal line representing the median, whiskers extending from minimum to maximum values, and all individual data points shown. Males are represented as circular symbols and females are depicted as squares. Comparisons were made by Spearman's rank correlation (A, E, I, M), Mann-Whitney test (B, D, F, H, J, L, N, P), or Jonckheere-Terpstra test for trend (C, G, K, O). SOFA, sequential organ failure assessment; ARDS, acute respiratory distress syndrome.

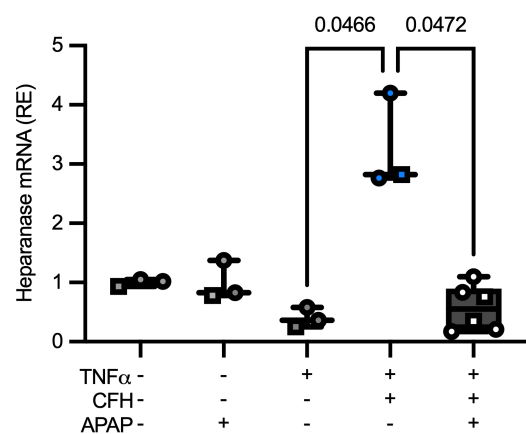
A



B



C



D

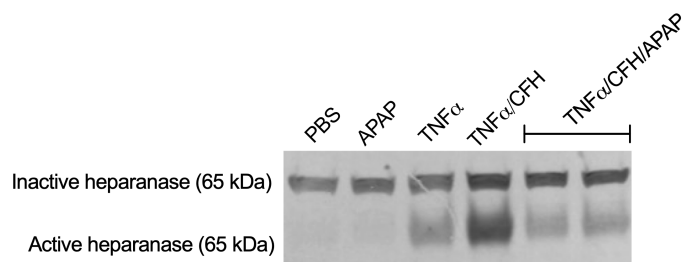
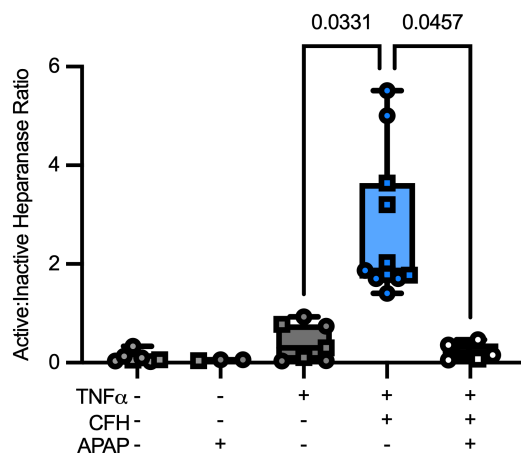


Figure 3. Cell-free hemoglobin redox cycling increases heparanase transcription and active enzyme synthesis during in vitro experimental sepsis. CFH significantly increases heparanase (A) transcription and (B) active enzyme levels during TNF α -induced experimental sepsis in primary human lung microvascular endothelial cells. Acetaminophen, a hemoprotein reductant, attenuates heparanase (C) expression and (D) active enzyme synthesis. $N = 3-10$ experimental replicates, with at least three unique donors per group. Timepoint: 24 hours. Data are presented as box-and-whisker plots, with boxes representing the interquartile range and the horizontal line representing the median, whiskers extending from minimum to maximum values, and all individual data points shown. Male donors are represented as circular symbols and female donors are depicted as squares. Comparisons were made by Kruskal-Wallis test. CFH, cell-free hemoglobin; TNF α , tumor necrosis factor-alpha; RE, relative expression; APAP, acetaminophen.

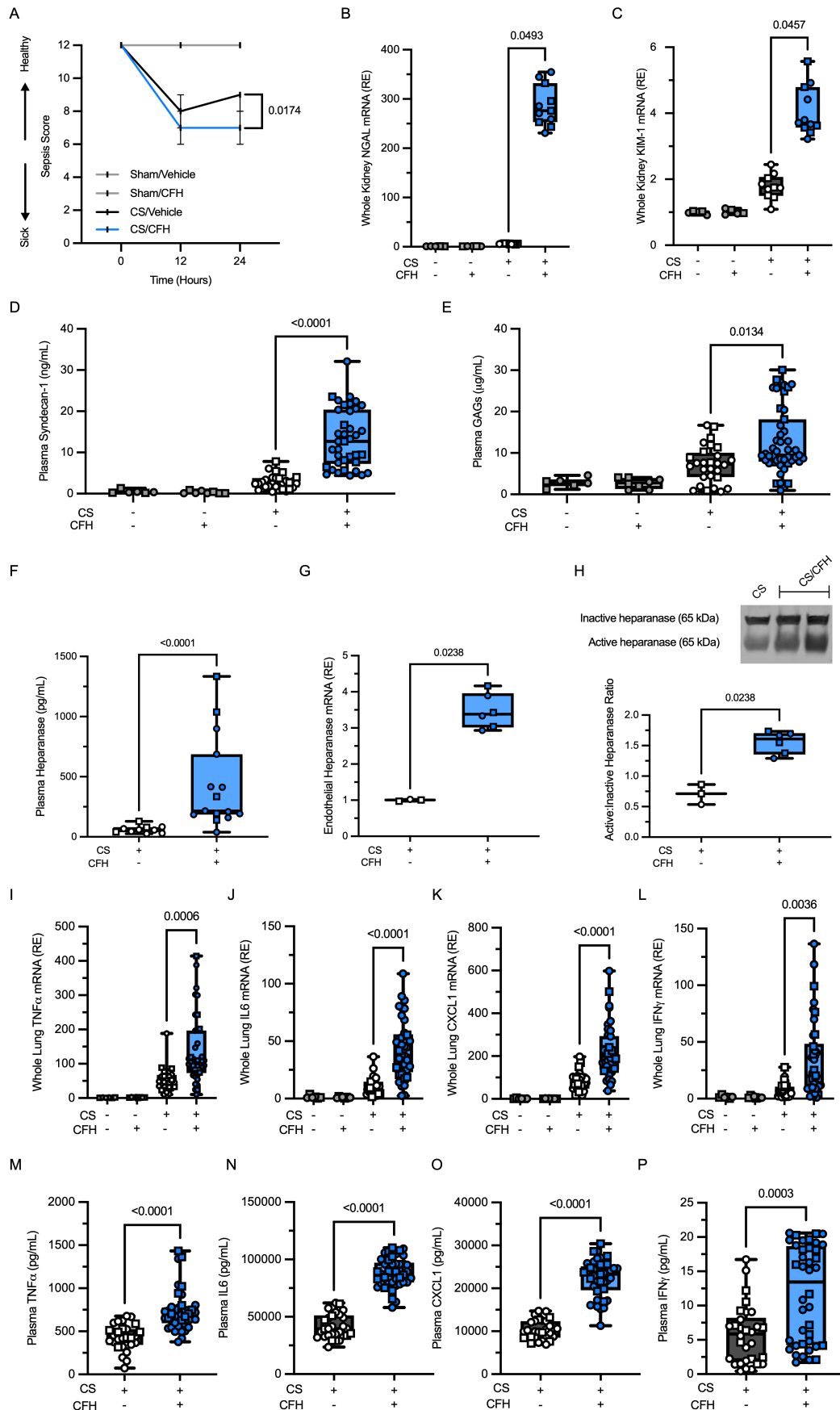


Figure 4. Cell-free hemoglobin exacerbates illness severity, renal damage, and glycocalyx shedding via heparanase upregulation during murine intraabdominal sepsis. (A) At 24 hours post-sepsis induction, wildtype septic mice treated with CFH had significantly lower sepsis scores compared to septic mice that were not exposed to excess CFH. The plots for the non-septic animals (i.e., Sham/Vehicle and Sham/CFH) are identical, as neither group demonstrated signs of illness. *N* = 3-51 per group. (B, C) Whole kidney expression of kidney injury biomarkers NGAL and KIM-1 were significantly higher in septic animals that received CFH. *N* = 3-11 per group. (D, E) Cell-free hemoglobin exposure resulted in significantly increased plasma syndecan-1 and GAG levels, both endothelial glycocalyx components. *N* = 6-47 per group. (F) Plasma heparanase levels were significantly elevated in CFH-treated mice. *N* = 10-15 per group. (G, H) Pulmonary endothelial cells isolated from septic animals given CFH had increased heparanase transcript abundance and active enzyme levels. *N* = 3-6 per group. (I-L) Whole lung expression of the proinflammatory cytokines TNF α , IL6, CXCL1, and IFN γ were significantly increased in septic wildtype mice that received CFH. *N* = 6-38 per group. (M-P) Circulating levels of these same cytokines were significantly higher in CFH-treated septic animals. *N* = 30-42 per group. Timepoint (B-P): 24 hours post-sepsis induction. Data in A are presented as median $\pm$ IQR. Data in B-P are presented as box-and-whisker plots, with boxes representing the interquartile range and the horizontal line representing the median, whiskers extending from minimum to maximum values, and all individual data points shown. Male mice are represented as circular symbols and female animals are depicted as squares. Comparisons were made by mixed-effects model with Tukey's post-hoc test for multiple comparisons at each

timepoint (A), Mann-Whitney test (F-H, M-P), or Kruskal-Wallis test (B-E, I-L). CS, cecal slurry; CFH, cell-free hemoglobin; NGAL, neutrophil gelatinase-associated lipocalin; KIM-1, kidney injury molecule-1; GAGs, glycosaminoglycans; RE, relative expression. $\text{TNF}\alpha$, tumor necrosis factor-alpha; IL6, interleukin-6; CXCL1, chemokine (C-X-C motif) ligand 1; $\text{IFN}\gamma$, interferon-gamma.

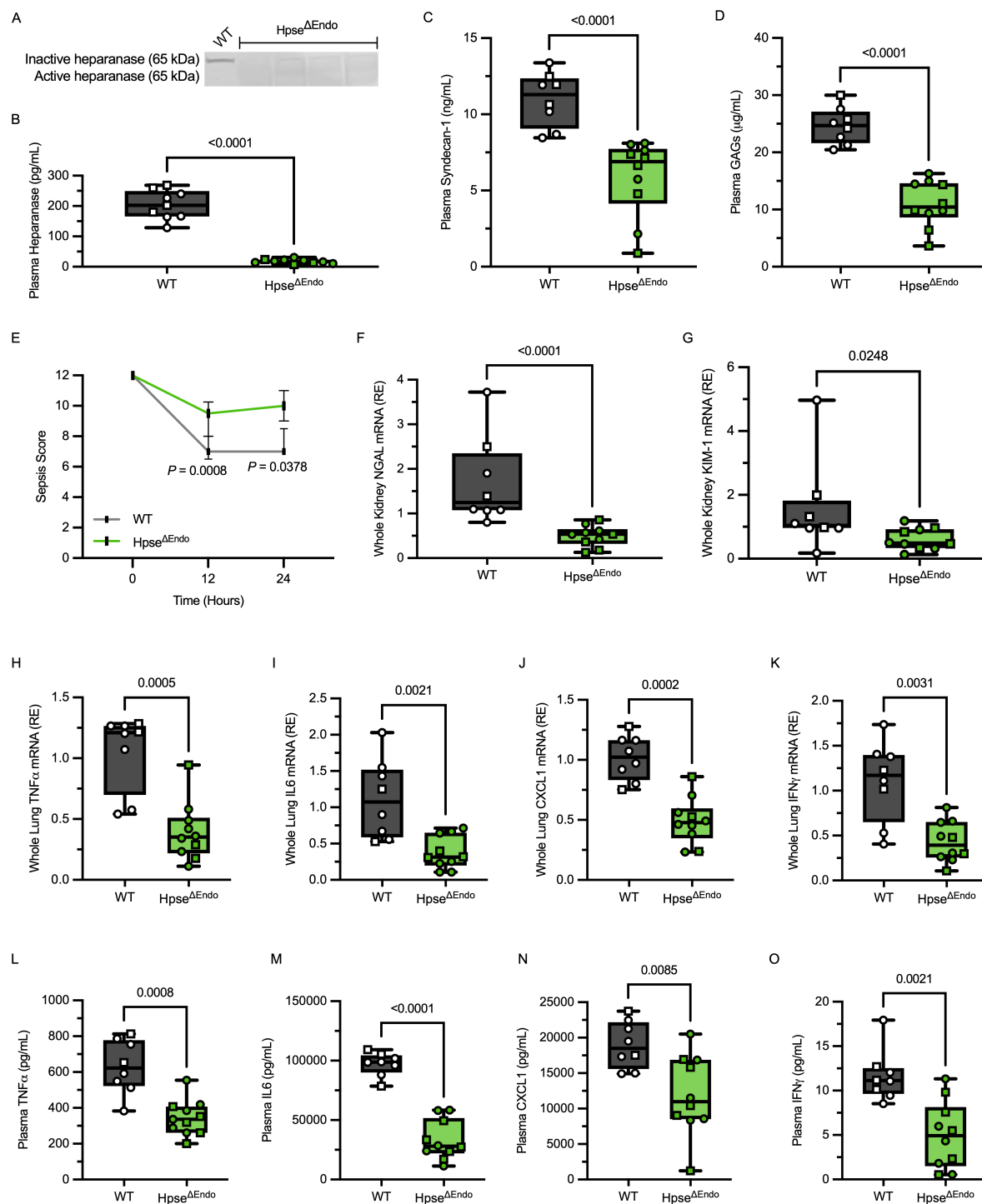


Figure 5. Heparanase mediates the adverse effects of cell-free hemoglobin.

Endothelial-specific heparanase knockout animals were generated using the CreLox system. Endothelial heparanase deletion was confirmed in healthy mice by (A) western

blot for heparanase in isolated pulmonary endothelial cells and **(B)** significantly reduced circulating levels of heparanase. **(C, D)** Plasma levels of the endothelial glyocalyx components syndecan-1 and GAGs were significantly decreased in septic Hpse^{ΔEndo} animals. **(E)** Septic mice that lack endothelial heparanase had significantly higher sickness scores at 12- and 24-hours post-sepsis induction. **(F, G)** Kidney injury markers NGAL and KIM-1 were significantly lower in septic Hpse^{ΔEndo} animals. **(H-K)** Endothelial-specific deletion of heparanase resulted in significantly reduced whole lung expression of the proinflammatory cytokines TNF α , IL6, CXCL1, and IFN γ in septic mice. **(L-O)** Circulating levels of these cytokines were significantly decreased as well. *N* = 8-10 per group. Timepoint (C, D, F-O): 24 hours post-sepsis induction. Data in B-D and F-O are presented as box-and-whisker plots, with boxes representing the interquartile range and the horizontal line representing the median, whiskers extending from minimum to maximum values, and all individual data points shown. Data in E are presented as median $\pm$ IQR. Male mice are represented as circular symbols and female animals are depicted as squares. Comparisons were made by mixed effects model with Tukey's post-hoc test for multiple comparisons at each timepoint (A) or Mann-Whitney test (B-D, F-O). WT, wildtype; Hpse^{ΔEndo}, endothelial-specific deletion of heparanase; GAGs, glycosaminoglycans; NGAL, neutrophil gelatinase-associated lipocalin; KIM-1, kidney injury molecule-1; TNF α , tumor necrosis factor-alpha; IL6, interleukin-6; CXCL1, chemokine (C-X-C motif) ligand 1; IFN γ , interferon-gamma.

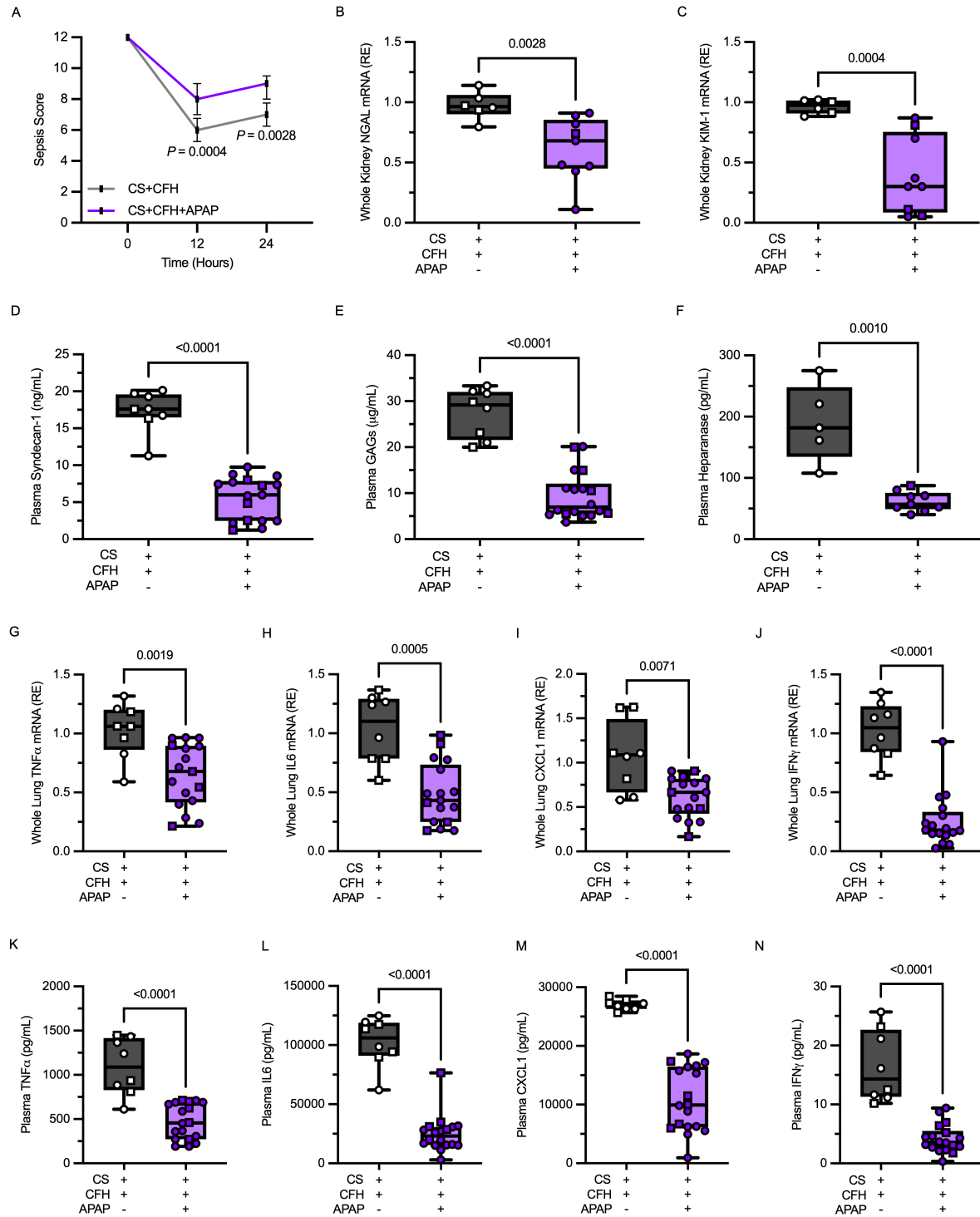


Figure 6. Acetaminophen, a hemoprotein reductant, attenuates the detrimental effects of cell-free hemoglobin. (A) Mice that received acetaminophen (APAP) had significantly higher sickness scores at 12- and 24-hours post-sepsis induction. (B, C)

Kidney injury markers NGAL and KIM-1 were significantly lower in APAP-treated animals. **(D, E)** Plasma levels of the endothelial glycocalyx components syndecan-1 and GAGs were significantly decreased in mice given APAP. **(F)** Circulating heparanase levels were significantly elevated in animals treated with APAP. **(G-J)** APAP significantly reduced whole lung expression of the proinflammatory cytokines $\text{TNF}\alpha$, IL6, CXCL1, and $\text{IFN}\gamma$. **(K-N)** Circulating levels of these cytokines were significantly decreased as well. *N* = 5-17 per group. Timepoint (B-N): 24 hours post-sepsis induction. Data in A are presented as median $\pm$ IQR. Data in B-N are presented as box-and-whisker plots, with boxes representing the interquartile range and the horizontal line representing the median, whiskers extending from minimum to maximum values, and all individual data points shown. Male mice are represented as circular symbols and female animals are depicted as squares. Comparisons were made by mixed effects model with Tukey's post-hoc test for multiple comparisons at each timepoint (A) or Mann-Whitney test (B-N). CS, cecal slurry; CFH, cell-free hemoglobin; APAP, acetaminophen; NGAL, neutrophil gelatinase-associated lipocalin; KIM-1, kidney injury molecule-1; GAGs, glycosaminoglycans; $\text{TNF}\alpha$, tumor necrosis factor-alpha; IL6, interleukin-6; CXCL1, chemokine (C-X-C motif) ligand 1; $\text{IFN}\gamma$, interferon-gamma.

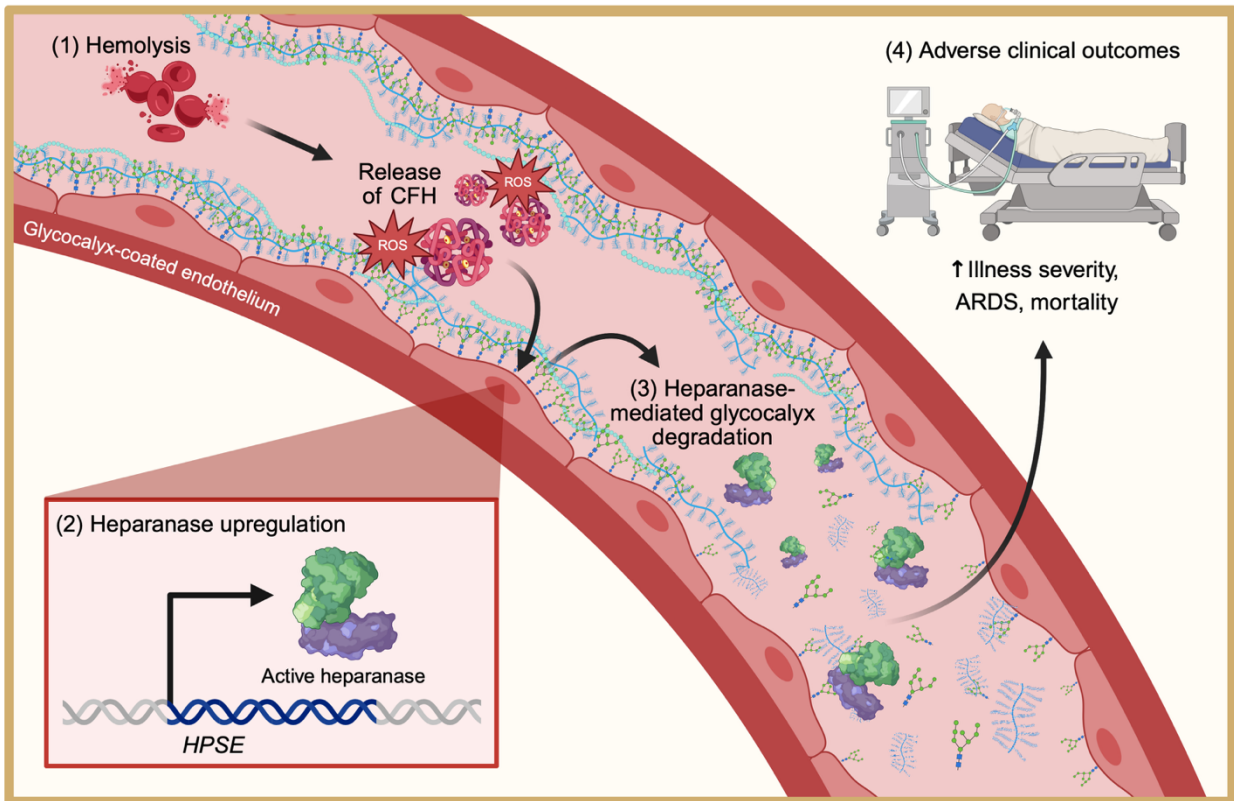


Figure 7. Proposed mechanism of cell-free hemoglobin-mediated injury to the endothelial glycocalyx during sepsis. (1) Increased red blood cell fragility results in hemolysis, releasing hemoglobin into the circulation (i.e., cell-free hemoglobin, CFH). (2) Redox cycling of CFH upregulates heparanase transcription and active enzyme synthesis in endothelial cells, leading to (3) endothelial glycocalyx degradation. (4) Loss of the endothelial glycocalyx results in more severe illness and adverse clinical outcomes including development of ARDS and death. Created in BioRender. Bogart, A. (2026) <https://BioRender.com/nt0pjni>.

Tables

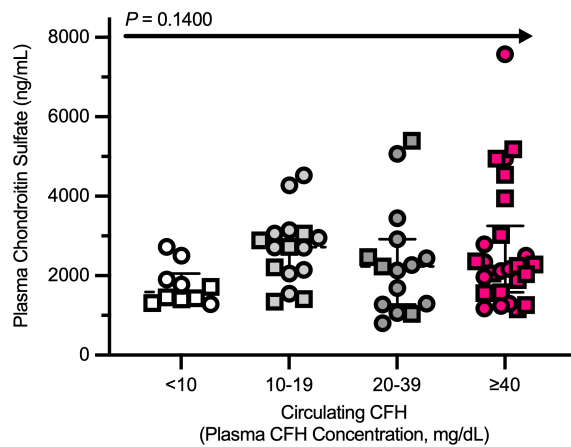
Characteristics	Total	Survivors	Non-Survivors	P-value
N	67	52	15	
Age ^A (years, median [IQR])	57.0 [44.5-62.0]	56.5 [46.8-62.0]	57.0 [36.0-67.0]	0.64
Sex ^B [F] (N, %)	32 (47.8)	25 (48.1)	7 (46.7)	>0.999
Race ^B [White] (N, %)	53 (94.0)	39 (75.0)	14 (93.3)	0.16
ARDS During Hospitalization ^B (N, %)	25 (37.3)	18 (34.6)	7 (46.7)	0.55
Lowest PaO ₂ :FiO ₂ During Admission ^A (mmHg, median, [IQR])	157.0 [92.0-222.7]	167.3 [93.1-268.7]	140.0 [66.0-194.9]	0.20
SOFA Score ^A (median [IQR])	10.0 [7.0-12.0]	9.5 [7.0-11.8]	12.0 [10.0-13.0]	0.055
ICU Free Days to day 28 ^A (days, median [IQR])	20.0 [9.0-23.0]	22.0 [19.0-24.0]	0.0 [0.0-0.0]	<0.0001
Mechanical Ventilation During Hospitalization ^B (N, %)	47 (70.1)	35 (67.3)	12 (80.0)	0.52

Table 1. Study population demographics and clinical features. Comparison of demographic and clinical data by hospital survivorship. Values are presented as

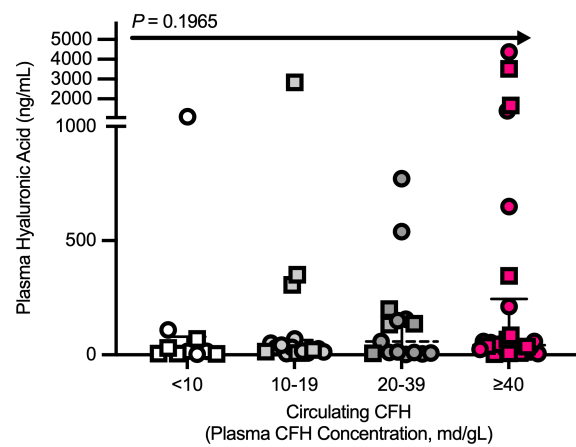
frequency (%) or median with the interquartile range. Comparisons were made by (A) Mann-Whitney test or (B) Chi-squared test. IQR, interquartile range; ARDS, acute respiratory distress syndrome; PaO₂, partial pressure of arterial oxygen; FiO₂, fraction of inspired oxygen; SOFA, sequential organ failure assessment; ICU, intensive care unit.

Supplemental Materials

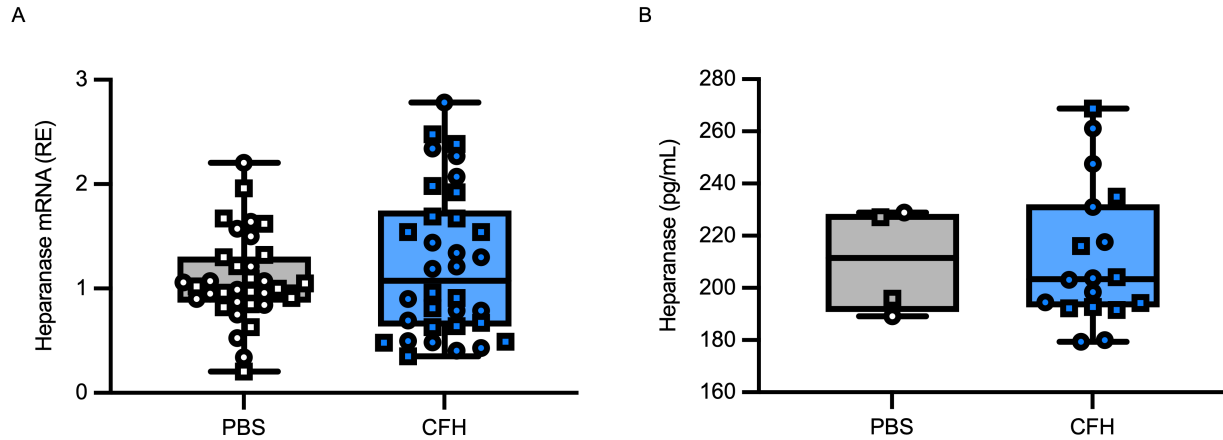
A



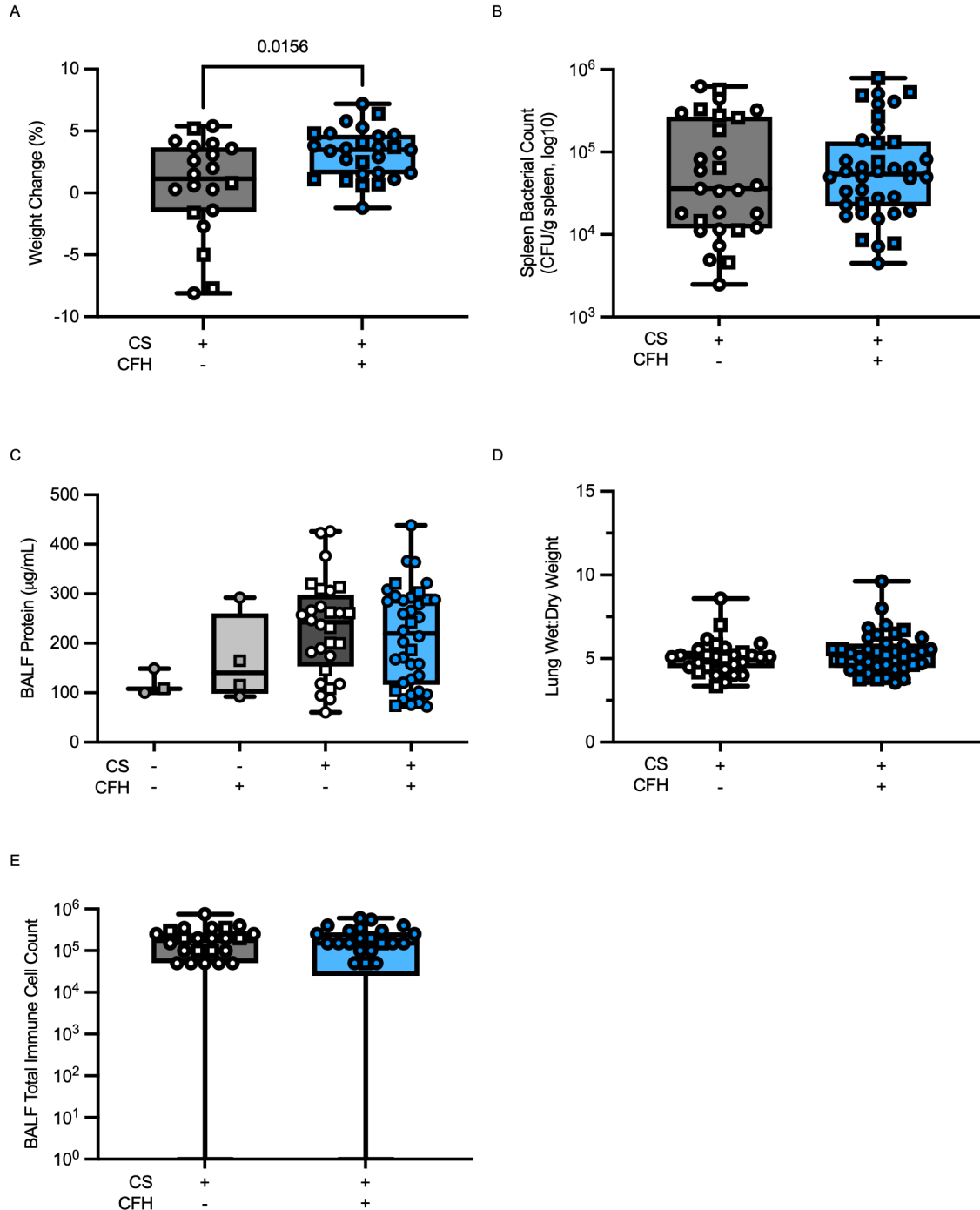
B



Supplemental Figure 1. There is no significant relationship between circulating CFH and plasma (A) chondroitin sulfate or (B) hyaluronic acid. $N = 10$ for CFH < 10 mg/dL, $N = 16$ for CFH 10-19 mg/dL, $N = 15$ for CFH 20-39 mg/dL, $N = 23$ for CFH ≥ 40 mg/dL. Data are presented as median $\pm$ IQR. Males are represented as circular symbols and females are depicted as squares. Comparisons were made by Jonckheere-Terpstra test for trend. CFH, cell-free hemoglobin.

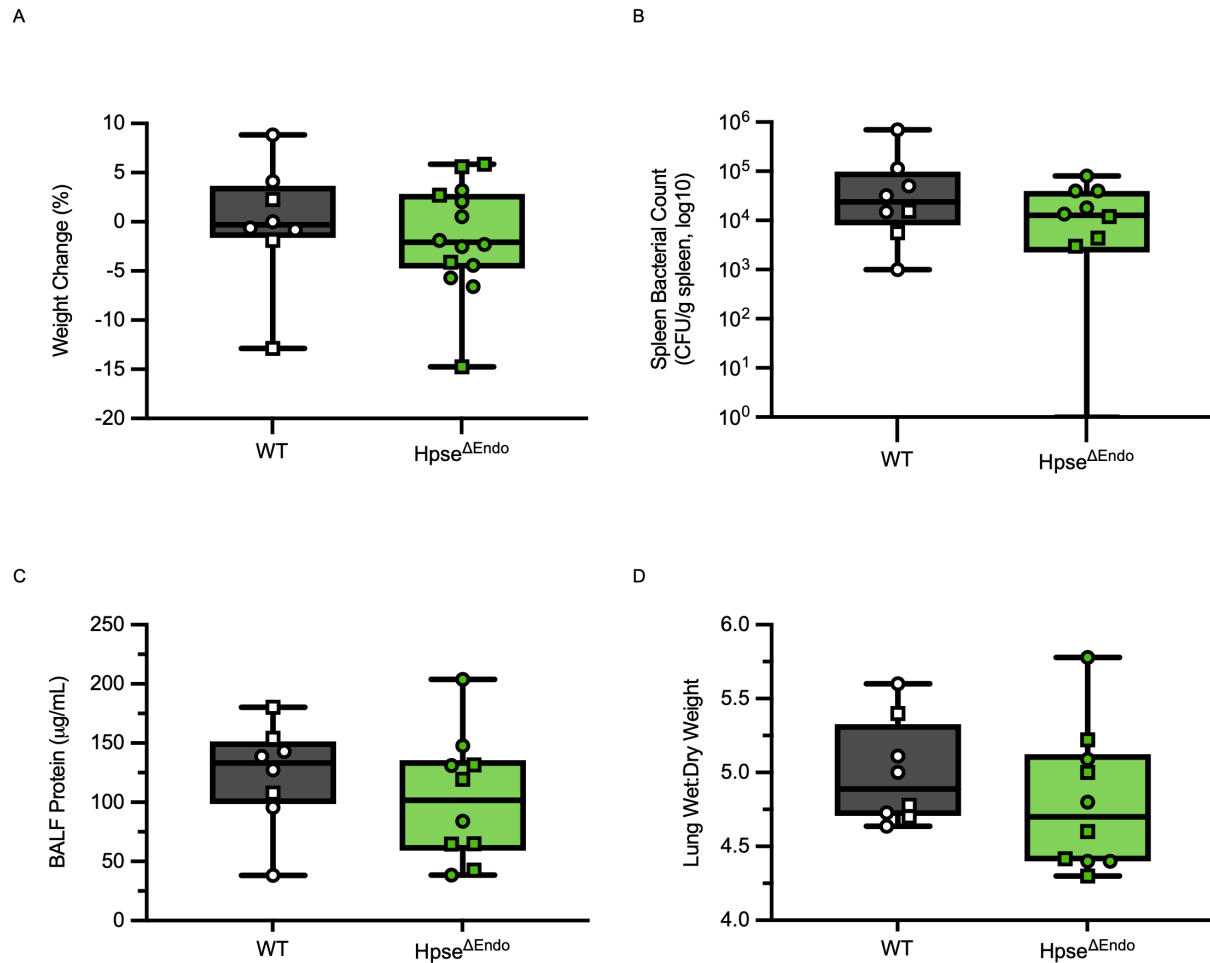


Supplemental Figure 2. Cell-free hemoglobin does not affect heparanase (**A**) transcription or (**B**) protein levels. $N = 4-18$ per group. Timepoint: 24 hours. Data are presented as box-and-whisker plots, with boxes representing the interquartile range and the horizontal line representing the median, whiskers extending from minimum to maximum values, and all individual data points shown. Male donors are represented as circular symbols and female donors are depicted as squares. Comparisons were made by Mann-Whitney test. CFH, cell-free hemoglobin; RE, relative expression.



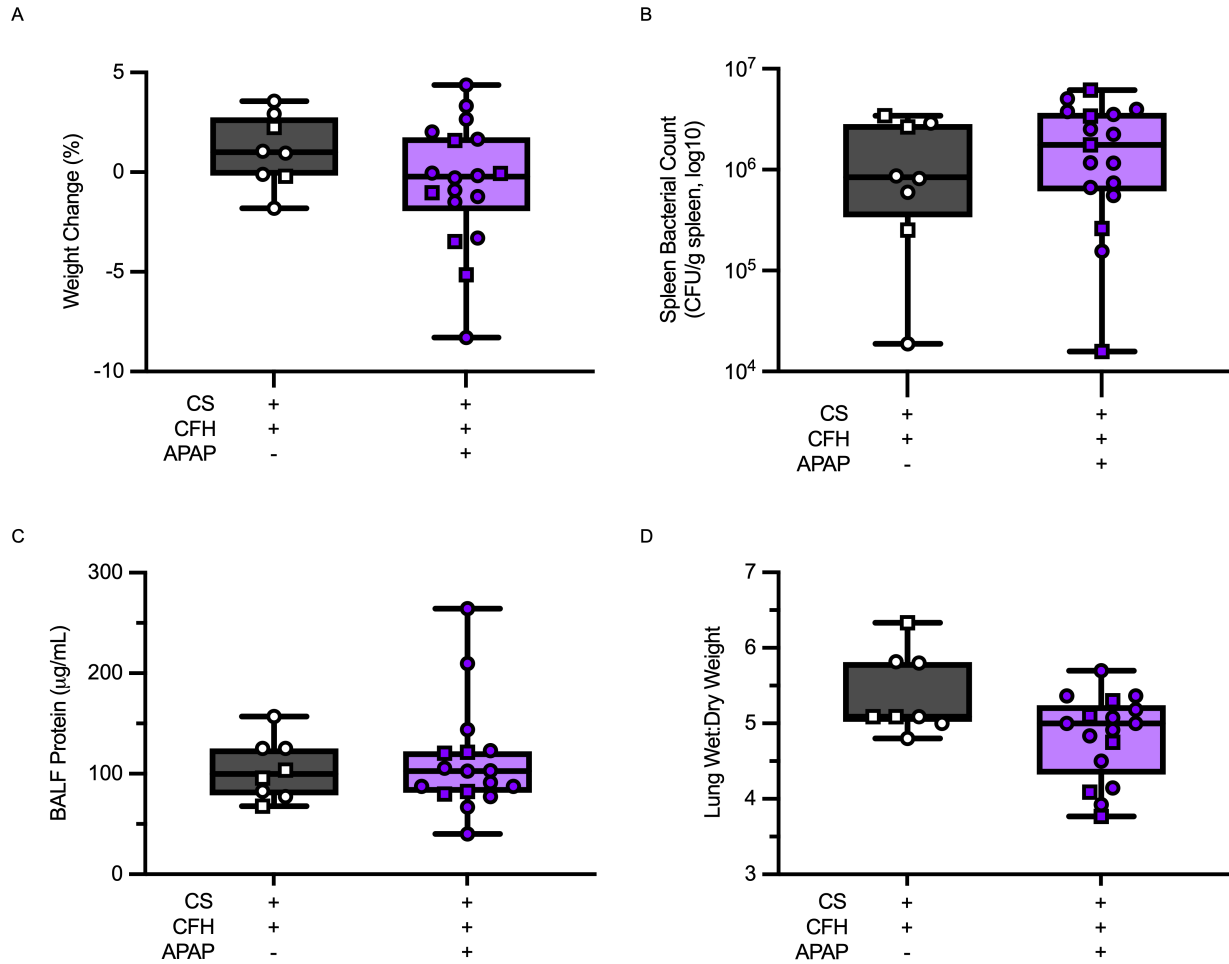
Supplemental Figure 3. (A) Septic mice treated with CFH gained significantly more weight over 24 hours. The addition of CFH to murine polymicrobial peritoneal sepsis does not impact (B) spleen bacterial counts, (C) BALF protein levels, (D) lung weight-to-dry weight ratios, or (E) BALF total immune cell counts. $N = 3-51$ per group. Timepoint:

24 hours post-sepsis induction. Data are presented as box-and-whisker plots, with boxes representing the interquartile range and the horizontal line representing the median, whiskers extending from minimum to maximum values, and all individual data points shown. Male mice are represented as circular symbols and female animals are depicted as squares. Comparisons were made by Mann-Whitney test (A-B, D-E) or Kruskal-Wallis test (C). CS, cecal slurry; CFH, cell-free hemoglobin; BALF, bronchoalveolar lavage fluid.



Supplemental Figure 4. There were no significant differences in **(A)** weight change over 24 hours, **(B)** spleen bacterial counts, **(C)** BALF protein levels, or **(D)** lung wet-to-dry weight ratios between $Hpse^{\Delta Endo}$ animals and their WT counterparts. $N = 8-10$ per group. Timepoint: 24 hours post-sepsis induction. Data are presented as box-and-whisker plots, with boxes representing the interquartile range and the horizontal line representing the median, whiskers extending from minimum to maximum values, and all individual data points shown. Male mice are represented as circular symbols and female animals are depicted as squares. Comparisons were made by Mann-Whitney test. WT, wildtype; $Hpse^{\Delta Endo}$, endothelial-specific deletion of heparanase; BALF, bronchoalveolar

lavage fluid.



Supplemental Figure 5. There were no significant differences in **(A)** weight change over 24 hours, **(B)** spleen bacterial counts, **(C)** BALF protein levels, or **(D)** lung wet-to-dry weight ratios between acetaminophen (APAP)-treated animals and their counterpart controls. $N = 7-17$ per group. Timepoint: 24 hours post-sepsis induction. Data are presented as box-and-whisker plots, with boxes representing the interquartile range and the horizontal line representing the median, whiskers extending from minimum to maximum values, and all individual data points shown. Male mice are represented as circular symbols and female animals are depicted as squares. Comparisons were made by Mann-Whitney test. CS, cecal slurry; CFH, cell-free hemoglobin; APAP,

acetaminophen; BALF, bronchoalveolar lavage fluid.

Target	Primer ID	Catalog Number	Manufacturer
GAPDH	Mm999999915	4331182	Thermo Fisher Scientific
TNF- α	Mm00443258	4331182	Thermo Fisher Scientific
IL-6	Mm00446190	4331182	Thermo Fisher Scientific
CXCL1	Mm04207460	4331182	Thermo Fisher Scientific
IFN- γ	Mm01168134	4331182	Thermo Fisher Scientific

Supplemental Table 1. Primer information for whole lung proinflammatory cytokine mRNA quantification. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF- α , tumor necrosis factor-alpha; IL-6, interleukin-6; CXCL1, chemokine (C-X-C motif) ligand 1; IFN- γ , interferon-gamma.

Target	Primer ID	Catalog Number	Manufacturer
GAPDH	Mm99999915	4331182	Thermo Fisher Scientific
NGAL	Mm01324470	4331182	Thermo Fisher Scientific
KIM-1	Mm00506686	4331182	Thermo Fisher Scientific

Supplemental Table 2. Primer information for whole lung proinflammatory cytokine mRNA quantification. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NGAL, neutrophil gelatinase-associated lipocalin; KIM-1, kidney injury molecule-1.