Acute respiratory distress syndrome (ARDS) is an inflammatory lung disorder that frequently complicates critical illness and commonly occurs in sepsis. Although numerous clinical and environmental risk factors exist, not all patients with risk factors develop ARDS, 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 predict worse outcomes. Excess CFH is rapidly scavenged by haptoglobin (Hp). A common HP genetic variant, HP2, is unique to humans and is common in many populations worldwide. HP2 haptoglobin has reduced ability to inhibit CFH-mediated inflammation and oxidative stress compared with the alternative HP1. We hypothesized that HP2 increases ARDS susceptibility during sepsis when plasma CFH levels are elevated. In a murine model of sepsis with elevated CFH, transgenic mice homozygous for Hp2 had increased lung inflammation, pulmonary vascular permeability, lung apoptosis, and mortality compared with wild-type mice. We then tested the clinical relevance of our findings in 496 septic critically ill adults, finding that HP2 increased ARDS susceptibility after controlling for clinical risk factors and plasma CFH. These observations identify HP2 as a potentially novel genetic ARDS risk factor during sepsis and may have important implications in the study and treatment of ARDS.
Haptoglobin-2 variant increases susceptibility to acute respiratory distress syndrome during sepsis

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Introduction

Acute respiratory distress syndrome (ARDS) is an inflammatory lung disorder that frequently complicates critical illness and commonly occurs in sepsis. Although numerous clinical and environmental risk factors exist, not all patients with risk factors develop ARDS, 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 predict worse outcomes. Excess CFH is rapidly scavenged by haptoglobin (Hp). A common HP genetic variant, HP2, is unique to humans and is common in many populations worldwide. HP2 haptoglobin has reduced ability to inhibit CFH-mediated inflammation and oxidative stress compared with the alternative HP1. We hypothesized that HP2 increases ARDS susceptibility during sepsis when plasma CFH levels are elevated. In a murine model of sepsis with elevated CFH, transgenic mice homozygous for Hap2 had increased lung inflammation, pulmonary vascular permeability, lung apoptosis, and mortality compared with wild-type mice. We then tested the clinical relevance of our findings in 496 septic critically ill adults, finding that HP2 increased ARDS susceptibility after controlling for clinical risk factors and plasma CFH. These observations identify HP2 as a potentially novel genetic ARDS risk factor during sepsis and may have important implications in the study and treatment 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 makes up 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 because Hp in patients with the HP2 variant would be predicted to have reduced ability to mitigate CFH-mediated oxidative stress and inflammation. Using transgenic mice with a murine homolog 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 with 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 potentially 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 1A). 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 1B).

**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 IV CFH, as evidenced by increased whole-lung myeloperoxidase activity (P = 0.014, Figure 2A) and CXCL1 mRNA expression (P = 0.022, Figure 2B). Hp2-2 mice also had increased CXCL1 levels in bronchoalveolar lavage (BAL) fluid compared with Hp1-1 mice (P = 0.011, Figure 2C).
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 retroorbital 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 24 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 colabeling in samples from wild-type mice (JA Bastarache et al., unpublished observations). Therefore, this suggests that lung endothelial apoptosis contributes to increased microvascular permeability in this model.

Adult sepsis cohort HP 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 2 methods depending on data and sample availability. For the 344 patients for 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; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.131206DS1). The observed HP genotype distribution was similar to expected allele frequencies for a majority-European ancestry cohort (P = 0.12 for difference from reference HP2 allele frequency by 2-sample binomial proportions test). The overall agreement between PCR and imputation in the 120 patients genotyped by both methods was 0.91 (Cohen’s κ 0.85, balanced accuracy 0.91, 95% CI 0.84–0.95) (Supplemental Table 1 and Supplemental Figure 2).

Adult sepsis cohort patient clinical characteristics. Patients with all 3 HP genotypes had similar baseline clinical characteristics, including age, sex, reported ethnicity, comorbid medical conditions, Acute Physiology and Chronic Health Evaluation II (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 3 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 CFH increases risk of ARDS. During the first 4 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).

HP2 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 for ARDS of 1.33, and HP2-2 had an odds ratio 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, and n = 414) and not in patients with undetectable levels of plasma CFH (P = 0.46, and n = 82) (Figure 7).

HP2 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 (odds ratio = 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 for 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, P = 0.015), of White race (n = 425, P = 0.020), with absence of chronic liver disease (n = 469, P = 0.042), and with severe sepsis (n = 475, P = 0.025) also demonstrated a statistically significant association between HP genotype and ARDS risk (data not shown).

HP2 variant and ventilator-free days. To assess whether 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 indicate more prolonged mechanical ventilation, indicating more severe respiratory failure (40). VFDs are a
commonly reported outcome in critical care trials because they capture 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 HP1-1 patients, 14.0 (± 1.1) days for HP2-1 patients, and 13.5 (± 1.2) days for HP2-2 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) or in 28-day survival (Supplemental Figure 7).

Discussion

We have identified the HP2 variant as a potentially 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 associated with increased risk of ARDS only 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 because 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 in animal models (41–48). Endothelial injury is also a key pathophysiological characteristic of ARDS (49, 50). By comparing Hp2-2 mice with 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 2 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 with 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 because of the chemical reactivity of the heme iron moiety. Heme-complexed iron can be oxidized from the ferrous (Fe²⁺) state to the more reactive ferric (Fe³⁺) and ferryl (Fe⁴⁺) states when outside.

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: 500 μm, scale bars on H&E images: 100 μm. (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, 25th and 75th percentiles), and whiskers represent the minimum and maximum values within 1.5 × IQR from the 25th and 75th percentiles. *P = 0.004 by Mann-Whitney U test.
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 atherosclerotic coronary artery disease (30, 31), type 2 diabetes (56), and diabetic nephropathy (32, 33). Our current study identifies a potentially 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 because 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 stud-
ies 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 both during 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

**Figure 5. ARDS risk increases with higher plasma CFH during sepsis.** Risk of developing ARDS during sepsis increased with higher enrollment plasma CFH levels. Patients are grouped by enrollment plasma CFH quartile; height of bars and numbers over bars indicate percentage of patients who developed ARDS during the study period. Number of patients per quartile = 82, 130, 156, and 128, respectively (N = 496 in total). P = 0.032 by Cochran-Armitage test for trend of increasing ARDS risk ordered by CFH quartile. The lower limit of detection for the assay is 10 mg/dL.

**Figure 6. HP 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 proportions of patients developing ARDS for each group. P = 0.029 by Cochran-Armitage test for increasing risk ordered by number of HP2 alleles.
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 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). Hp2-2 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 RBC 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

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds ratio</th>
<th>95% CIs</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP2 allele count</td>
<td>1.41</td>
<td>[1.06, 1.88]</td>
<td>0.018</td>
</tr>
<tr>
<td>Age (per 10 years)</td>
<td>0.76</td>
<td>[0.67, 0.87]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>APACHE II (per 5 units)</td>
<td>1.49</td>
<td>[1.31, 1.70]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Male sex</td>
<td>1.02</td>
<td>[0.69, 1.51]</td>
<td>0.52</td>
</tr>
<tr>
<td>Non-White race</td>
<td>0.52</td>
<td>[0.29, 0.95]</td>
<td>0.033</td>
</tr>
<tr>
<td>Plasma CFH (per 50 mg/dL)</td>
<td>1.12</td>
<td>[0.91, 1.37]</td>
<td>0.29</td>
</tr>
<tr>
<td>Chronic liver disease</td>
<td>1.62</td>
<td>[0.70, 3.74]</td>
<td>0.26</td>
</tr>
</tbody>
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Number of subjects: 496. APACHE, Acute Physiology and Chronic Health Evaluation score.
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) because all study patients were enrolled before the publication of the more recent Third International Consensus definition of sepsis (76). Because 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. Last, 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 potentially 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 because HP2 is the more common variant in many human populations. This study identifies a large clinical subpopulation 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 a murine homolog of human HP2 (Hp2-2) were a gift from Rafael Tamargo of the Department of Neurosurgery of the Johns Hopkins University School of Medicine (67). We used a previously reported murine model of polymicrobial sepsis (77, 78). We prepared a CS from 6-week-old female C57BL/6 donor mice purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). In brief, cecal contents were collected from euthanized donor mice, resuspended in 5% dextrose at 80 mg/mL, vortexed for 15 seconds, and filtered through a 25-gauge needle. We administered an intraperitoneal injection CS at 2.0 mg/g body weight (BW) and a retroorbital injection of CFH at 0.15 mg/g BW to recipient 8- to 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 4 or 24 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 retroorbital puncture in heparinized syringes and centrifuged at 2000 g for 10 minutes. BAL 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 the time of analysis.

**Plasma circulating CFH in mice.** Plasma CFH was measured at 24 hours in mouse plasma using the HemoCue Plasma/Low Hb System (HemoCue America, Brea, California, USA).

**Lung inflammation biomarkers in mice.** We measured CXCL1/KC in duplicate using an electrochemiluminescence assay (MesoScale Discovery, Gaithersburg, Maryland, USA) in BAL samples according to the manufacturer’s recommendations. For mRNA expression, we extracted mRNA from flash-frozen whole lungs using Qiagen RNeasy Plus Mini Kit (Hilden, Germany). We generated cDNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, California, USA) and quantified CXCL1 mRNA expression level by quantitative PCR, normalized to GAPDH expression using TaqMan primer probes (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

**MPO activity.** Frozen lungs were homogenized in 50 mM potassium phosphate (pH 6.0), 0.5% hexadecyltrimethyl ammonium bromide, and 5 mM EDTA, then sonicated, centrifuged, and diluted at 1:30 dilution in 100 mM potassium phosphate (pH 6.0), 0.3% hydrogen peroxide (MilliporeSigma, St. Louis, Missouri, USA), and 1 mg/mL o-dianisidine (MilliporeSigma). We recorded absorbance at 460 nm using a spectrophotometer at 1 and 3 minutes and calculated MPO activity according to the equation (81):

\[
\text{Activity} = \frac{([A_{460\text{ nm, } 1\text{ min}} - A_{460\text{ nm, } 3\text{ min}}] \times 13.5)}{\text{weight}_{\text{mouse}}}
\]

**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 formalin-fixed sections of mouse lung in paraffin following deparaffinization and antigen retrieval. Costaining of endothelial cells was performed by incubating with Mouse Thrombomodulin/BDCA-3 polyclonal goat IgG antibody (AF3894, R&D Systems, Minneapolis, Minnesota, USA) at 1:200 overnight at 4°C and Alexa Fluor 568 anti-goat IgG secondary antibody (A-11057, Invitrogen, Carlsbad, California, USA) at 1:200 for 30 minutes. Lung sections were blinded and 10 non-overlapping images at original magnification ×20 were taken on an Olympus IX51 microscope using an Olympus DP70 camera (Shinjuku, Tokyo, Japan). A trained reviewer who was blinded to each sample's Hp genotype examined lung histology slides and identified the number of TUNEL-positive cells per low-power field. Counts over the 10 fields were averaged for each mouse.

**Pulmonary vascular permeability by AngioSense assay.** In selected experiments, we administered 100 μL AngioSense 750EX near-infrared fluorescent imaging agent (2 nmol/100 μL, PerkinElmer, Waltham, Massachusetts, USA) to each study mouse via retroorbital injection. We measured extravascular accumulation of the imaging agent of excised lungs at 24 hours using a LI-COR Pearl small-animal imaging camera (LI-COR Biosciences, Lincoln, Nebraska, USA).

**Human study population and clinical data collection.** We studied patients greater than 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 American College of Chest Physicians/Society of Critical Care Medicine 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 before 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 before enrollment and daily for the first 4 days after enrollment.

**Human sample 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, California, USA) and Hp (Abcam, Cambridge, Massachusetts, USA) in plasma as previously described (15). There was insufficient plasma available to measure Hp levels in 152 patients. We have previously reported some of the hemoglobin and Hp 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 –80°C until genotyping.
**HP 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 PCR as previously described (84). We calculated the change in threshold cycle (ΔCt) for each sample as ΔCt HP5′ − ΔCt HP2 and calculated the HP2/HP5′ ratio of each sample as 2−ΔCt sample. We defined HP1-1 by an HP2/HP5′ ratio of 0, 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 Hp phenotype (85) using study personnel blinded to the PCR genotyping results.

**HP genotype imputation.** In 152 White 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 GWAS 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 (San Diego, California, USA). 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-Mb 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 50 iterations of the phasing algorithm and 25 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 an HP2 genotype probability of 0.0–0.5 corresponding to a predicted HP genotype of HP1-1, a genotype probability of 0.5–1.5 corresponding to HP2-1, and a genotype probability of 1.5–2.0 corresponding to HP2-2.

**Statistics for experimental mouse studies.** For the survival study using the 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.

**Statistics for human studies.** The primary outcome of the human study was prevalence of ARDS defined by the Berlin Criteria (82) on at least 1 of the first 4 ICU study days. We performed a secondary analysis focused on patients with detectable plasma CFH levels defined as at least 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 HP allele. Differences in continuous outcomes between HP genotypes was tested using 1-way ANOVA for variables with normal distributions and the Kruskal-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 Hp synthetic function. We calculated the number of 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 3 × 3 confusion matrix and calculated sensitivity, specificity, and F statistics for all 3 possible imputed genotypes. We also calculated overall accuracy and unweighted Cohen's κ 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 reviewed and approved all animal study protocols. The Vanderbilt University Medical Center Institutional Review Board 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 because of 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 or edited the manuscript. VEK designed and implemented computer scripts for haptoglobin imputation. HN, JBM, SRL, NDP, WKY, 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.

Acknowledgments
Research reported in this publication was supported by the National Institutes of Health (NIH) under award numbers NIH T32GM108554 and T15LM007450 (VEK), NIH R01HL135849 (LBW and JAB), NIH K24HL103836 (LBW), and NIH K08HL136888 (CMS). This work was also supported in part by the Parker B. Francis Foundation (CMS) and Vanderbilt Faculty Research Scholars (CMS). The project publication described was supported by Clinical and Translational Science Awards award UL1TR002243 from the National Center for Advancing Translational Sciences. The authors would like to acknowledge Linda Boettger and Steven McCarroll of the Broad Institute of MIT and Harvard for providing the reference panels for Hp imputation and their advice on implementation, Neil Zheng for assistance with implementation of Hp imputation, and Rafael Tamargo for providing the transgenic mice used in this study. The contents of this project are solely the responsibility of the authors and do not necessarily represent official views of the National Center for Advancing Translational Sciences or the NIH.

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