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The acute respiratory distress syndrome (ARDS) is an inflammatory lung disorder that frequently complicates critical illness, and most commonly occurs in the setting of sepsis. Although a number of clinical and environmental risk factors for ARDS have been described, not all patients with risk factors develop the syndrome, raising the possibility of genetic underpinnings for ARDS susceptibility. We have previously reported that circulating cell-free hemoglobin (CFH) is elevated during sepsis, and higher levels are associated with worse outcomes. CFH is rapidly scavenged by the plasma protein haptoglobin (Hp). A common *HP* genetic variant *HP2* is unique to humans and represents 60% of the *HP* allele frequency in populations of European ancestry. The *HP2* gene product has reduced ability to inhibit CFH-mediated inflammation and oxidative stress compared to the alternative *HP1*. We hypothesized that the *HP2* variant increases ARDS susceptibility during sepsis when plasma CFH levels are elevated. In a murine model of sepsis with elevated CFH levels, transgenic mice homozygous for *Hp2* had increased lung inflammation, pulmonary vascular permeability, lung apoptosis, and mortality compared to mice homozygous for the alternative allele *Hp1*. We then tested the clinical relevance of our findings in a prospective observational cohort study of 496 septic critically ill adults, and found that the *HP2* variant was significantly associated with increased ARDS susceptibility (odds ratio 1.41 per […]



# Haptoglobin-2 variant increases susceptibility to the acute respiratory distress syndrome during sepsis

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## Abstract

The acute respiratory distress syndrome (ARDS) is an inflammatory lung disorder that frequently complicates critical illness, and most commonly occurs in the setting of sepsis. Although a number of clinical and environmental risk factors for ARDS have been described, not all patients with risk factors develop the syndrome, raising the possibility of genetic underpinnings for ARDS susceptibility. We have previously reported that circulating cell-free hemoglobin (CFH) is elevated during sepsis, and higher levels are associated with worse outcomes. CFH is rapidly scavenged by the plasma protein haptoglobin (Hp). A common *HP* genetic variant *HP2* is unique to humans and represents 60% of the *HP* allele frequency in populations of European ancestry. The *HP2* gene product has reduced ability to inhibit CFH-mediated inflammation and oxidative stress compared to the alternative *HP1*. We hypothesized that the *HP2* variant increases ARDS susceptibility during sepsis when plasma CFH levels are elevated. In a murine model of sepsis with elevated CFH levels, transgenic mice homozygous for *Hp2* had increased lung inflammation, pulmonary vascular permeability, lung apoptosis, and mortality compared to mice homozygous for the alternative allele *Hp1*. We then tested the clinical relevance of our findings in a prospective observational cohort study of 496 septic critically ill adults, and found that the *HP2* variant was significantly associated with increased ARDS susceptibility (odds ratio 1.41 per *HP2* allele, 95% confidence interval 1.06 – 1.88, $P = 0.018$) after controlling for clinical risk factors and plasma CFH. This relationship between the *HP2* genetic variant and ARDS risk was only seen in patients with elevated plasma CFH levels. These observations identify the *HP2* variant as a novel genetic ARDS risk factor during sepsis, and may have important implications in the study and treatment of ARDS.

## Introduction

The acute respiratory distress syndrome (ARDS) is an acute inflammatory lung disorder that frequently complicates critical illness and affects over 200,000 adults per year in the United States (1–3). Despite decades of research, currently there are no disease-modifying treatments that reduce ARDS mortality (4–6). The majority of patients with ARDS clinical risk factors do not develop the syndrome, and there is substantial variability in disease severity among patients who do develop ARDS (4, 7). This suggests that additional factors including inheritable genetic factors may contribute to ARDS susceptibility (4, 8).

Sepsis, a clinical syndrome characterized by dysregulated inflammation and organ dysfunction in response to infection, is the most common cause of ARDS (1–3, 9, 10). Patients with sepsis have alterations in red blood cell (RBC) membrane stability resulting in hemolysis (11–14). Hemoglobin released from RBCs, termed cell-free hemoglobin (CFH), is detectable in the plasma of 80% of critically ill sepsis patients (15), and higher plasma CFH levels are associated with increased organ dysfunction and mortality during sepsis (15, 16). CFH causes organ injury via scavenging of nitric oxide in vascular beds (17), injury to the vascular endothelium (18), neutrophil activation (19), and oxidation of lipid cell membranes (20, 21). Therefore, elevated plasma CFH during sepsis may contribute to development of ARDS.

The plasma protein haptoglobin (Hp) serves as the primary endogenous scavenger for CFH in mammals (22). Hp binds irreversibly to CFH, and the resultant CFH-Hp complex binds with high affinity to the CD163 receptor present on monocytes and macrophages (23), resulting in

endocytosis and clearance of CFH from the circulation (24, 25). Humans have a unique *HP* genetic variant *HP2* (26), which comprises 45% of the *HP* allele frequencies in African-American and west African populations, 60% in European populations, and 75% in East and South Asian populations (27). Hp from subjects homozygous for the *HP2* variant (*HP2-2* genotype) has reduced ability to inhibit CFH-mediated inflammation and oxidative stress compared with Hp from subjects homozygous for the alternative allele *HP1* (*HP1-1* genotype) (25, 28, 29). The *HP2-2* genotype has been associated with increased risk of atherosclerotic coronary artery disease (30, 31), diabetic nephropathy (32, 33), and worse outcomes after subarachnoid hemorrhage (34, 35).

We hypothesized that the *HP2* variant increases susceptibility to ARDS in the setting of sepsis with elevated CFH, as Hp in patients with the *HP2* variant would be predicted to have reduced ability to mitigate CFH-mediated oxidative stress and inflammation. Utilizing transgenic mice with a murine homologue of human *HP2*, we determined the mechanistic effects of *HP2* on acute lung injury in an experimental model of polymicrobial sepsis, finding that *Hp2-2* mice experienced increased lung inflammation, pulmonary vascular endothelial injury, and mortality compared to wild-type *Hp1-1* mice. We then validated our observations in a prospective observational cohort study of septic critically ill adults, finding that the *HP2* variant was significantly and independently associated with increased susceptibility to ARDS in humans. These findings identify the *HP2* variant as a novel genetic risk factor for ARDS during sepsis.

## Results

### *Hp2-2* mice have decreased survival during experimental sepsis

We first tested the effect of *Hp* genotype on survival in a murine model of polymicrobial sepsis with elevated CFH levels. Following injection of intraperitoneal cecal slurry (CS) and intravenous CFH, *Hp2-2* mice had decreased survival compared with *Hp1-1* mice ($P = 0.03$ by log-rank test, **Figure 1, Panel A**). Although median plasma CFH levels were higher in *Hp2-2* mice compared with *Hp1-1* mice, these differences did not reach statistical significance ($P = 0.09$ by Mann-Whitney *U* test, **Figure 1, Panel B**).

### *Hp2-2* mice have increased lung inflammation during experimental sepsis

We next assessed the effect of *Hp* genotype on lung inflammation in the mouse polymicrobial sepsis model. *Hp2-2* mice had increased lung inflammation compared with *Hp1-1* mice in response to intraperitoneal CS and intravenous CFH, as evidenced by increased whole lung myeloperoxidase activity ($P = 0.014$, **Figure 2, Panel A**) and CXCL-1 mRNA expression ($P = 0.022$, **Figure 2, Panel B**). *Hp2-2* mice also had increased CXCL-1 levels in BAL fluid compared with *Hp1-1* mice ($P = 0.011$, **Figure 2, Panel C**).

### *Hp2-2* mice have increased pulmonary vascular permeability and lung apoptosis during experimental sepsis

We next tested the effects of *Hp2-2* genotype on the pulmonary vascular endothelium, hypothesizing that CFH may impair pulmonary vascular barrier function during sepsis. We tested microvascular barrier integrity by retro-orbital injection of AngioSense®, a 70-kDa near-infrared fluorescent macromolecule that accumulates in sites of increased vascular

permeability. *Hp2-2* mice had increased lung accumulation of AngioSense® compared with *Hp1-1* mice twenty-four hours after intraperitoneal CS and IV CFH ($P$ = 0.037, **Figure 3**). This finding indicates that *Hp2-2* mice have worsened pulmonary vascular permeability during sepsis compared with *Hp1-1* mice. Excised lungs from *Hp2-2* mice also had increased apoptosis by TUNEL staining compared with lungs from *Hp1-1* mice ($P$ = 0.004, **Figure 4**). We have previously determined that 80% of apoptotic lung cells in this CS + CFH model are endothelial using co-labeling in samples from wild type mice (Bastarache JA, et al., unpublished observations). Therefore, this suggests that lung endothelial apoptosis contributes to increased microvascular permeability in this model.

**Adult sepsis cohort haptoglobin genotyping**

To examine the clinical implications of the mechanistic findings from the mouse polymicrobial sepsis model, we tested the association between *HP* genotype and ARDS in a prospective observational cohort of critically ill adults hospitalized with sepsis. We determined *HP* genotype by two different methods depending on data and sample availability. For the 344 patients in whom DNA was available, we directly genotyped *HP* using real-time PCR. In an additional 152 patients, we used prior GWAS level genotyping to impute *HP* genotype using a previously reported algorithm (36). To verify the accuracy of the imputation method in our cohort, we determined genotype by both methods in 120 patients. The observed *HP* genotype distribution in the entire study cohort ($N$ = 496) was 15% *HP1-1*, 45% *HP2-1*, and 40% *HP2*-2 (**Supplemental Figure 1**). The observed *HP* genotype distribution was similar to expected allele frequencies for a majority-European ancestry cohort (27) ($P$ = 0.12 for difference from reference *HP2* allele frequency by two-sample binomial proportions test). The overall

agreement between PCR and imputation in the 120 patients genotyped by both methods was 0.91 (Cohen's Kappa 0.85, balanced accuracy 0.91, 95% CI [0.84, 0.95]) (**Supplemental Table 1, Supplemental Figure 2**).

### Adult sepsis cohort patient clinical characteristics

Patients with all three *HP* genotypes had similar baseline clinical characteristics including age, sex, reported ethnicity, comorbid medical conditions, APACHE II scores, mechanical ventilation on enrollment, and organ failures as measured by Brussels score (37) (**Table 1**). Plasma CFH levels measured on ICU day 2 were similar across all three genotypes, while plasma Hp levels were significantly decreased in *HP2-1* and *HP2-2* patients compared with *HP1-1* ($P$ = 0.0008 by Kruskal-Wallis test, **Table 1**, **Supplemental Figure 3**), consistent with prior epidemiological studies of serum Hp levels among different *HP* genotypes (38, 39).

### Elevated circulating cell-free hemoglobin increases risk of ARDS

During the first four ICU days, 181 (36.5%) patients developed ARDS. To test the hypothesis that *HP* genotype modulates the effect of CFH on ARDS susceptibility, we first tested the association between plasma CFH levels and ARDS susceptibility. We observed a dose-response relationship between plasma CFH and increased risk for ARDS ($P$ = 0.032 by Cochran-Armitage test for increasing ARDS risk by CFH quartile, **Figure 5**).

### Haptoglobin 2 variant increases ARDS risk in critically ill adults with sepsis

The distribution of ARDS cases among genotypes was *HP1-1* 28.9% ($N$ = 22), *HP2-1* 35.3% ($N$ = 79), and *HP2-2* 40.8% ($N$ = 80). In the unadjusted analysis, ARDS risk significantly

increased with increasing number of *HP2* alleles. Compared with *HP1-1* genotype, *HP2-1* had an odds ratio (OR) for ARDS of 1.33, and *HP2-2* had an OR for ARDS of 1.68, with $P = 0.029$ by the Cochran-Armitage test for increasing ARDS risk ordered by number of *HP* alleles (**Figure 6**). To test whether the relationship between *HP* genotype and ARDS was mediated by CFH, we assessed the impact of *HP* genotype in the presence and absence of detectable CFH. The association between *HP* genotype and ARDS susceptibility was present only in patients with detectable levels of plasma CFH ($P = 0.026$, $N = 414$), and not in patients with undetectable levels of plasma CFH ($P = 0.46$, $N = 82$) (**Figure 7**).

**Haptoglobin 2 variant is an independent ARDS risk factor**

To control for prespecified clinical confounders, we tested the association of *HP* genotype with ARDS using a multivariable logistic regression model. *HP* genotype remained independently associated with ARDS risk (OR = 1.41 per *HP2* allele, 95% CI [1.06, 1.88], $P = 0.018$) when controlling for age, sex, ethnicity, severity of illness, plasma CFH, and presence of chronic liver disease (as a surrogate for reduced hepatic Hp synthesis, **Table 2**, **Figure 8**). In a sensitivity analysis limited to the 344 patients in whom plasma was available for measurement of Hp levels, a statistically significant association between *HP* genotype and ARDS risk remained (**Supplemental Table 2**, **Supplemental Figure 4**). Additional subgroup analyses restricted to patients with detectable CFH levels ($N = 414$), Caucasian race ($N = 425$), absence of chronic liver disease ($N = 469$), and patients with severe sepsis ($N = 475$) also demonstrated a statistically significant association between *HP* genotype and ARDS risk (data not shown).

## Haptoglobin 2 variant and ventilator-free days

To assess if the *HP2* variant affected patient-centered outcomes, we tested the effect of *HP* genotype on ventilator-free days (VFDs), defined as the number of days alive and not receiving mechanical ventilation from ICU day 1 to ICU day 28 (40). Lower VFD values indicates more prolonged mechanical ventilation, indicating more severe respiratory failure (40). VFDs is a commonly reported outcome in critical care trials as it captures both duration of respiratory failure as well as mortality as a competing outcome (40). Although the number of VFDs decreased with each copy of *HP2*, with a mean (± SEM) of 15.7 (± 2.0) days for HP 1-1 patients; 14.0 (± 1.1) days for HP 2-1 patients; and 13.5 (± 1.2) days for HP 2-2 patients, these differences were not significant (P = 0.89 by Kruskal-Wallis H test, **Supplemental Figure 5**). We did not observe a difference in in-hospital mortality in ARDS patients between *HP* genotypes (**Supplemental Figure 6**), nor in 28-day survival (**Supplemental Figure 7**).

## Discussion

We have identified the *HP2* variant as a novel genetic risk factor for ARDS during sepsis. In a mouse polymicrobial sepsis model, *Hp2-2* mice exposed to experimental sepsis had increased lung inflammation, pulmonary vascular injury, and mortality compared with *Hp1-1* mice. In a cohort of septic critically ill adults, the *HP2* variant was independently associated with increased ARDS susceptibility. Moreover, the *HP2* variant was only associated with increased risk of ARDS in patients with elevated plasma CFH, supporting a mechanistic role for the CFH-Hp axis in ARDS pathogenesis during sepsis. These findings have significant clinical implications as the *HP2* variant is more common than *HP1* in many populations of European, African, South Asian, and East Asian ancestry (27), potentially affecting over 100,000 ARDS patients per year in the United States alone (1–3).

Endothelial injury with increased microvascular permeability is a defining pathogenic feature of sepsis that leads to shock, organ failure, and death both in human studies and animal models (41–48). Endothelial injury is also a key pathophysiologic characteristic of ARDS (49, 50). By comparing *Hp2-2* mice to *Hp1-1* mice during experimental sepsis, we found that the *HP2* variant was associated with increased lung microvascular injury and increased disruption of the alveolar-capillary barrier, in part due to increased apoptosis. The mechanistic impact of *HP2* on ARDS during sepsis may be explained by the reduced ability of the *HP2* gene product to limit the injurious effects of CFH.

The *HP2* variant is a partial copy number variant of *HP1* and contains two additional exons, one of which encodes a second multimerization domain (36, 51). In humans, Hp from

individuals with the *HP1-1* genotype circulates in plasma as a dimer, whereas Hp from individuals with *HP2-1* and *HP2-2* genotypes aggregates into progressively larger multimers (27, 28, 36, 52, 53). The larger Hp2-2 multimers have reduced ability to prevent CFH-mediated lipid peroxidation compared with Hp1-1 dimers, despite similar binding capacities for CFH (25, 29). CFH-Hp2-2 complexes are also cleared more slowly from the extracellular space by CD163 compared to CFH-Hp1-1 complexes (54), despite a greater binding affinity for the CD163 receptor (23, 54). Release of CFH into the circulation during hemolysis represents a significant source of oxidative stress due to the chemical reactivity of the heme iron moiety. Heme-complexed iron can be oxidized from the ferrous ($Fe^{2+}$) state to the more reactive ferric ($Fe^{3+}$) and ferryl ($Fe^{4+}$) states when outside of the reducing environment of the RBC cytoplasm (21). Therefore, because Hp2-2 may have less capacity to regulate CFH-mediated oxidative stress, experimental mice with *Hp2* may have increased susceptibility to end-organ injury when plasma CFH levels are elevated during sepsis (15, 55).

We tested the clinical relevance of our experimental findings in a large prospective observational cohort of critically ill septic adults. The *HP2* variant independently associated with increased ARDS susceptibility, with an odds ratio for ARDS of 1.41 per *HP2* allele after controlling for clinical factors and plasma CFH. Furthermore, *HP* genotype affected ARDS susceptibility only in the subgroup of patients with elevated plasma CFH. This finding extends our prior observation that higher CFH levels are associated with increased risk of organ dysfunction and death during sepsis (15). In addition, our findings support the hypothesis that *HP* genotype and the CFH-Hp axis have a mechanistic role in the pathogenesis of ARDS during sepsis, as observed in our experimental animal model studies. Other investigators have reported associations between *HP2-2* genotype and increased risk of many chronic

diseases such as artherosclerotic coronary artery disease (30, 31), type II diabetes (56), and diabetic nephropathy (32, 33). Our current study identifies a novel clinical effect of *HP2* affecting risk for a common acute clinical illness with high mortality.

The association between the *HP2* variant and increased risk of ARDS may be leveraged in several ways to inform ongoing clinical and experimental research in ARDS. Genotyping the *HP2* variant in patients with sepsis may be useful for risk-stratification, to identify specific subpopulations at increased risk for ARDS. It may also have use in clinical trial enrichment, as patients with the *HP2* variant may be more likely to benefit from pharmacological agents that target the CFH-Hp axis. Furthermore, these findings may affect studies using human Hp as a therapeutic agent. Human plasma-derived Hp is approved in Japan for the treatment of severe hemolysis during extracorporeal cardiopulmonary bypass, severe burn injuries, or massive transfusion following trauma (57–61), but this product is not approved in the United States. Additional experimental studies have reported that Hp reduces CFH-induced vasoconstriction, hemoglobinuria, and renal dysfunction following massive transfusion (18, 62, 63); cardiotoxicity following lipopolysaccharide and CFH administration (64); and organ dysfunction following experimental *Staphylococcus aureus* pneumonia with massive exchange transfusion (65). These experimental studies used either mixed pooled human Hp (18, 62) or specifically *HP2-1* and *HP2-2* human Hp (63–65), and the clinical trials used commercial pooled human Hp derived from populations with a high prevalence of *HP2-1* and *HP2-2* genotypes (27, 57–61). Our current study demonstrated differential effects of *HP2-2* and *HP1-1* genotypes during both experimental murine polymicrobial sepsis and in septic critically ill humans. Therefore future studies using human Hp as a novel therapeutic agent may need to consider the Hp phenotype in their study design to maximize clinical benefit.

Our study has several strengths. We used transgenic mice that expressed either the wild-type *Hp1* or an engineered murine *Hp2* variant homologous to the human *HP2* variant (66, 67). This novel murine *Hp2* variant has the same size and function as human *HP2 (66)*, thus making it a good model to study the functional effects of *HP* genotype. *Hp2-2* mice exhibit many of the same phenotypes as humans with *HP2-2* genotype including increased coronary atherosclerosis (66) and increased cerebral vasospasm after subarachnoid hemorrhage (68). Given the strong differences we have observed between *Hp2*-2 and *Hp1-1* mice in response to intraperitoneal sepsis, we propose that this model is ideal to study the molecular mechanisms of the CFH-Hp axis in sepsis. Our human cohort study draws from a large well-phenotyped cohort of critically ill adults hospitalized with sepsis. All clinical data were collected prospectively (69). The study was sufficiently sized to determine that the association between the *HP2* variant and ARDS risk was not simply due to higher severity of illness or other clinical confounders. Furthermore, the size of the cohort allowed us to test the association between the *HP2* variant and ARDS risk by subgroups of patients both with and without detectable plasma CFH, providing further support that CFH mediates the pulmonary effects of the *HP2* variant during sepsis.

Although our study advances understanding of the roles of Hp and plasma CFH in the pathogenesis of ARDS, there are some limitations. We do not yet fully understand the mechanisms through which CFH contributes to risk of ARDS. While the TUNEL staining suggests a role for CFH-induced apoptosis, this assay is nonspecific and can also indicate other forms of cell death such as necroptosis or necrosis (70). Our data suggesting endothelial apoptosis is supported by previous reports showing increased apoptosis of

pulmonary microvascular endothelial cells in other murine sepsis models as assessed by caspase activation or flow cytometry (48). We did not examine the role of CD163 in our experimental sepsis model. CD163 is the primary receptor for monocyte-mediated endocytosis of CFH-Hp complexes (23). Hp*2-2* has different binding affinity and clearance kinetics via CD163 compared with Hp1-1 (23, 54). Therefore, differential clearance of CFH-Hp complexes from the circulation may also contribute to the observed differences in our model between *Hp1-1* and *Hp2-2* mice. We also did not examine the role of nitric oxide (NO) in our experimental sepsis model. CFH consumes NO leading to peripheral vasoconstriction in patients with sickle cell anemia (71), and NO depletion is a common feature of both chronic pulmonary vascular disease (72) and red blood cell transfusion (17), a known etiology of ARDS (55, 73). Hp binding does not appear to attenuate CFH-mediated NO scavenging (62, 74), however the slower clearance kinetics of CFH-Hp2-2 complex (54, 74) could also affect the rate of NO depletion in the pulmonary vascular endothelium of *HP2-2* individuals during sepsis. The cellular mechanisms by which CFH causes disruption of the pulmonary vascular endothelial barrier will require further study. We identified sepsis using the “Sepsis-2” definition (75) as all study patients were enrolled prior to the publication of the more recent Third International Consensus definition of sepsis (76). Since we enrolled patients exclusively from ICUs, the overwhelming majority (*N* = 475, 95.7%) of sepsis patients in our cohort also had “severe sepsis” (defined as sepsis with concomitant organ dysfunction or hypoperfusion), which is analogous to the more recent “Sepsis-3” definition (76). We found similar results when limiting our analyses to patients with severe sepsis. Lastly, although we noted a high correlation between imputed *HP* genotype and PCR *HP* genotype in our human study without any evidence of systematic bias, we cannot exclude the possibility of some misclassification among imputed genotypes, particularly between *HP2-1* and *HP2-2*. Reassuringly, we did not

observe any misclassification in patients predicted to have *HP1-1* by imputation, although the sample size of this group was relatively small.

In summary, we have demonstrated for the first time that the *HP2* variant represents a novel genetic risk factor for ARDS susceptibility during sepsis. In an experimental polymicrobial sepsis model, *Hp2-2* mice had increased lung inflammation, pulmonary microvascular permeability, lung apoptosis, and death. In critically ill patients with sepsis, each additional *HP2* allele was associated with an increased risk of ARDS, independent of potential confounders. These findings have important clinical implications as *HP2* is the more common variant in many human populations. This study identifies a large clinical sub-population of sepsis patients who are genetically predisposed to develop ARDS, and has important implications for further research into the role of the CFH-Hp axis during critical illness.

## Methods

### Transgenic murine model of polymicrobial sepsis

Transgenic mice with murine homologues of human *Hp1-1* and *Hp2-2* were a kind gift from Dr. Rafael Tamargo of the Johns Hopkins University School of Medicine (67). We used a previously reported murine model of polymicrobial sepsis (77, 78). We prepared a cecal slurry (CS) from six-week-old female C57BL/6 donor mice purchased from The Jackson Laboratory (Bar Harbor, ME). In brief, cecal contents were collected from euthanized donor mice, resuspended in 5% dextrose at 80 mg/mL, vortexed for fifteen seconds, and filtered through a 25 gauge needle. We administered an intraperitoneal injection CS at 2.0 mg / gram body weight (BW) and a retro-orbital injection of CFH at 0.15 mg / gram BW to recipient 8-12-week-old male and female transgenic mice as previously reported (79). The CFH injection was included to increase plasma CFH levels to levels observed in human sepsis (79). We monitored the study mice for 72 hours during survival studies. Mice were monitored closely for signs of pain following induction of experimental sepsis. Antibiotics were not administered in this model of sepsis to allow robust bacterial growth and dissemination, which better reflects the natural history of sepsis in patients before seeking medical care. For all other studies, we euthanized the study mice with pentobarbital at four or twenty-four hours after CS administration for collection of samples. The Vanderbilt Institutional Animal Care and Use Committee approved all animal experiments.

### Mouse sample collection

Blood was collected by retro-orbital puncture in heparinized syringes and centrifuged at 2,000x g for ten minutes. Bronchoalveolar lavage fluid was collected as previously described

(80), as were excised whole lungs which were immediately flash frozen in liquid nitrogen. We stored all samples at -80 ˚C until time of analysis.

**Plasma circulating cell-free hemoglobin in mice**

Plasma CFH was measured at twenty-four hours in mouse plasma using the HemoCue® Plasma/Low Hb System (HemoCue America, Brea, CA).

**Lung inflammation biomarkers in mice**

We measured chemokine ligand 1 (CXCL1/KC) in duplicate using an electrochemiluminescence assay (MesoScale Discovery, Gaithersburg, MD) in BAL samples according to the manufacturer's recommendations. For mRNA expression, we extracted mRNA from flash frozen whole lungs using Qiagen RNAeasy Plus Mini Kit (Hilden, Germany). We generated cDNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA), and quantified CXCL1 mRNA expression level by quantitative PCR, normalized to GAPDH expression using TaqMan® primer probes (Thermo Fisher Scientific, Waltham, MA).

**Myeloperoxidase activity**

Frozen lungs were homogenized frozen in 50 mM potassium phosphate (pH 6.0), 0.5% hexadecyltrimethylammonium bromide, and 5mM EDTA, then sonicated, centrifuged, and diluted at 1:30 dilution in 100 mM potassium phosphate (pH 6.0), 0.3% hydrogen peroxide (Sigma-Aldrich, St Louis, MO), and 1 mg/ml *o*-dianisidine (Sigma-Aldrich). We recorded absorbance at 460 nm using a spectrophotometer at one and three minutes, and calculated MPO activity according to the equation (81):

$$\text{Activity} = [A_{460\ nm,\ 1\ min} - A_{460\ nm,\ 3\ min}] \times 13.5 / \text{weight}_{lung}$$

**Lung apoptosis**

At the time of organ harvest, we perfused lungs with 10% formalin. Cell apoptosis was ascertained using the Fluorescein In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) on paraffin fixed sections of mouse lung following deparaffinization and antigen retrieval. A trained reviewer who was blinded to each sample's *Hp* genotype examined lung histology slides and identified the number of cells undergoing apoptosis per low power field. Using endothelial co-labeling in samples from wild type mice, we have previously determined that 80% of apoptotic lung cells in this model are endothelial (Bastarache JA, et al., unpublished observations).

**Pulmonary vascular permeability by AngioSense assay**

In selected experiments, we administered 100 µl AngioSense® 750EX near-infrared fluorescent imaging agent (2 nmol/ 100 µl) to each study mouse via retro-orbital injection. We measured extravascular accumulation of the imaging agent of excised lungs at twenty-four hours using a LI-COR Pearl® small-animal imaging camera (LI-COR Biosciences, Lincoln, NE).

**Human study population and clinical data collection**

We studied patients ≥ 18 years of age enrolled in the Validating Acute Lung Injury markers for Diagnosis (VALID) study, a prospective observational cohort study of critically ill patients at high risk for ARDS and other acute organ dysfunction (69). We included patients with sepsis

on admission to the ICU as defined by the ACCP/SCCM Consensus (“Sepsis-2”) Criteria (75) with plasma samples available for CFH measurement and DNA samples available for genotyping. Sepsis was defined using the Sepsis-2 criteria because all patients included in the study were enrolled prior to the publication of the Third International Consensus definition of sepsis (76). Study personnel collected clinical data including medications, vital signs, laboratory studies, severity of illness scores, organ failures by the Brussels criteria (37), and chest radiographs for ARDS phenotyping (82) for the 24 hours prior to enrollment and daily for the first four days after enrollment.

**Human samples collection and assays**

Study personnel collected blood samples on the morning of ICU day 2 at the time of enrollment, and preferentially drew blood through a central venous catheter to minimize hemolysis. We measured CFH (HemoCue® Plasma/Low Hb System, HemoCue America, Brea, CA) and haptoglobin (Abcam®) in plasma as previously described (15). There was insufficient plasma available to measure haptoglobin levels in 152 patients. We have previously reported some of the hemoglobin and haptoglobin levels (15, 83). We extracted genomic DNA from buffy coat peripheral blood leukocytes using Gentra PureGene blood kit (Qiagen) according to manufacturer protocols and stored at -80C until genotyping.

**Haptoglobin genotyping**

We genotyped *HP* by measuring the ratio of HP5´ (a region common to both the *HP1* and *HP2* variants) to HP2 (a region specific to *HP2* variant) via TaqMan® based real time polymerase chain reaction (RT-PCR) as previously described (84). We calculated the change

in threshold cycle (ΔCt) for each sample as $Ct_{HP5´}$ - $Ct_{HP2}$, and calculated the HP2/HP5´ ratio of each sample as $2^{-\Delta Ct\ sample}$. We defined *HP1-1* by a HP2/HP5´ ratio of zero, *HP2-1* by a ratio between 0 and 0.60, and *HP2-2* by a ratio above 0.60. We confirmed the accuracy of our PCR genotyping method in a subset of samples with gel electrophoresis to determine haptoglobin phenotype (85) using study personnel blinded to the PCR genotyping results.

**Haptoglobin genotype imputation**

In 152 Caucasian patients, we imputed *HP* genotype from genome-wide microarray data. Genotyping was performed at the W.M. Keck facility at Yale University as part of previously reported genome-wide association study of acute kidney injury (86) using the Illumina HumanOmni1 Quad v1.0 BeadChip. We used the genotyping v1.9.4 module clustering algorithm from Illumina GenomeStudio™ software for SNP calling (Illumina, San Diego, CA). Patients with sample genotype call rate under 97% and patients with a discrepancy between X-chromosome zygosity and reported sex were excluded (86). We extracted SNPs within a 2 megabase region surrounding the *HP* gene (Hg19 chr16:71,036,975-73,063,764), then phased the target region and imputed *HP* genotype using the Beagle version 3.3.2 algorithm (87) with a phased reference panel provided by Boettger et al. (36). The reference panel consisted of 274 unrelated individuals of European ancestry from the 1000 Genomes Project (88) and HapMap3 project (89) who underwent genotyping on several GWAS platforms as well as droplet-digital PCR *HP* genotyping (36). We used the default parameters for Beagle with fifty iterations of the phasing algorithm and twenty-five haplotypes sampled for each individual during each iteration. We used the calculated genotype posterior-probabilities for number of *HP2* alleles as the surrogate for *HP* genotype, with a *HP2* genotype probability of

0.0 to 0.5 corresponding to a predicted *HP* genotype of *HP1-1*, a genotype probability of 0.5 to 1.5 corresponding to *HP2-1*, and a genotype probability of 1.5 to 2.0 corresponding to *HP2-2*.

**Statistical analysis for experimental mouse studies**

For the survival study using the experimental sepsis model, we estimated the survival function using the method of Kaplan and Meier and assessed differences in survival using the Mantel-Cox log rank test. For all biomarker studies, data are presented as the median ± IQR. We used the Mann-Whitney-U test for comparisons of continuous variables between *Hp1-1* and *Hp2-2* mice.

**Statistical analysis for human studies**

The primary outcome of the human study was prevalence of ARDS defined by the Berlin Criteria (82) on at least one of the first four ICU study days. We performed a secondary analysis focused on patients with detectable plasma CFH levels defined as ≥ 10 mg/dL to test the hypothesis that the effect of *HP* genotype is magnified when plasma CFH levels are elevated. Differences in genotype distributions were tested using the Binomial proportion test for observed *HP2* allele frequencies. We used the Cochran-Armitage Test for trend to test categorical outcomes versus CFH quartiles and *HP* genotype, using the alternative hypothesis that risk increases with each *HP2* allele. Differences in continuous outcomes between *HP* genotypes was tested using one-way ANOVA for variables with normal distributions and the Kruskall-Wallis *H* test for variables with non-normal distributions. We also tested the association between *HP* genotype and ARDS using multivariable logistic regression

to control for potential clinical and biochemical confounders including age, sex, ethnicity, severity of illness (by APACHE II score) (90), plasma CFH levels, and presence of chronic liver disease as a surrogate for hepatic haptoglobin synthetic function. We calculated number of ventilator-free days (VFDs) during the first 28 days using an accepted definition (40): 0 if the patient died during the first 28 days following enrollment in the study, or 28 – *x* if the patient was successfully weaned from mechanical ventilation, where *x* was the number of days receiving mechanical ventilation after enrollment in the study. Categorical outcomes are presented as percentage (number with outcome). Continuous outcomes are presented as mean ± SEM for outcomes with normal distributions and for VFDs (40), and median ± IQR for all other non-normally distributed outcomes. A *P* value of less than 0.05 was considered significant.

To assess the accuracy of imputation-based *HP* genotyping, we compared imputed *HP* genotype with PCR-determined *HP* genotype as the gold-standard in 120 patients with data available for both methods. We constructed a 3x3 confusion matrix and calculated sensitivity, specificity, and F-statistics for all three possible imputed genotypes. We also calculated overall accuracy and unweighted Cohen's kappa correlation statistic. We used R version 3.5.1 (91) using the packages DescTools (92) and rms (93) for statistical testing and RStudio version 1.0.147 (94) with the package ggplot2 (95) for data visualization.

**Study Approval**

The Vanderbilt University Medical Center Institutional Animal Care and Use Committee (Nashville, TN) reviewed and approved all animal study protocols. The Vanderbilt University

Medical Center Institutional Review Board (Nashville, TN) reviewed and approved the VALID study protocol (IRB #051065). Study personnel obtained informed consent from the patient or the patient's surrogate decision maker whenever possible. The IRB approved a waiver of consent when the patient could not give consent due to severity of medical illness and no surrogate decision-maker was available.

## Author Contributions

VEK, JAB, CMS, and LBW designed the study, provided data analysis and figure generation, performed statistical analyses, and wrote / edited the manuscript. VEK designed and implemented computer scripts for haptoglobin imputation. HN, JBN, SRL, NDP, WKU, JJ, NEW, TNS, and DRJ performed relevant experiments. LBW, CRP, and EDS provided genetic microarray data and performed quality control. All authors reviewed and approved the final version of the manuscript.

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## Figures

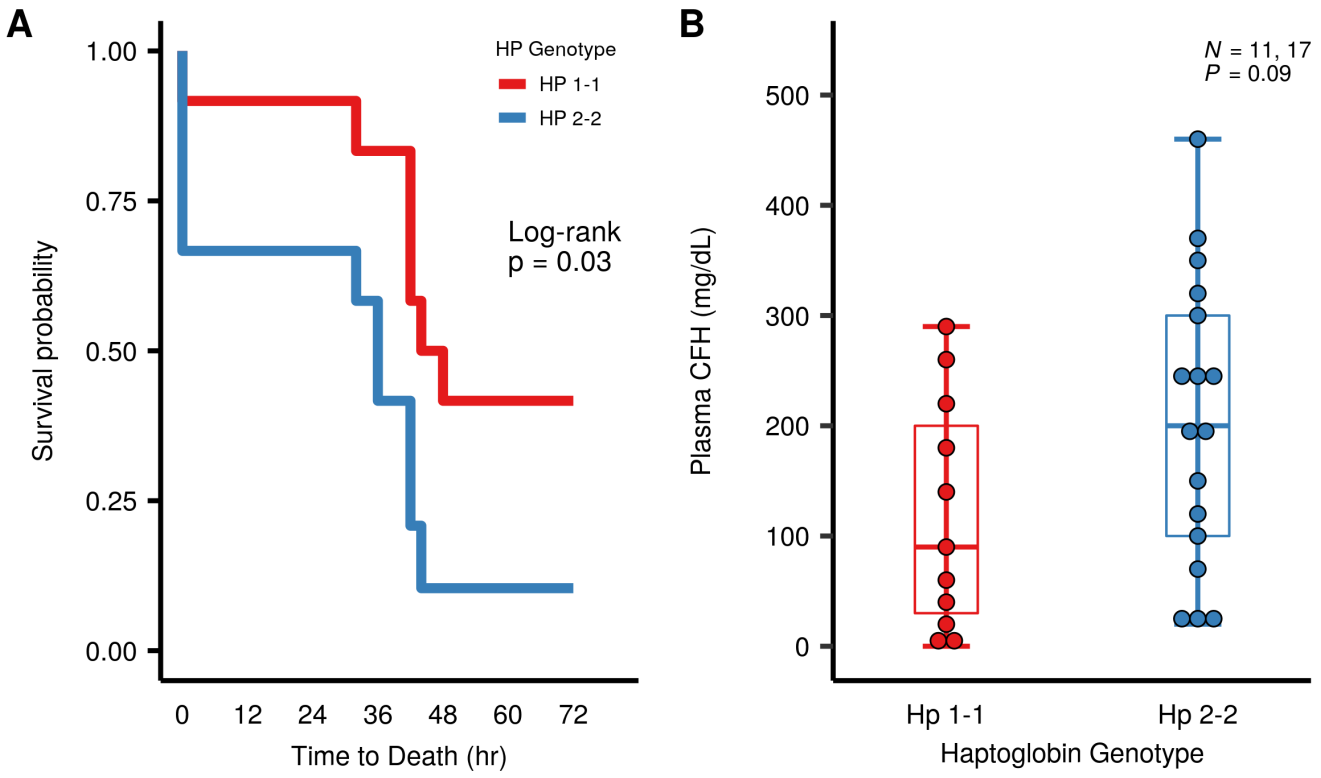



**Figure 1. *Hp2-2* mice have decreased survival over 72 hours following experimental sepsis**

*Hp1-1* (red) and *Hp2-2* (blue) mice (*n* = 12 each group) were treated with intraperitoneal injection of 2.0 mg cecal slurry per gram body weight and intravenous injection of 100 µl of 0.15 mg/mL CFH, then monitored for survival over 72 hours. (**A**) Survival curves showing survival was significantly worse in *Hp2-2* mice. **P* = 0.03 by the Mantel-Cox log rank test. (**B**) Plasma CFH levels were measured at 24 hours in *Hp1-1* (*n* = 11) and *Hp2-2* (*n* = 17) mice treated with CS and IV CFH. Dots represent individual values. In the box plots, the thick horizontal bars represent the median, boxes represent the interquartile range (IQR, 25$^{th}$ and

548 75$^{th}$ percentiles), and whiskers represent the minimum and maximum values within 1.5 * IQR

549 from the 25$^{th}$ and 75$^{th}$ percentiles. *P* = 0.09 by Mann-Whitney *U* test.

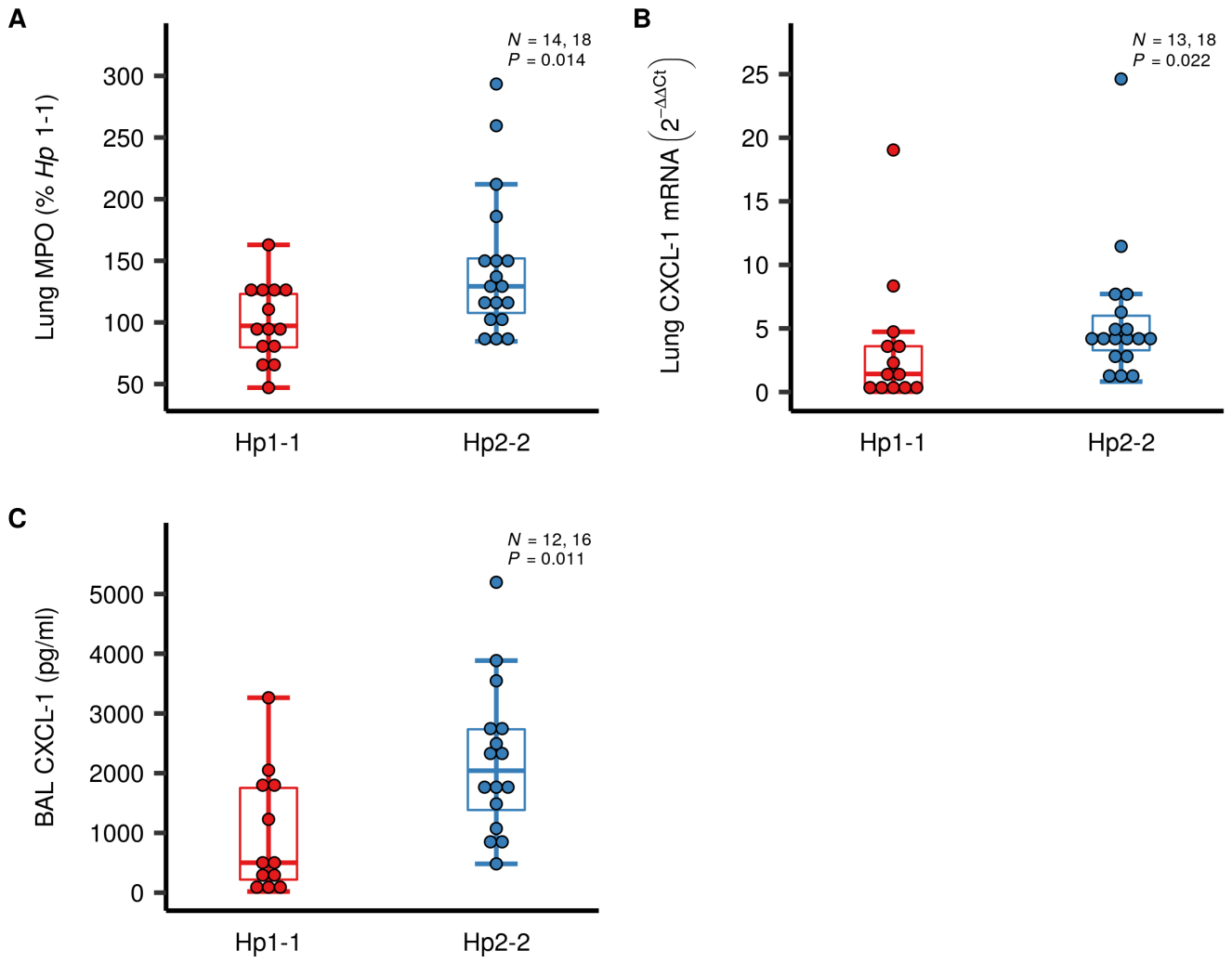
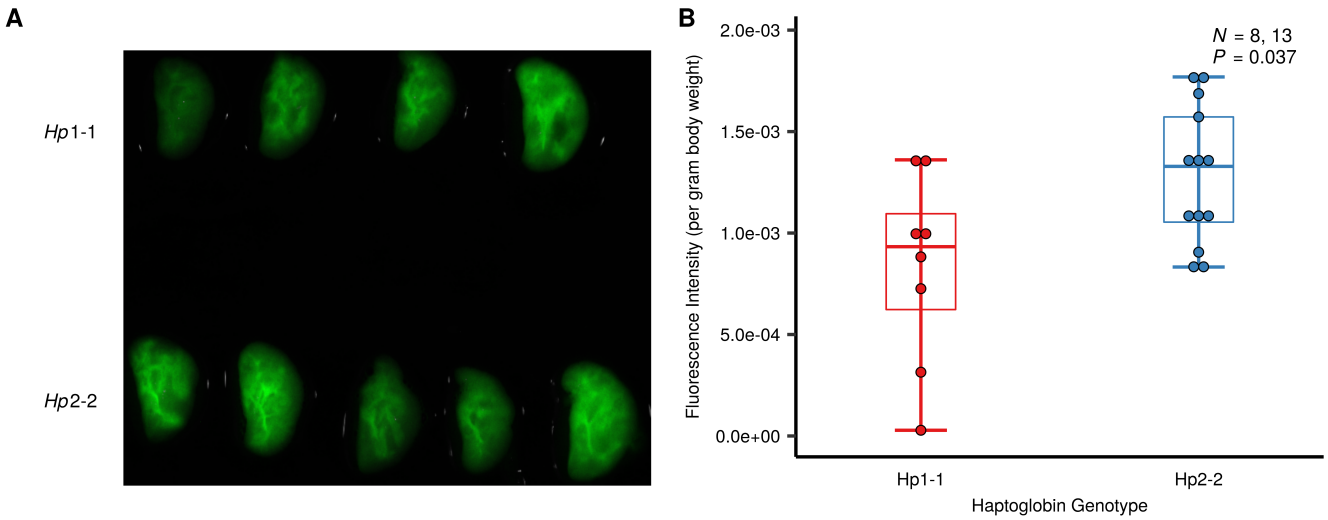



**Figure 2. *Hp2-2* mice have increased markers of lung inflammation.**

*Hp1-1* (red) and *Hp2-2* (blue) mice were treated with CS and IV CFH. (**A**) Myeloperoxidase (MPO) activity was measured enzymatically in whole lungs. Reported values are normalized to the mean value for *Hp1*-1 mice. $P = 0.014$ by Mann-Whitney *U* test. (**B**) Whole lung mRNA was extracted for CXCL-1 and expression measured by real-time PCR. Values are reported as fold-change relative to GAPDH expression. $P = 0.022$ by Mann-Whitney *U* test. (**C**) CXCL-1 protein levels were measured by ELISA in BAL samples. $P = 0.011$ by Mann-Whitney *U* test. Dots represent individual values. For the box plots, the horizontal bars represent the median,

559 boxes represent the interquartile range (IQR, 25$^{th}$ and 75$^{th}$ percentiles), and whiskers
560 represent the minimum and maximum values within 1.5 * IQR from the 25$^{th}$ and 75$^{th}$
561 percentiles.

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563 **Figure 3. *Hp2-2* increases lung microvascular permeability.**

564 Angiosense, a fluorescent 70kD macromolecule, was injected retro-orbitally at the time of
565 treatment. (**A**) After twenty-four hours, whole lungs were excised and imaged using a high
566 sensitivity CCD camera. (**B**) Fluorescent signal per lung was normalized to body weight and
567 quantified. Dots represent individual values. For the box plots, thick horizontal bars represent
568 the median, boxes represent the interquartile range (IQR, 25$^{th}$ and 75$^{th}$ percentiles), and
569 whiskers represent the minimum and maximum values within 1.5 * IQR from the 25$^{th}$ and 75$^{th}$
570 percentiles. *$P$ = 0.037 by Mann-Whitney $U$ test.

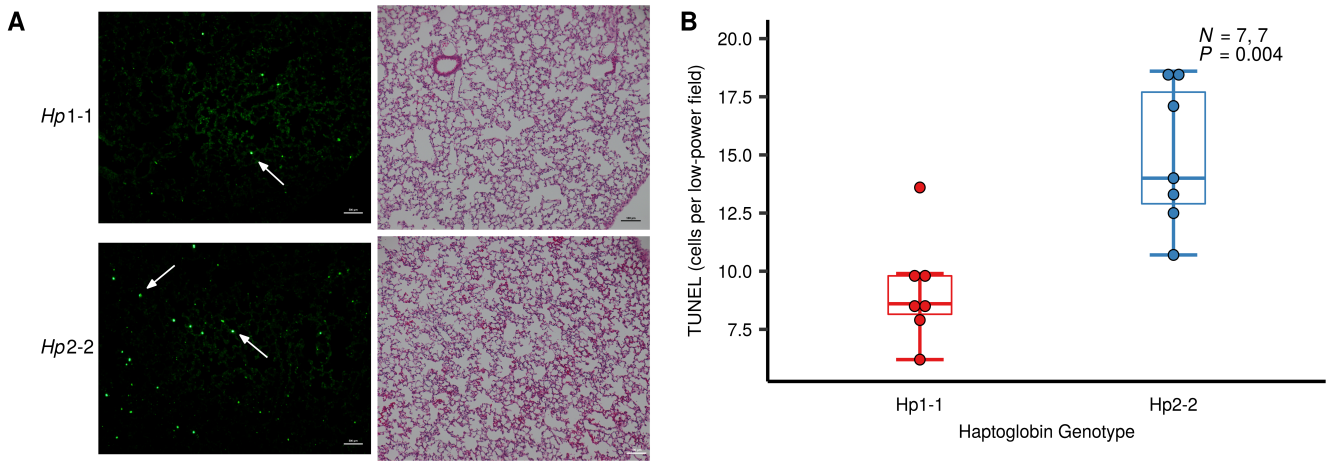
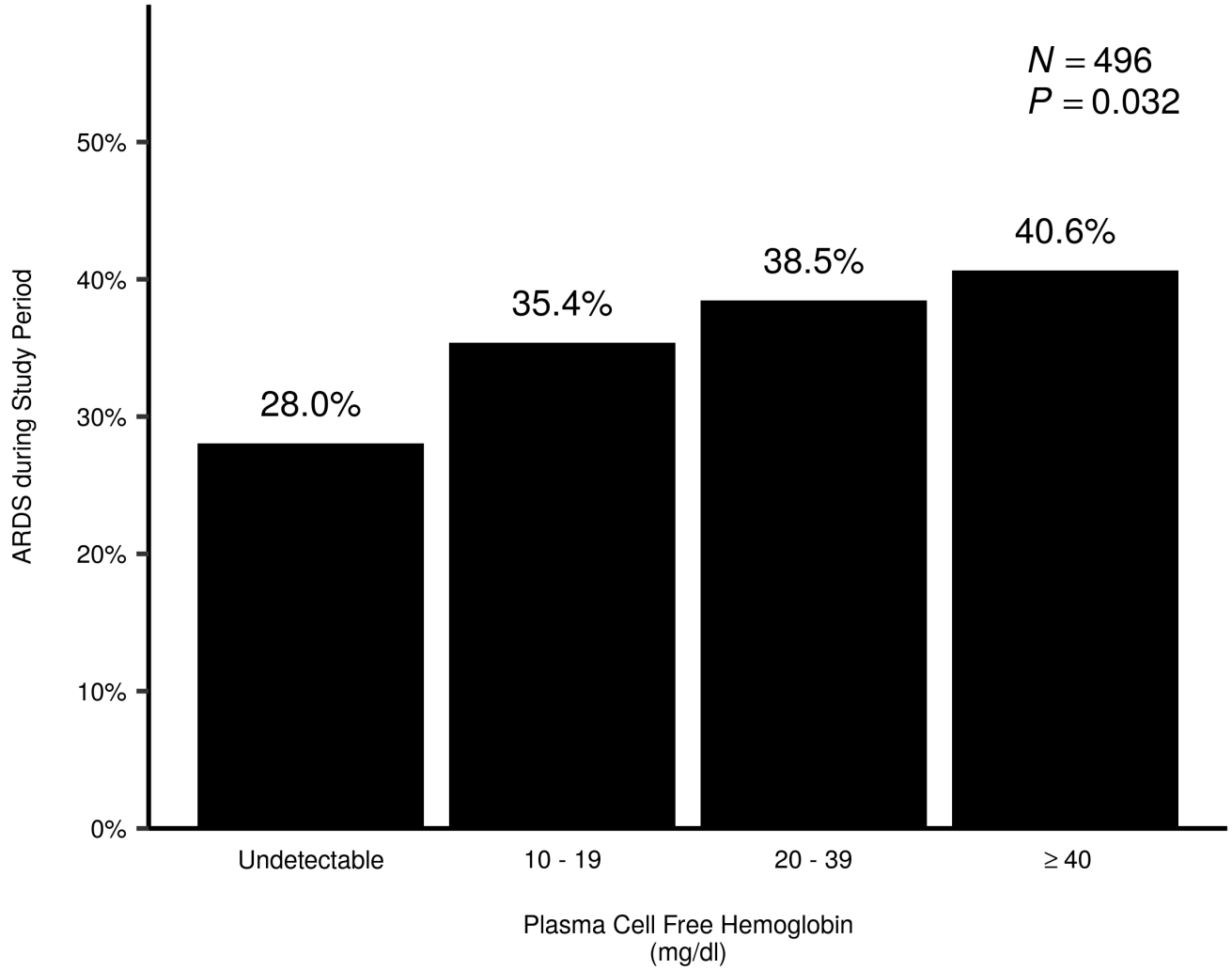



**Figure 4. *Hp2-2* mice have increased pulmonary apoptosis**

*Hp1-1* and *Hp2-2* mice were treated with CS and IV CFH. Lungs were harvested at 4 hours and examined for apoptotic cells by the TUNEL assay by a trained reviewer blinded to genotype. (**A**) Representative TUNEL stain images (left images) show increased number of TUNEL-positive cells (white arrows) in *Hp2-2* mouse lungs compared with *Hp1-1* mouse lungs. H&E stained sections from the same lung (right images). Scale bars on TUNEL images: 500μm, scale bars on H&E images: 100μm. (**B**) *Hp2-2* mouse lungs (blue) demonstrated increased apoptosis of pulmonary cells following sepsis compared with *Hp1-1* mice (red). Dots represent individual values. For the box plots, thick horizontal bars represent the median, boxes represent the interquartile range (IQR, 25$^{th}$ and 75$^{th}$ percentiles), and whiskers represent the minimum and maximum values within 1.5 * IQR from the 25$^{th}$ and 75$^{th}$ percentiles. *$P$ = 0.004 by Mann-Whitney $U$ test.

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585 **Figure 5. ARDS risk increases with higher plasma cell-free hemoglobin during sepsis**

586 Risk of developing ARDS during sepsis increased with higher enrollment plasma CFH levels.
587 Patients are grouped by enrollment plasma CFH quartile, height of bars and numbers over
588 bars indicate percentage of patients who developed ARDS during the study period. Number of
589 patients per quartile = 82, 130, 156, 128 respectively (*N* = 496 in total). *P* = 0.032 by Cochran-
590 Armitage test for trend of increasing ARDS risk ordered by CFH quartile. The lower limit of
591 detection for the assay is 10 mg/dL.

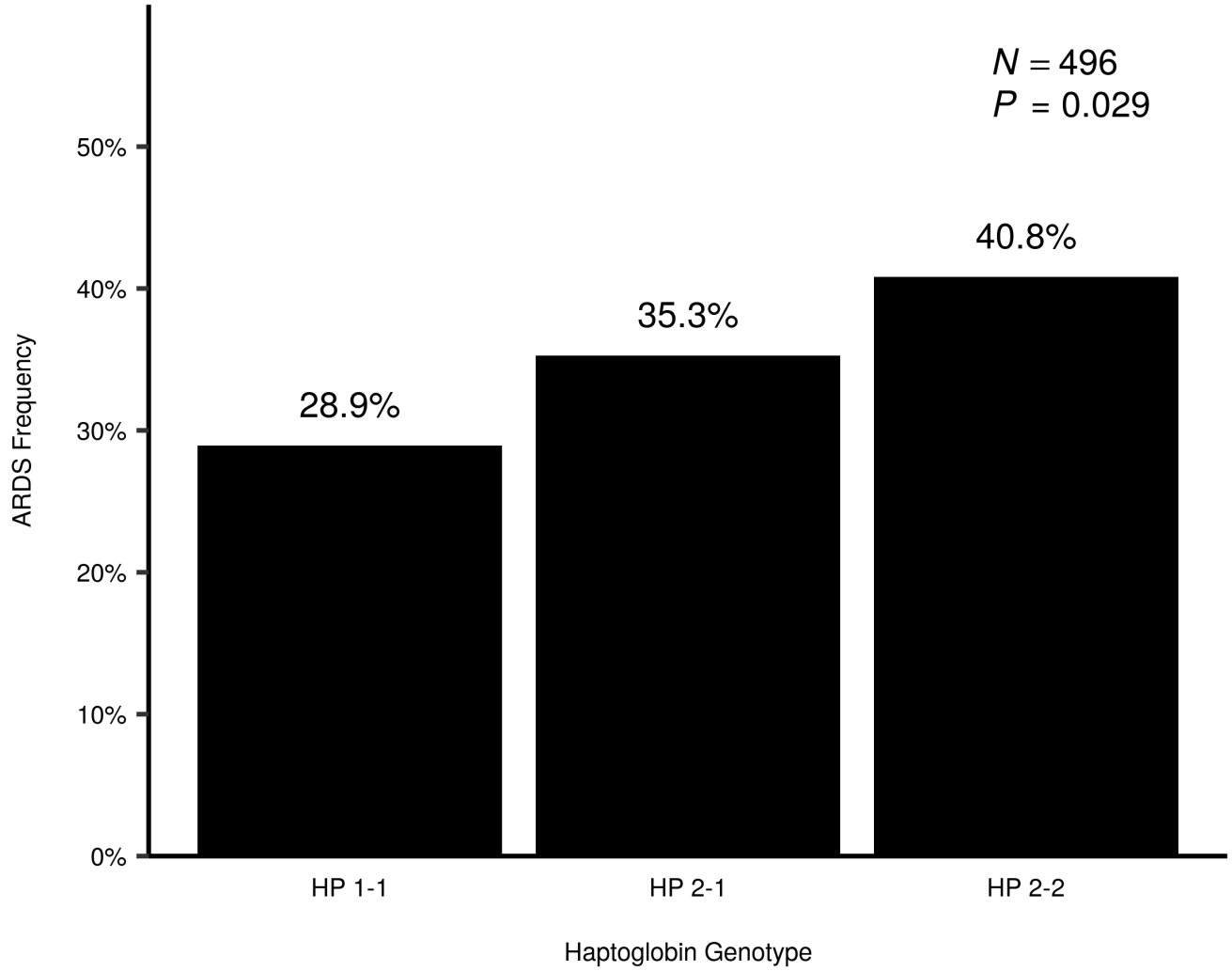
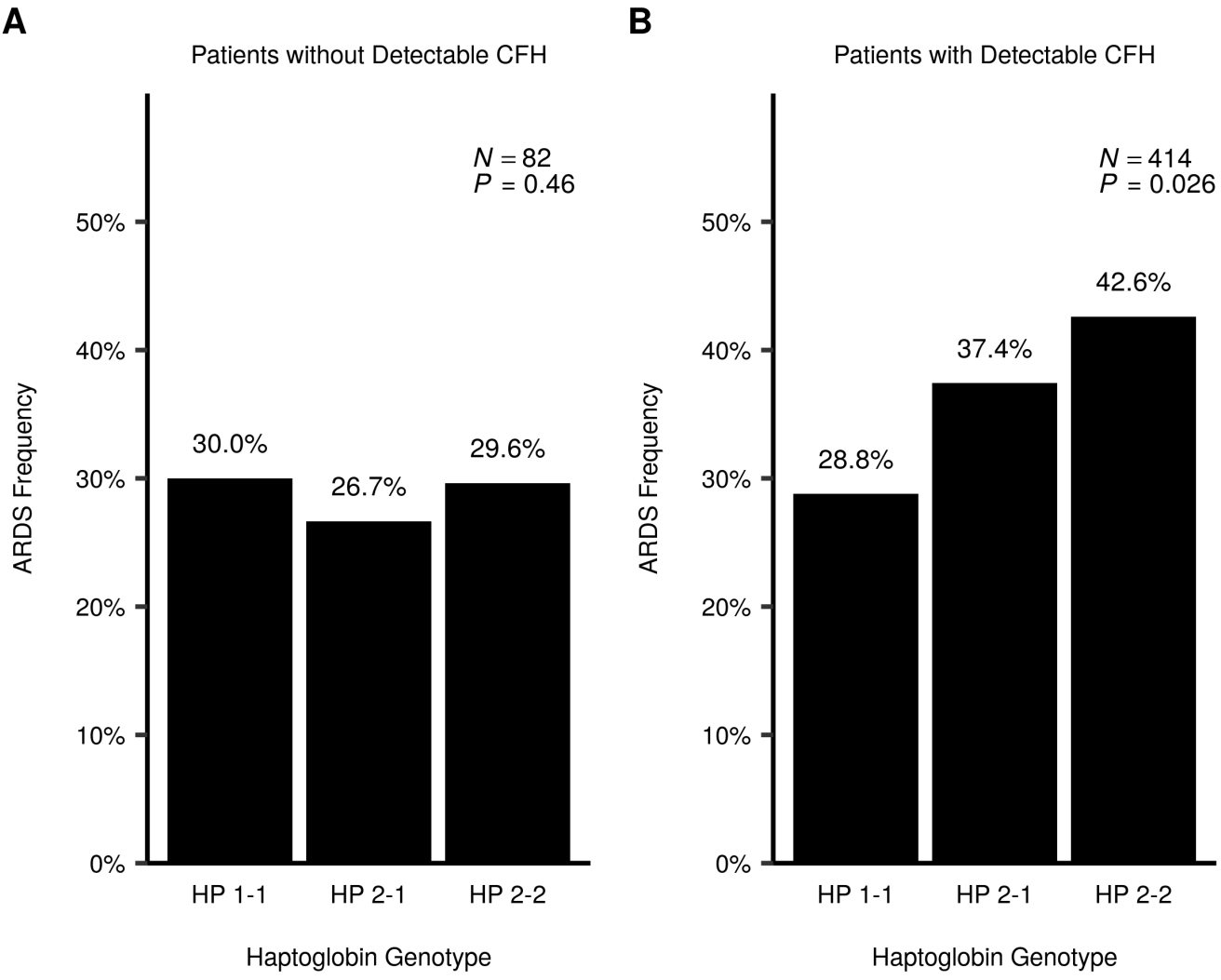



**Figure 6. Haptoglobin genotype increases ARDS risk in septic adults**

In the entire study cohort (*N* = 496), *HP2-2* patients and *HP2-1* patients had increased risk of developing ARDS during the study period. Height of bars and numbers over bars indicate proportion of patients developing ARDS for each group. *P* = 0.029 by Cochran-Armitage test for increasing risk ordered by number of *HP2* alleles.




598

599 **Figure 7. Haptoglobin genotype affects ARDS risk only in patients with detectable**

600 **plasma cell-free hemoglobin.**

601 (**A**)There was no association between *HP* genotype and ARDS risk in patients with

602 undetectable plasma cell-free hemoglobin (CFH). *N* = 82, *P* = 0.46 by Cochran-Armitage test.

603 (**B**) An association between haptoglobin (*HP*) genotype and ARDS risk was only observed in

604 patients with detectable CFH. *N* = 414, *P* = 0.026 by Cochran-Armitage test.

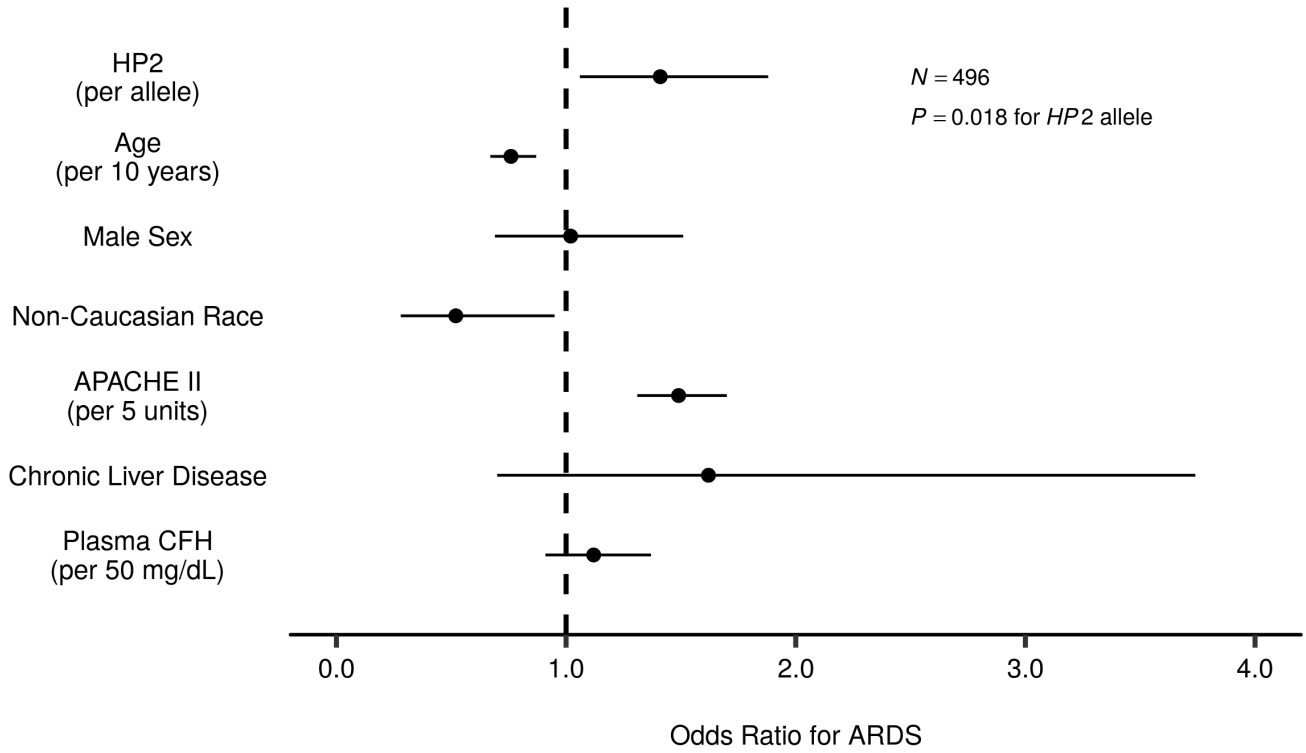



**Figure 8. Haptoglobin 2 variant increases ARDS risk in septic adults when controlling for clinical factors**

Multivariable logistic regression model for ARDS in study population (*N* = 496). Circles represent point estimate odds ratios and horizontal lines represent 95% confidence intervals for each variable included in the model. Black dashed vertical line indicates odds ratio of 1.0 (no change in ARDS risk). *P* = 0.018 for *HP2* variant. APACHE II – Acute physiology and chronic health evaluation II. *HP2* – Haptoglobin 2. CFH – cell-free hemoglobin.

## Tables

### Table 1. Demographic and clinical characteristics of study population

| | *HP1-1* | *HP2-1* | *HP2-2* | P[A] |
|---|---|---|---|---|
| **Demographics** | | | | |
| Number | 76 | 224 | 196 | |
| Age (years) | 56 (16) | 59 (15) | 56 (16) | 0.077 |
| Male Sex | 37 (49%) | 117 (52%) | 111 (57%) | 0.444 |
| Caucasian Race | 62 (82%) | 193 (86%) | 170 (87%) | 0.204 |
| | | | | |
| **Comorbid Medical Conditions** | | | | |
| Chronic Kidney Disease | 21 (28%) | 40 (18%) | 41 (21%) | 0.188 |
| Chronic Liver Disease | 5 (7%) | 16 (7%) | 6 (3%) | 0.165 |
| Diabetes | 23 (30%) | 75 (34%) | 60 (31%) | 0.778 |
| | | | | |
| **ICU Characteristics** | | | | |
| Mechanical Ventilation on Enrollment | 47 (62%) | 140 (63%) | 128 (65%) | 0.793 |
| APACHE II Score | 28 (7) | 28 (8) | 26 (8) | 0.066 |
| In-Hospital Mortality | 15 (20%) | 49 (22%) | 42 (21%) | 0.925 |
| Ventilator-Free Days | 18 (11) | 18 (11) | 18 (11) | 0.918 |
| ARDS During Study Period | 22 (29%) | 79 (35%) | 80 (41%) | 0.029[B] |
| | | | | |
| **Brussels Organ Failures on Enrollment** | | | | |
| Circulatory Failure | 57 (75%) | 162 (72%) | 132 (67%) | 0.363 |
| Coagulation Failure | 12 (16%) | 28 (13%) | 23 (12%) | 0.661 |
| Hepatic Failure | 15 (20%) | 40 (18%) | 36 (18%) | 0.935 |
| Renal Failure | 29 (38%) | 84 (38%) | 72 (37%) | 0.973 |
| Any Organ Failure | 65 (86%) | 189 (84%) | 158 (81%) | 0.487 |
| | | | | |
| **Plasma Biomarkers** | | | | |
| Plasma Cell-Free Hemoglobin (mg/dL) | 20 [10, 50] | 20 [10, 30] | 20 [10, 30] | 0.251 |
| Haptoglobin (µg/dL) | 1600 [800, 3880] | 1260 [570, 3300] | 860 [330, 1940] | 0.001 |

Data are presented n (% ) for categorical variables, mean (standard deviation) for continuous variables with normal distributions, and median [interquartile range] for continuous variables with non-normal distributions.

[A] Group-wise comparison testing performed using Chi-squared test for categorical variables, one-way

ANOVA for continuous variables with normal distributions, and Kruskal-Wallis *H* Test for continuous variables with non-normal distributions. [B]By Cochran-Armitage test for trend with alternative hypothesis that risk increases with each *HP2* allele.

**Table 2. Multivariable logistic regression model of ARDS in septic ICU patients**

| Variable | Odds Ratio | 95% Confidence Intervals | *P* |
|---|---|---|---|
| *HP2* Allele Count | 1.41 | [ 1.06; 1.88 ] | 0.018 |
| Age (per 10 years) | 0.76 | [ 0.67; 0.87 ] | < 0.0001 |
| APACHE II (per 5 units) | 1.49 | [ 1.31; 1.70 ] | < 0.0001 |
| Male Sex | 1.02 | [ 0.69; 1.51 ] | 0.92 |
| Non-Caucasian Race | 0.52 | [ 0.29; 0.95 ] | 0.033 |
| Plasma CFH (per 50 mg/dL) | 1.12 | [ 0.91; 1.37 ] | 0.29 |
| Chronic Liver Disease | 1.62 | [ 0.70; 3.74 ] | 0.26 |

Number of subjects: 496

APACHE: Acute Physiology and Chronic Health Evaluation score.

CFH: Circulating free hemoglobin. *HP2*: Haptoglobin-2 variant