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Alterations in Systemic Extracellular Heme and Hemopexin Are Associated With Adverse Clinical Outcomes in Ugandan Children With Severe Malaria

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Background. Malaria remains a major cause of global mortality. Extracellular heme, released during malaria-induced hemolysis, mediates a number of pathogenic processes associated with vascular and organ injury. Hemopexin (hpx) facilitates the degradation of extracellular heme. In this study, we explore the hypothesis that dysregulation of the heme-hpx axis is associated with disease severity, acute kidney injury (AKI), and outcome.

Methods. Plasma levels of hemin and hpx (at admission, day 3, and day 14) were assessed in children with severe malaria in Jinja, Uganda.

Results. The ratio of heme to hpx was higher at admission and decreased with recovery (median, 0.043 [interquartile range {IQR}, 0.007–0.239] on day 1, 0.024 [IQR, 0.005–0.126] on day 3, and 0.008 [IQR, 0.002–0.022] on day 14; $P < .001$). Ratios of heme to hpx at admission were higher in children with as compared to those without severe anemia (median, 0.124 [IQR, 0.024–0.431] vs 0.016 [IQR, 0.003–0.073]; $P < .0001$), children with as compared to those without respiratory distress (median, 0.063 [IQR, 0.017–0.413] vs 0.020 [IQR, 0.004–0.124]; $P < .01$), and children with as opposed to those without stage 3 AKI (median, 0.354 [IQR, 0.123–2.481] vs 0.037 [IQR, 0.005–0.172], $P < .01$). The heme to hpx ratio at admission was associated with 6-month mortality (median, 0.148 [IQR, 0.042–0.500] vs 0.039 [IQR, 0.007–0.172]; $P = .012$).

Conclusions. The ratio of heme to hpx is associated with disease severity and adverse clinical outcomes in Ugandan children, and dysregulation of the heme axis may contribute to malaria pathogenesis.

Keywords. severe malaria; heme; hemopexin; metabolic acidosis; anemia; respiratory distress; acute kidney injury; pediatric.

Malaria caused an estimated 214 million cases and 438 000 deaths in 2015 [1]. Artemisinin-based therapies have improved survival, but mortality rates for severe malaria remain high (10%–22%) [2, 3]. A detailed understanding of malaria pathogenesis may suggest new targets for intervention to further improve outcome [4, 5].

Severe disease in children manifests primarily as cerebral malaria, severe malarial anemia, and/or respiratory distress [6]. There is emerging evidence that acute kidney injury (AKI), thought to occur primarily in adults [7], also occurs commonly in young children with severe malaria and is associated with increased mortality [8, 9].

Malaria parasite infection is characterized by hemolysis of both infected and uninfected erythrocytes [7]. Erythrocyte lysis results in the release of cell-free hemoglobin with secondary release of heme. Heme is a toxic molecule that mediates a range of pathogenic effects, including oxidative stress [10], endothelial activation [10–12], and inflammation [11–13], and has been implicated in malaria pathogenesis [14] and kidney injury [15]. To mitigate heme-mediated damage, host hemopexin (hpx) functions as part of an endogenous protective mechanism. hpx is an acute-phase reactant that binds extracellular heme and transports it for degradation by heme oxygenase 1 [14].

Preclinical animal models have causally implicated extracellular heme in malaria pathogenesis [14, 16], with hpx having a protective role [17] in severe malaria. Studies of adults in India [18] and children in Africa [17] with severe malaria have reported increased circulating levels of plasma heme and low levels of hpx at admission. This study extends these findings in a well-characterized prospective cohort of Ugandan children

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presenting with severe malaria and undergoing longitudinal assessment of host biomarkers over the course of acute illness and long-term follow-up.

METHODS

This study is a secondary analysis of a randomized, double-blinded, placebo-controlled trial evaluating inhaled nitric oxide versus placebo as an adjunctive therapy for children with severe malaria in Jinja, Uganda [19]. Children (age, 1–10 years) were eligible for enrollment if they had a 3-band (histidine rich protein 2 [HRP-2], and *Plasmodium* lactate dehydrogenase) rapid diagnostic test positive for *Plasmodium falciparum* and had the following clinical manifestations of severe malaria: impaired consciousness (Blantyre coma score, <5), prostration, repeated seizures (≥2 in 24 hours), and/or respiratory distress/acidosis as previously defined [19]. Inclusion criteria were based on clinical assessment at presentation and, therefore, did not include some World Health Organization criteria requiring additional laboratory investigations. Exclusion criteria included severe malnutrition, hemoglobinopathy, known chronic illness, or clinical suspicion of acute bacterial meningitis [19]. Pneumonia with incidental parasitemia could not be excluded owing to the unavailability of chest radiography at this site. All patients were treated with intravenous artesunate as per international guidelines [19].

Within the classification of severe malaria, children were further subclassified by the primary manifestations of severe malaria: cerebral malaria, severe malarial anemia, and/or respiratory distress. Cerebral malaria was defined by coma (Blantyre coma score, <3), after exclusion of hypoglycemia and postictal state as a cause for decreased consciousness. Severe malarial anemia was defined as an admission hemoglobin level of <5.0 g/dL. Respiratory distress was defined as age-related tachypnea with deep breathing, nasal flaring, or subcostal retractions.

Heme and hpx were measured in plasma collected from all children at admission, prior to treatment (n = 179), and then levels were measured only in children in the placebo arm on day 3 (n = 83) and day 14 (n = 78). Since we could not exclude the possibility that the intervention influenced heme levels, we only measured levels in children who had not received inhaled nitric oxide. Samples were assessed for hpx by enzyme-linked immunosorbent assay (ELISA; Immunology Consultants Laboratory, Portland, OR) and for extracellular heme by a colorimetric assay measuring hemin, an oxidized form of heme (Biovision, Milpitas, CA). The lower limits of detection for the assays were 19.5 μg/mL for hpx and 0.93 μM for hemin. The following biomarkers were measured by ELISA, according to manufacturer's instructions (Duosets, R&D Biosystems, Minneapolis, MN): C-reactive protein (CRP), angiopoietin 2 (Ang-2), soluble Tie2 receptor (sTie2), and soluble fms-like tyrosine kinase-1 (sFlt-1).

Assessment and classification of AKI in this study was conducted using KDIGO guidelines [20] as described elsewhere [9]. Briefly, children were classified as having stage 3 AKI if they had a >3-fold increase in serum creatinine level from an estimated baseline creatinine level, a single serum creatinine level measurement of ≥354 μmol/L, or an estimated glomerular filtration rate of <35 mL/minute/1.73 m^2. At admission, creatinine and blood urea nitrogen (BUN) levels were measured at the bedside, using the i-STAT device (CHEM8+ or Crea cartridges, Abbott Laboratories, Saint-Laurent, Canada). BUN measurements were unavailable for 48 children due to unavailable cartridges.

Ethics

Ethical approval was granted from the Uganda National Council for Science and Technology, Makerere University Research Ethics Committee (Uganda), and by the Toronto Academic Health Science Network. Written, informed consent was provided by the parent of or caregiver for all study participants.

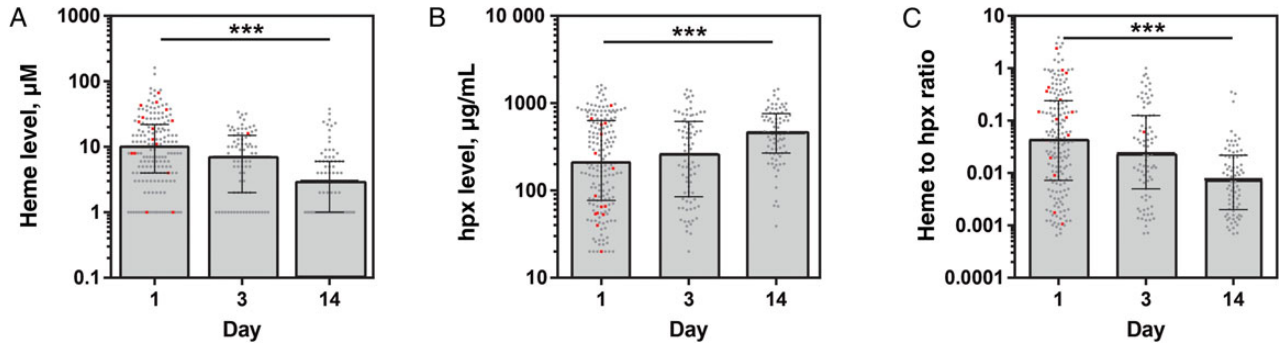

Figure 1. Decreases in levels of extracellular heme and increases in hemopexin (hpx) are associated with clinical recovery in children with severe malaria. *A* and *B*, Levels of extracellular heme were significantly higher at admission and decreased with recovery (*A*), while levels of hpx were significantly lower at admission and increased over time (*B*). *C*, Owing to the importance of the relative concentrations of extracellular heme to hpx, a ratio of extracellular heme to hpx was also analyzed and showed a significant decrease associated with clinical recovery. Plasma levels of extracellular heme and hpx were measured at admission (day 1), during hospitalization (day 3), and during follow-up (day 14). Children who died within 14 days of admission are indicated by a red square. Data are median values and interquartile ranges. ***$P < .001$ by the Friedman test.

Statistical Analysis

Longitudinal analysis was performed using the Friedman test. Continuous variables were analyzed by the Mann–Whitney test, and categorical variables were analyzed using χ^2 analysis. All statistical analysis was performed using IBM SPSS Statistics, version 22, and GraphPad Prism 6.

RESULTS

Kinetics of Free Heme and hpx During Acute Infection and Convalescence

Heme and hpx were measured in plasma samples from all children with severe malaria at admission (day 1 before therapy; n = 179) and from the placebo recipients during hospitalization (day 3; n = 83) and at convalescence (day 14; n = 78). The median age was 2.0 years, and 102 of 179 study participants (57%) were male. Longitudinal analysis of plasma heme levels showed that there was a significant decrease in extracellular heme levels with clinical recovery (median, 10 μM [interquartile range {IQR}, 4–22 μM] on day 1, 7 μM [IQR, 2–15 μM] on day 3, and 3 μM [IQR, 1–6 μM] on day 14; $P < .001$; Figure 1*A*). Conversely, hpx levels significantly increased with recovery (median, 209 μg/mL [IQR, 77–633 μg/mL] on day 1, 261 μg/mL [IQR, 85–620 μg/mL] on day 3, and 455 μg/mL [IQR, 268–762 μg/mL] day 14; $P < 0.001$; Figure 1*B*). Owing to the biological relationship between extracellular heme and hpx, we also analyzed the ratio of extracellular heme to hpx over time. Similar to extracellular heme levels, the ratio of extracellular heme to hpx decreased with recovery (median, 0.043 [IQR, 0.007–0.239] on day 1, 0.024 [IQR, 0.005–0.126] on day 3, and 0.008 [IQR, 0.002–0.022] on day 14; $P < 0.001$; Figure 1*C*). Given the critical interplay between extracellular heme and hpx, the remainder of the analyses are presented as a ratio of these factors.

Clinical Characteristics and Their Association With the Heme to hpx Ratio

To examine the association between various clinical markers and ratios of heme to hpx at admission, the ratios were analyzed by quartiles. Children with heme to hpx ratios in the highest quartile (quartile 4) were distinct from other enrolled children with respect to a number of measured parameters and therefore were compared to the children in the other quartiles (quartiles 1–3). There were no differences observed in age, sex, or weight between children in quartiles 1–3 versus those in quartile 4. Compared with children in quartiles 1–3, those in quartile 4 had evidence of metabolic acidosis, with higher respiratory rates, higher lactate levels, lower $HC0_3^-$ levels, and a larger base excess ($P < .01$; Table 1). Children in quartile 4 also had higher white blood cell counts and lower axillary temperatures, parasitemia levels, and hemoglobin levels ($P < .01$; Table 1).

Children Presenting With Severe Malarial Anemia or Respiratory Distress Have Higher Ratios of Heme to hpx

The most common manifestations of severe malaria in children are cerebral malaria, respiratory distress/metabolic acidosis, and severe anemia [6]. Ratios of heme to hpx were higher at admission and day 3 of hospitalization in children admitted with severe anemia, compared with children who were not (median, 0.124 vs 0.016 on day 1 [$P < .0001$] and 0.047 vs 0.007 on day 3 [$P < .001$]; Figure 2*A*). Children with respiratory distress also had higher heme to hpx ratios at admission (median, 0.063 vs 0.020; $P < .01$; Figure 2*B*), and ratios trended toward being higher on day 3 of hospitalization (median, 0.045 vs 0.013; $P = .053$), compared with children without respiratory distress. Similarly, children admitted with acidosis (lactate level, >5 mmol/L) had higher heme to hpx ratios at admission and

Table 1. Patient Characteristics at Admission, by Quartile of Extracellular Heme to Hemopexin (hpx) Ratio

Variable	Quartiles 1–3 (n = 135)	Quartile 4 (n = 44)	*P* Value[a]
Demographic characteristic			
Age, y	2.0 (1.0–3.0)	2.0 (1.0–3.0)	.659
Male sex, %	75 (56)	27 (61)	.499
Weight, kg	11.0 (9.4–13.0)	11.0 (9.0–13.5)	.991
Clinical characteristic			
Temperature, °C	38.1 (37.1–39.0)	37.6 (36.8–38.0)	**.006**
Pulse rate, beats/minute	162 (143–179)	162 (150–176)	.983
Blood pressure, mm Hg			
Systolic	110 (100–120)	110 (100–120)	.950
Diastolic	60 (50–70)	53 (40–68)	.075
Respiratory rate, breaths/minute	46 (36–60)	56 (44–70)	**.004**
WBC count, $\times 10^9$ cells/L	10.7 (6.7–18.1)	17.6 (10.5–23.2)	**.001**
Hemoglobin level, g/dL	5.3 (3.5–6.8)	4.0 (2.8–4.78)	**<.001**
Platelet count, $\times 10^9$ platelets/L	69.0 (37.5–122.0)	91.0 (40.0–234.0)	.132
Parasitemia level, parasites/μL	34 540 (5380–102 800)	3320 (480–33 000)	**<.001**
Base excess, mmol/L[3]	−6.0 (−11.5 to −4.0)	−12.0 (−16.0 to −9.0)	**<.001**
Lactate level, mmol/L	3.0 (2.0–4.9)	6.7 (3.8–10.1)	**<.001**
HCO_3^- level, mmol/L	18.5 (14.8–20.2)	12.8 (10.1–15.3)	**<.001**
Complication at admission			
Convulsions	112 (83)	31 (70)	.072
Coma	79 (59)	27 (61)	.739
Shock	8 (6)	8 (18)	**.013**
Deep breathing	55 (41)	31 (70)	**.001**
Jaundice	13 (10)	15 (35)	**<.001**
Hemoglobinuria	15 (11)	17 (39)	**<.001**
Biomarker level			
CRP, μg/mL	188 (125–275)	194 (109–253)	.688
Ang-2, ng/mL	2.50 (1.29–4.45)	5.70 (3.41–8.62)	**<.001**
sTie2, ng/mL	58.4 (44.5–71.9)	74.2 (51.8–91.6)	**.001**
sFlt-1, ng/mL	1.63 (0.67–3.45)	3.43 (1.95–6.12)	**<.001**

Bold values indicate a significance level $P < .05$.

Data are median (interquartile range) or no. (%) of subjects in the 3 quartiles with the lowest ratio of heme to hpx (quartiles 1–3) and subjects in the quartile with the highest ratio (quartile 4).

Abbreviations: Ang-2, angiopoietin 2; CRP, C-reactive protein; WBC, white blood cell.

[a] Continuous variables were analyzed by the Mann–Whitney test, and dichotomous variables were analyzed by the Pearson χ^2 test.

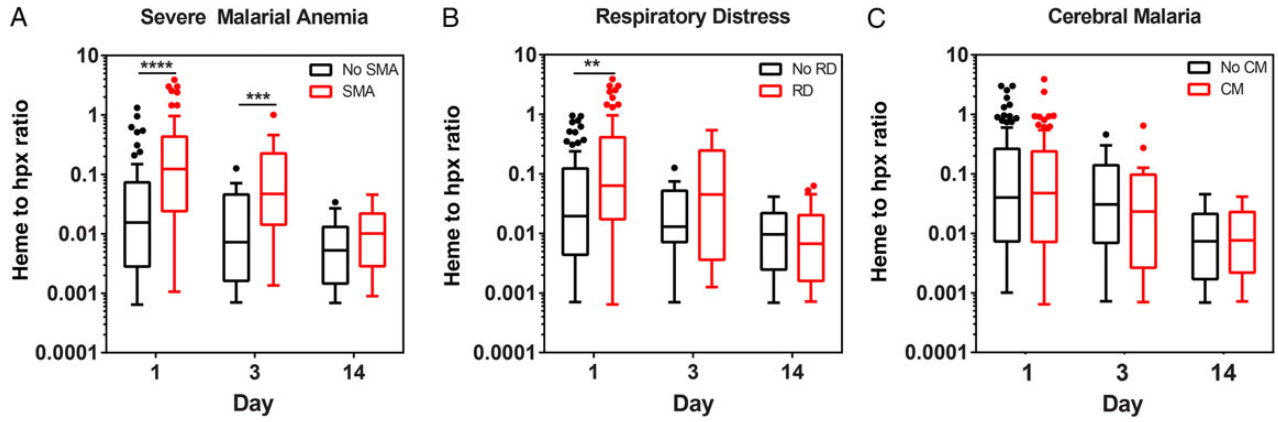

Figure 2. Ratios of extracellular heme to hemopexin (hpx) at admission were associated with common disease presentations of severe malaria. *A* and *B*, Children who presented with severe malarial anemia (SMA; *A*) or respiratory distress (RD; *B*) had significantly higher ratios of heme to hpx at admission than children without these presentations, and these differences resolved with clinical recovery. *C*, There was no significant difference in ratios of heme to hpx between children who presented with cerebral malaria (CM) and those who did not; however, the majority of children enrolled in this study presented with altered consciousness. Children often presented with >1 manifestation of the disease. Plasma levels of extracellular heme and hemopexin were measured at admission (day 1), during hospitalization (day 3), and during follow-up (day 14). Data are presented as Tukey box plots. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$, by the Mann–Whitney test.

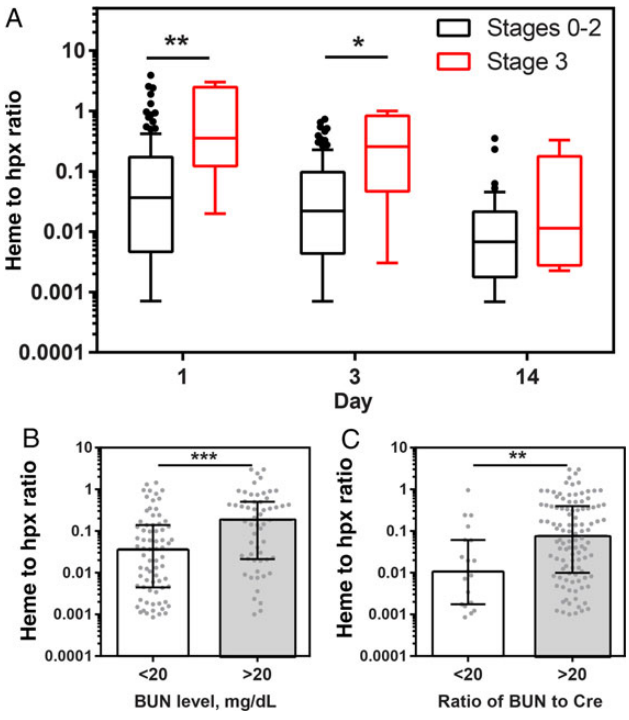

Figure 3. Ratios of extracellular heme to hemopexin (hpx) were associated with acute kidney injury in children with severe malaria. *A*, Children in the placebo arm were classified using KDIGO guidelines as having either stages 0–2 or stage 3 acute kidney injury, and their ratios of heme to hpx were compared over time. Children with stage 3 acute kidney injury had significantly higher ratios of heme to hpx at admission and on day 3 than those classified as having stage 0–2. Differences between the 2 groups resolved by day 14 of follow-up. Children with admission levels of blood urea nitrogen (BUN) of >20 mg/dL (*B*) or a ratio of BUN to creatinine (Cre) of >20 (*C*) had significantly larger ratios of heme to hpx. Data are presented as Tukey box plots or median values with interquartile ranges. Mann–Whitney, $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$, by the Mann–Whitney test.

day 3 of hospitalization than those admitted without acidosis (median, 0.183 vs 0.024 on day 1 [$P < .001$] and 0.115 vs 0.011 on day 3 [$P < .001$]). By follow-up at day 14, there was no significant differences in the heme to hpx ratio between any of the groups. There was no difference in ratios of heme to hpx between children with cerebral malaria versus those without cerebral malaria at any of the times measured ($P > .05$; Figure 2*C*).

Acute Kidney Injury Is Associated With Elevated Ratios of Heme to hpx

To investigate a potential role for the heme axis in AKI, we compared ratios of heme to hpx in children with the most severe AKI (stage 3; n = 8) to those in children with less severe or no AKI (stages 0–2; n = 83). Owing to the association of AKI with the use of inhaled nitric oxide observed in the trial [9], only children in the placebo arm were included in this analysis. Children classified as having stage 3 AKI had significantly higher ratios of heme to hpx at admission and on day 3 of hospitalization as compared to children with stage 0–2 AKI (median, 0.354 vs 0.037 [$P < .01$] on day 1 and 0.256 vs 0.022 on day 3 [$P < .05$]; Figure 3*A*). By follow-up day 14, the difference between groups had resolved (median, 0.011 vs 0.007; $P = .426$).

Elevated BUN level (> 20 mg/dL) has previously been linked to increased mortality in children with severe malaria [8]. In this study, ratios of heme to hpx were significantly higher in children with a BUN level of > 20 mg/dL on admission, compared with those with a BUN level of ≤20 mg/dL (median, 0.185 vs 0.037; $P < .001$; Figure 3*B*). Similarly, children with ratios of BUN to creatinine of >20 had significantly higher ratios of heme to hpx than children with BUN to creatinine ratios of <20 (median, 0.077 vs 0.011; $P < .01$; Figure 3*C*).

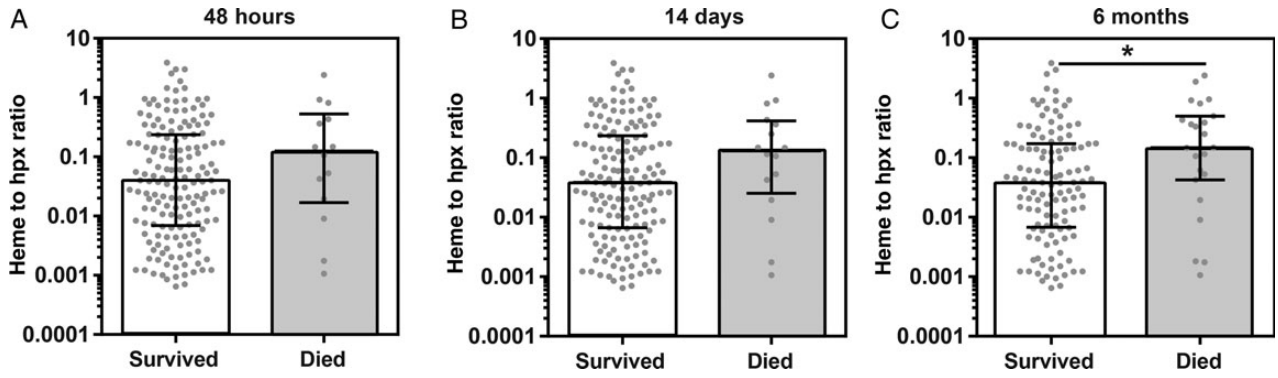

Figure 4. Admission ratios of extracellular heme to hemopexin (hpx) were associated with mortality in children with severe malaria. Ratios of heme to hpx trended toward being higher at admission in children who died within 48 hours (*A*) or 14 days (*B*) of admission and were significantly higher in children who died within six months of admission (*C*). Data are median values and interquartile ranges. **P* < .05 by the Mann–Whitney test.

Admission Ratio of Heme to hpx Is Associated With Increased All-Cause Mortality at 6 Months

Last, we assessed the association between heme to hpx ratios at admission and short-term and long-term mortality. Children who died of infection within either 48 hours or 14 days had an approximately 3-fold higher ratio of heme to hpx at admission than children who survived these periods, but the ratios of heme to hpx at admission did not reach statistical significance in predicting mortality at 48 hours (median, 0.126 among 14 who died vs 0.040 among 165 who survived; $P = .232$; Figure 4*A*) or day 14 (median, 0.130 among 16 who died vs 0.039 among 163 who survived; $P = .139$; Figure 4*B*). However, a higher heme to hpx ratio at presentation was associated with increased long-term mortality, with a median ratio that was approximately 4-fold higher at presentation in children who went on to die within 6 months, compared with those who survived (0.148 among 23 who died vs 0.038 among 120 who survived; $P = .012$; Figure 4*C*). There were no differences in mortality outcomes between the 2 trial arms [19]; therefore, admission ratios of heme to hpx in both treatment arms were assessed. If only children from the placebo arm were included, then there was a trend toward increased admission heme to hpx ratios and all-cause mortality at 6 months (median, 0.130 among 12 who died vs 0.040 among 59 who survived; $P = .060$). However, this latter analysis is likely underpowered.

Ratios of Heme to hpx Are Associated With Plasma Markers of Endothelial Dysfunction

Extracellular heme has previously been reported to cause endothelial activation [11, 12]. To determine whether there was an association between heme and endothelial dysfunction in the context of severe malaria, we compared levels of endothelial activation markers, previously shown to be associated with disease severity and outcome [21], across the heme to hpx ratio quartiles. Children with the highest ratios of heme to hpx had higher levels of Ang-2, sTie2, and sFlt-1 ($P < .01$; Table 1), but there was no difference in CRP levels between children with heme to hpx ratios in quartile 4 and those with ratios in quartiles 1–3 ($P > .05$; Table 1).

DISCUSSION

Children presenting with severe malaria in this study had increased levels of plasma heme and decreased levels of hpx, and these levels improved with clinical recovery. Increased ratios of heme to hpx at admission were associated with severe malarial anemia, respiratory distress, and metabolic acidosis. Moreover, dysregulation of the balance of extracellular heme to hpx at admission was associated with AKI and long-term mortality.

Ratios of heme to hpx were associated with several markers previously linked to acute mortality in children with severe malaria. Markers of endothelial dysfunction are established prognostic factors in children with severe malaria [21]. Levels of the endothelial activation markers sFlt-1, Ang-2, and sTie2 were significantly elevated in the children with the highest ratios of heme to hpx, supporting an association between increased extracellular heme and endothelial dysfunction, a key pathogenic process in severe malaria [4]. Heme has been directly implicated in the mediation of endothelial activation. It is associated with exocytosis of Weibel-Palade bodies, both in vitro [12] and in vivo [11], which results in the release of P-selectin, Ang-2, and von Willebrand factor. Endothelial cells exposed to heme downregulate expression of complement regulatory proteins and have the ability to directly activate complement, leading to enhanced endothelial dysfunction and permeability [12]. Endothelial cell adhesion molecules are also upregulated in response to heme exposure [11], potentially contributing to enhanced sequestration of parasitized erythrocytes within the microvasculature.

Ratios of heme to hpx were strongly associated with the presence of both respiratory distress and severe anemia. In children

with severe malaria, respiratory distress is primarily due to metabolic acidosis [8]. Children with the highest ratios of heme to hpx had evidence of metabolic acidosis, including a significantly larger base excess and increased lactate and decreased bicarbonate levels. Increased lactate level and larger base excess are known predictors of mortality in children with severe malaria [8, 22]. Collectively, these findings support a hypothesis whereby increased extracellular heme induces endothelial activation [10–12], leading to increased sequestration of parasitized erythrocytes, microvasculature obstruction, and tissue hypoxia, further impairing endothelial function [23, 24].

Of note, children in the quartile with the highest ratios of heme to hpx had an almost 10-fold lower level of peripheral parasitemia, compared with children in the lower 3 quartiles. This may be due to increased sequestration of parasitized erythrocytes within the microvasculature associated with extracellular heme. This contention is supported by Dalko et al, who observed a significant positive correlation between heme and parasite burden, as measured by HRP-2 levels [18]. Conversely, there is evidence that extracellular heme may limit the permissibility of red blood cells to merozoite invasion, thereby leading to lower parasitemia counts in both in vitro culture and in vivo mouse models [25]. This may also explain, at least partly, the association between severe malarial anemia and lower parasite burdens [21, 26]. Additional investigations into the association between high heme to hpx ratio and peripheral parasitemia are warranted.

Despite strong associations with both severe malarial anemia and respiratory distress, in this study the heme:hpx axis was not associated with neurologic manifestations of severe malaria. There were no observed differences in ratios of heme to hpx in children presenting with or without cerebral malaria or seizures. This was somewhat unexpected given that hpx has been implicated in neuroprotection in vitro [27, 28] and in in vivo animal models [29, 30]. Furthermore, in adults with severe malaria, increasing hpx concentrations were previously linked to improved Glasgow coma scores [18]. It is possible that differences in the function of the blood brain barrier between adults and children [31] may alter the neurological response to heme and/or hpx in children. However, it is important to note that the majority of children in this study (161 of 179 [90%]) had a decreased level of consciousness (Blantyre coma score, $\leq$3), which makes it challenging to determine whether there is an association between neurological symptoms and the ratio of heme to hpx in this pediatric cohort.

There has been increasing interest in the prevalence and role of AKI in children with severe malaria [8]. The levels of AKI were recently reported for our patient population [9], with 46% of children classified as having some form of AKI (stage 1–3), of whom 26% had stage 3 AKI [9]. AKI was associated with an increased risk of mortality [9]. Owing to the well-known role of extracellular heme/hemoglobin as potential mediators of kidney injury, the role of extracellular heme was investigated in this cohort. KDIGO classification is based on fold-changes in creatinine level, and it has previously been reported in adults with severe malaria that hpx is negatively correlated with levels of creatinine [18]. In agreement with this observation, children in this study with the highest fold changes in creatinine (ie, stage 3 AKI) had significantly elevated ratios of heme to hpx. Additionally, ratios of heme to hpx were significantly elevated in children with elevated levels of BUN, a marker previously associated with mortality [8, 9].

Extracellular heme may mediate AKI by promoting endothelial dysfunction and impaired renal perfusion. Children with the highest ratios of heme to hpx also had elevated levels of Ang-2 and sFlt-1, both of which have been implicated in renal injury [32–35]. Heme is also able to induce the expression of endothelial adhesion markers, including intercellular adhesion molecule 1 [11], which could lead to sequestration of parasitized erythrocytes within the renal microvasculature, further impairing renal perfusion [36].

Prerenal azotemia is a common putative mechanism of AKI in this pediatric population; however, BUN levels were only available at admission, and we were unable to determine whether sustained hypoperfusion resulted in intrarenal injury. Sequestration of parasitized erythrocytes within the renal vasculature would result in local hemolysis and the release of heme, where increased local concentrations may contribute to renal endothelial and tubular cell death [37] and permanent renal injury. Further mechanistic studies of the role of free heme in the pathophysiology of the AKI in the context of pediatric severe malaria are warranted.

AKI, even after full recovery, is associated with the development of chronic kidney disease [38]. Furthermore, repeated episodes of AKI, as may occur in malaria-endemic settings, further increase the risk of chronic kidney disease [38]. Other causes of increased extracellular heme, including hemoglobinopathies common in sub-Saharan Africa, likely contribute to heme-mediated AKI. With an increasing prevalence of chronic kidney disease in Africa [39], it is important to understand the role for malaria-induced AKI in the development of chronic kidney injury later in life.

In our study, increased ratios of heme to hpx at presentation were associated with increased all-cause 6-month mortality. Increased heme released during malaria parasite infection may increase the susceptibility of erythrocytes to subsequent rupture through multiple mechanisms, including changes in pH [40]. Erythrocytes become increasingly rigid in the presence of heme [41], leading to increased microvascular obstruction, increased rupture within the microvasculature, and increased clearance by the spleen. Following hemolysis, compensatory pathways are activated, including the production of erythropoietin, to promote the production of new erythrocytes. Despite increases in erythropoietin, patients with severe malaria have

inadequate erythropoiesis [42]. Similarly, mice injected with heme show both elevated erythropoietin levels and inadequate erythropoiesis, as indicated by decreased numbers of reticulocytes [43]. This suggests that heme may be playing an inhibitory role downstream of erythropoietin, potentially via marrow suppression. Hemozoin, with its ability to generate oxidative stress, has been shown to mediate erythroid cell death [44]. We hypothesize that heme may also promote loss of erythroid cells in a prooxidant manner. Additionally, an increased risk of bacteremia following malaria parasite infection has been linked with hemolysis and impaired neutrophil function, further contributing to the risk of posthospital mortality [45]. Collectively, our findings add additional mechanistic insights to those of Phiri et al, who reported higher postdischarge mortality in Malawian children with severe anemia [46].

Strengths of our study included its ability to assess both extracellular heme and hpx levels longitudinally in a well-annotated prospective study of children with severe malaria during the course of acute infection and clinical recovery. This allowed us to evaluate the kinetics of levels of extracellular heme in relationship to hpx levels and determine their association with disease severity, AKI, response to treatment, and long-term mortality. Limitations include the lack of premorbid assessment of measures of the heme axis and renal function. These measurements would have permitted an assessment of risk related to circulating hpx levels at baseline or whether there was an inability to compensate in response to hemolysis. It is interesting to note that levels of heme and hpx remained altered, with higher heme and lower hpx levels, by day 14 after infection, compared with levels observed in children with uncomplicated malaria, in a previous study (median values, 3 vs 1 μM for heme and 455 vs 993 μg/mL for hpx) [17], suggesting that there is incomplete recovery of the axis by day 14, which may contribute to sustained endothelial activation and long-term mortality in this cohort. As such, it would be of interest to determine the recovery period for the heme axis following severe infection. Additionally, levels of HRP-2 in these children would have provided insight into the relationship between heme and parasite burden, especially considering the lower peripheral parasitemia levels observed in children with the highest ratios of heme to hpx.

In conclusion, we show a strong association between extracellular heme and hpx and microvascular and organ dysfunction that directly contribute to the pathogenesis and mortality of severe malaria, including severe malarial anemia, metabolic acidosis, and AKI. The ratio of extracellular heme to hpx illustrates the biological interaction between a pathological mediator and a physiologically protective response to mitigate toxicity. Interventions that aim to normalize this ratio, either by decreasing levels of extracellular heme or increasing levels of circulating hpx, may contribute to improved outcomes in children with severe malaria.

Notes

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Potential conflicts of interest. In accordance with institutional regulations, H. S. W. declared use of hemopexin to the Massachusetts General Hospital as a molecule to treat inflammation, and the institution has obtained patent protection. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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