Diagnosis, prognosis and treatment of severe falciparum malaria in African children
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Chapter 5

Diagnosing severe falciparum malaria in parasitaemic African children; a prospective evaluation of plasma PfHRP2 measurement

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Summary

Background
In African children, distinguishing severe falciparum malaria from other severe febrile illnesses with coincidental Plasmodium falciparum parasitaemia is a major challenge. P. falciparum histidine-rich protein-2 (PfHRP2) is released by mature sequestered parasites and can be used to estimate the total parasite burden. We investigated the prognostic significance of plasma PfHRP2 and used it to estimate the malaria-attributable fraction in African children diagnosed with severe malaria.

Methods and findings
Admission plasma PfHRP2 was measured prospectively in African children (from Mozambique, The Gambia, Kenya, Tanzania, Uganda, Rwanda, and the Democratic Republic of the Congo) aged 1 month to 15 years with severe febrile illness and a positive Plasmodium lactate dehydrogenase (pLDH)-based rapid test in a clinical trial comparing parenteral artesunate versus quinine (the AQUAMAT trial, ISRCTN 50258054). In 3826 severely ill children, plasma PfHRP2 was higher in patients with coma (p=0.0209), acidosis (p<0.0001), and severe anaemia (p<0.0001). Admission geometric mean (95% CI) plasma PfHRP2 was 1611 (1350-1922) ng/mL in fatal cases (n=381) versus 1046 (991-1104) ng/mL in survivors (n=3445; p<0.0001), without differences in parasitaemia as assessed by microscopy. There was a U-shaped association between log_{10} plasma PfHRP2 and risk of death. Mortality increased 20% per log_{10} increase in PfHRP2 above 174 ng/mL (adjusted odds ratio [AOR] 1.21, 95% CI 1.05-1.39; p=0.009). A mechanistic model assuming a PfHRP2 independent risk of death in non-malaria illness closely fitted the observed data and showed malaria-attributable mortality less than 50% with plasma PfHRP2 ≤174 ng/mL. The odds ratio (OR) for death in artesunate versus quinine-treated patients was 0.61 (95% CI 0.44-0.83; p=0.0018) in the highest PfHRP2 tertile, whereas there was no difference in the lowest tertile (OR 1.05, 95% CI 0.69-1.61; p=0.82). A limitation of the study is that some conclusions are drawn from a mechanistic model, which is inherently dependent on certain assumptions. However, a sensitivity analysis of the model indicated that the results were robust to a plausible range of parameter estimates. Further studies are needed to validate our findings.

Interpretation
Plasma PfHRP2 has prognostic significance in African children with severe falciparum malaria and provides a tool to stratify the risk of “true” severe malaria-attributable disease as opposed to other severe illnesses in parasitaemic African children.
Introduction

Severe falciparum malaria in children presents a major diagnostic challenge in malaria-endemic countries where a high proportion of children is parasitaemic at any time. A positive malaria blood smear is therefore not specific for severe malaria, and neither are clinical signs which are similar to those of other severe childhood infections.\(^1\)\(^-\)\(^3\) Overdiagnosis of falciparum malaria in severely ill children is an important problem in sub-Saharan Africa.\(^4\)\(^,\)\(^5\) Misdiagnosis is associated with increased mortality.\(^6\) Autopsy studies in children dying with “slide-positive” cerebral malaria show an alternative diagnosis in up to 23% of cases.\(^4\) The central pathological process in severe falciparum malaria is sequestration of trophozoite- and schizont-stage infected erythrocytes in venules and capillaries, which compromise microcirculatory flow to vital organs.\(^7\) The circulating young ring-form parasites do not sequester and therefore do not reflect accurately the sequestered parasite burden. Thus peripheral parasite counts have weak prognostic significance,\(^8\)\(^,\)\(^9\) although this can be improved by assessing the stage of development of these peripheral blood parasites or counting the numbers of malaria pigment-containing neutrophils which reflects recent schizogony.\(^10\)\(^,\)\(^11\)

*Plasmodium falciparum* histidine-rich protein-2 (*Pf*HRP2) is a water-soluble protein found inside the malaria parasite and host erythrocyte, and that circulates free or bound to proteins or antibodies in the plasma compartment.\(^12\)\(^,\)\(^13\) *Pf*HRP2 production peaks during the trophozoite stage, and approximately 90% is released during schizont rupture.\(^14\) Since released *Pf*HRP2 is distributed through the total plasma volume, plasma *Pf*HRP2 can be considered a measure of total parasite burden of the preceding 48-hour asexual parasite life cycle.\(^14\)\(^,\)\(^15\) Studies in Asian adults have shown a strong correlation between plasma *Pf*HRP2, disease severity and outcome.\(^15\)\(^,\)\(^16\)

In the current study we assessed the prognostic significance of plasma *Pf*HRP2 in African children with severe malaria and tested the hypothesis that its assessment could distinguish children with “true” severe malaria, in need of urgent antimalarial treatment, from those with non-malarial severe febrile illness and coincidental peripheral blood parasitaemia, in whom alternative diagnoses and additional treatment need to be considered.
Methods

The study was part of a large multinational trial comparing quinine and artesunate for the treatment of severe malaria in African children (“AQUAMAT”, ISRCTN 50258054), undertaken between October 2005 and July 2010. Ethics approval was granted by the Oxford Tropical Research Ethics Committee and the countries’ ethics review boards. Full details of this trial have been described elsewhere. In brief, children with signs of severe malaria confirmed by a positive *P. falciparum* lactate dehydrogenase (pLDH)-based rapid diagnostic test were included, provided their parents or carers gave full written informed consent. Severity was defined by clinical criteria (see Text S1). Patients were excluded if treated parenterally for >24 hours before admission. Patients were randomised to treatment with either parenteral artesunate or quinine. A venous blood sample was taken for peripheral blood slide, haematocrit (Hct), *Pf* HRP2, biochemistry, and acid-base parameters (EC8+ cartridge for i-STAT handheld blood analyser). Slide reading was performed by expert microscopists at the Mahidol-Oxford Tropical Medicine Research Unit, and parasites/µL was calculated from thin film (count/1000 RBC x 125.6 x Hct) or thick film (count/200 WBC x 40). Plasma *Pf* HRP2 was assessed blinded to patient outcomes from freeze-thawed EDTA plasma samples by a commercial sandwich ELISA kit (Celisa, Cellabs, Sydney, Australia), according to the manufacturer’s instructions with minor modifications. Pooled reference plasma from 20 subjects with *P. falciparum* parasitaemia >200 0000/µl was calibrated with recombinant *Pf* HRP2 standard (kindly provided by D. Sullivan, John Hopkins School of Public Health, Baltimore, USA) and used to construct standard curves. Concentrations in duplicate plasma dilutions (1/25 to 1/3125 in PBS/0.01%Tween) were determined according to the linear segment of the standard curve, with re-assay in cases where duplicates differed by more than 50%. Plasma samples for *Pf* HRP2 were received from 9 of the 11 AQUAMAT research sites in 7 countries (Mozambique, The Gambia, Kenya, Tanzania, Uganda, Rwanda and the Democratic Republic of the Congo). The study site in Ghana did not collect samples and the samples from Nigeria defrosted during transportation.

Individual patient estimation of parasite burden

Estimation of the total body parasite burden from plasma *Pf* HRP2 has been described in detail in Asian adults with severe malaria and requires incorporation of an elimination half-life estimate.
This was assessed separately in African children because clearance might be dependent on immunity (antibodies against \( Pf/HRP2 \)), which is greater in high transmission settings, and \( Pf/HRP2 \) production is parasite strain dependent.\(^{20} \) Plasma \( Pf/HRP2 \) half-life was assessed in 30 patients from Tanzania from samples taken on admission and after 3 and 7 days following treatment. Separate ethical approval for this sub-study was obtained from the Ethics Committee of the National Institute for Medical Research, Tanzania (NIMR/HQ/R.8c/Vol.I/60). These data were analysed using WinNonlin statistical package (Pharsight, Mountain View, California, USA). Individual \( Pf/HRP2 \) concentration-time curves were fitted according to a first-order elimination model. From this, a mean (95% CI) plasma elimination half-life (\( t_{1/2} \)) was estimated as 1.10 (0.91–1.29) days, or 0.55 erythrocytic cycles. Half-life was not significantly different between treatment arms, and was not correlated with renal function (estimated by blood urea nitrogen). A parasite multiplication factor of 3 immediately before peak parasitaemia was assumed, based on in-vitro and Saimiri monkey studies of African parasite strains causing severe malaria.\(^{21,22} \) Higher multiplication rates were explored in a sensitivity analysis.\(^{23,24} \) The formula for total parasite burden is: \( P_{\text{tot}} = 7.3 \times Pf/HRP2 \times (1-Hct) \times \text{body weight} \times 10^{13} \), with \( Pf/HRP2 \) in g/L.\(^{15} \) The differences in the current formula with the one used earlier in adult Asian patients result from the different estimates for plasma \( Pf/HRP2 \) half-life and parasite multiplication rates. The circulating parasite burden was calculated from the peripheral blood parasites/\( \mu \)L x \( 10^6 \) x blood volume (=0.08 x weight [kg]).\(^{15} \) The sequestration index was calculated as total parasite burden/circulating burden.\(^{25} \)

**Statistical analysis**

Data were analysed with STATA, version 10 (StataCorp, TX, USA). Categorical variables were compared between survivors and fatal cases with \( \chi^2 \) or Fisher’s exact test. Normally distributed or \( \log_{10} \)-normalized variables were compared using a Student’s \( t \) test, the remainder by Wilcoxon rank-sum test. For lowest, middle, and highest tertiles of plasma \( Pf/HRP2 \), comparisons were made between peripheral blood parasitaemia, sequestration index, and treatment effect (mortality) following artesunate versus quinine treatment.

To determine the prognostic significance of plasma \( Pf/HRP2 \), a logistic regression model was constructed with in-hospital death as the dependant variable and \( Pf/HRP2 \) as the independent variable. Since the risk of death showed a non-linear association with \( \log_{10} Pf/HRP2 \) (Figure 2A), both first- and second- degree fractional polynomial functions were explored to find the optimal fit. A quadratic polynomial function provided the best fit using the likelihood ratio test and by comparison of the areas under the curve (AUCs). The regression model was stratified for study site and adjusted for treatment and other
established predictors of death, including coma, convulsions, prostration, hypoglycaemia, respiratory distress, shock (combined compensated and decompensated), parasitaemia (parasites/µL), haemoglobin (Hb; g/dl), blood urea nitrogen (BUN; mg/dL), and base excess (BE; mmol/L).8,9 Using a stepwise approach, only covariates that were significant at \( P < 0.01 \) were retained in the final model. Fit of the final logistic regression model was confirmed using the Hosmer-Lemeshow goodness-of-fit test after ordering the data on predicted probabilities and then regrouping the data into 10 nearly equal-sized groups.26 Any interaction by transmission intensity regarding associations between plasma \( PfHRP2 \) and survival was checked and accounted for, if significant. Study sites in Mozambique and The Gambia were defined as low transmission; Rwanda, Tanzania, and Kenya as intermediate; and study sites in Uganda and the Democratic Republic of the Congo as high transmission.

**Modelling malaria-attributable mortality based on plasma \( PfHRP2 \)**

A mechanistic model was constructed to describe the observed U-shaped relationship between \( PfHRP2 \) strata and probability of in-hospital death (Figure 2A) making the following assumptions: (1) an exponential increase of malaria-attributable mortality with plasma \( PfHRP2 \), which describes the right side of the curve in Figure 2A:

\[
Pr_{\text{death|malaria}} = -1 + \exp (k_1 \log PfHRP2^{k_2});
\]

(2) a probability of severe febrile illness due to non-malaria which decreased exponentially with increasing \( \log PfHRP2 \):

\[
Pr_{\text{non-malaria}} = \exp (-k_4 \log PfHRP2);
\]

(3) a risk of death in patients with non-malaria infection equal to 0.3, independent of plasma \( PfHRP2 \): \( Pr_{\text{death|non-malaria}} = 0.3 \);5,27 and (4) that 20% of all deaths were due to non-malaria illness: \( \text{Death}_{\text{non-malaria}}/\text{Death}_{\text{total}} = 0.2 \).4 The number of non-malarial deaths according to \( PfHRP2 \) stratum is then given by:

\[
\text{Death}_{\text{non-malaria}} = Pr_{\text{death|non-malaria}} \times Pr_{\text{non-malaria}} \times \text{Cases}_{\text{total}}
\]

and the number of deaths due to malaria by:

\[
\text{Death}_{\text{malaria}} = Pr_{\text{death|malaria}} \times (1-Pr_{\text{non-malaria}}) \times \text{Cases}_{\text{total}}
\]

For more details, see Text S2. The effects of assumptions 3 and 4 were explored in a sensitivity analysis.

**Results**

**Patient characteristics**

Of the 5425 children with pLDH-based rapid diagnostic test (RDT) confirmed falciparum malaria included in the AQUAMAT trial, plasma \( PfHRP2 \) was measured in 3826 patients. \( PfHRP2 \) could not be measured in 1600 (29%) patients either because the sample was either not collected or not received in optimal condition. Patients without \( PfHRP2 \)
data did not differ from the remainder regarding malaria slide positivity rate, geometric mean parasitaemia, or case fatality rate. Baseline clinical and laboratory characteristics according to outcome are summarized in Table 1. Although many clinical and laboratory variables associated with severity differed between survivors and fatal cases, admission parasitaemia did not.

**Table 1.** Demographic, clinical and laboratory characteristics of children diagnosed with severe falciparum malaria according to outcome

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Survivors (n=3445)</th>
<th>Fatal cases (n=381)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female sex</td>
<td>1692 (49%)</td>
<td>188 (49%)</td>
<td>0.93</td>
</tr>
<tr>
<td>Age, y</td>
<td>2.7 (1.5–4)</td>
<td>2.3 (1.4–4)</td>
<td>0.055</td>
</tr>
<tr>
<td>Fever before enrolment, d</td>
<td>3 (2–4)</td>
<td>3 (2–4)</td>
<td>0.54</td>
</tr>
<tr>
<td>Coma before enrolment, h</td>
<td>4 (2–8)</td>
<td>5 (3–8)</td>
<td>0.020</td>
</tr>
<tr>
<td><strong>Complications on admission</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coma (GCS ≤10 or BCS ≤2)</td>
<td>983 (29%)</td>
<td>247 (65%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Convulsions</td>
<td>1176 (34%)</td>
<td>186 (49%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Severe acidosis (BE &lt; -8 mmol/L)</td>
<td>1132 (41%)</td>
<td>251 (80%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Severe anaemia (Hb &lt;5 g/dl)</td>
<td>841 (29%)</td>
<td>117 (34%)</td>
<td>0.030</td>
</tr>
<tr>
<td>Hypoglycaemia</td>
<td>317 (9%)</td>
<td>136 (36%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Respiratory distress</td>
<td>466 (14%)</td>
<td>103 (27%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Shock (combined)</td>
<td>470 (14%)</td>
<td>100 (26%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Black Water Fever</td>
<td>126 (4%)</td>
<td>18 (5%)</td>
<td>0.30</td>
</tr>
<tr>
<td>Jaundice</td>
<td>75 (2%)</td>
<td>16 (4%)</td>
<td>0.014</td>
</tr>
<tr>
<td>Hyperparasitaemia</td>
<td>778 (25%)</td>
<td>101 (30%)</td>
<td>0.046</td>
</tr>
<tr>
<td><strong>Laboratory assessments</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. falciparum</em> slide positive</td>
<td>99%</td>
<td>98%</td>
<td>0.088</td>
</tr>
<tr>
<td>Parasitaemia, geometric mean (range)</td>
<td>45 008 (0-1 858 880)</td>
<td>39 589 (0-1 252 227)</td>
<td>0.33</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/dL)</td>
<td>15 (11)</td>
<td>23 (16)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Haemoglobin g/dL</td>
<td>6.9 (2.8)</td>
<td>6.5 (2.9)</td>
<td>0.015</td>
</tr>
<tr>
<td>pH</td>
<td>7.38 (0.11)</td>
<td>7.24 (0.19)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HCO3 (mmol/L)</td>
<td>17.0 (5.4)</td>
<td>11.3 (5.8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Base excess (mmol/L)</td>
<td>-8 (7)</td>
<td>-16 (8)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Data are No. (%) of patients, median (IQR), or mean (SD), unless otherwise indicated.

Abbreviations: BCS, Blantyre coma scale; BE, base excess; GCS, Glasgow coma scale; Hb, haemoglobin
**Plasma PfHRP2 in relation to disease severity**

*Pf*HRP2 was detectable in 3800/3826 (99%) patients with severe malaria. A detectable plasma *Pf*HRP2 (geometric mean 450 ng/mL, 95% CI 209–966 ng/mL) with a negative blood slide result (but positive malaria RDT) was found in 36 (0.9%) children. Geometric mean plasma *Pf*HRP2 (95% CI) in survivors was 1046 ng/mL (991–1104 ng/mL) versus 1611 ng/mL (1350–1922 ng/mL) in fatal cases (p<0.0001; Table 2). There was no heterogeneity by stratification for transmission intensity in the difference of plasma *Pf*HRP2 concentrations between survivors and fatal cases (p=0.1). Plasma *Pf*HRP2 concentrations in relation to established features of severe falciparum malaria are summarized in Table 2. Plasma *Pf*HRP2 was significantly higher in patients with coma, acidosis, and severe anaemia but not in those with shock.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Trait</th>
<th>n</th>
<th>Plasma <em>Pf</em>HRP2</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outcome</td>
<td>Fatal</td>
<td>381</td>
<td>1611 (1350–1922)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Surviving</td>
<td>3445</td>
<td>1046 (991–1104)</td>
<td></td>
</tr>
<tr>
<td>Coma (GCS ≤10 or BCS ≤2)</td>
<td>Yes</td>
<td>1230</td>
<td>1193 (1079–1320)</td>
<td>0.0209</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>2596</td>
<td>1047 (986–1111)</td>
<td></td>
</tr>
<tr>
<td>Acidosis (BE &lt; -8 mmol/L)</td>
<td>Yes</td>
<td>1383</td>
<td>1494 (1382–1614)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>1692</td>
<td>969 (896–1047)</td>
<td></td>
</tr>
<tr>
<td>Severe anaemia (Hb &lt; 5 g/dL)</td>
<td>Yes</td>
<td>958</td>
<td>1585 (1458–1722)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>2306</td>
<td>1044 (975–1118)</td>
<td></td>
</tr>
<tr>
<td>Shock</td>
<td>Yes</td>
<td>570</td>
<td>1193 (1051–1355)</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>3256</td>
<td>1075 (1016–1138)</td>
<td></td>
</tr>
</tbody>
</table>

*Abbreviations: BCS, Blantyre coma scale; BE, base excess; GCS, Glasgow coma scale; Hb, haemoglobin
* Data are geometric mean (95% CI)
* BE available for n=3075 and Hb available for n=3264 due to missing i-STAT values
* Compensated and decompensated shock combined

**Estimated total body parasite burden**

Geometric mean (95% CI) *Pf*HRP2-derived total parasite burden was 7.5x10¹¹ (7.2x10¹¹ to 7.9x10¹¹) parasites/body (n=3800); this was greater in fatal cases (1.2x10¹² [1.0x10¹²–1.5x10¹²], n=327) than in survivors (7.2x10¹¹ [6.8x10¹¹–7.6x10¹¹], n=3070; p<0.0001; Figure 1). In contrast, the total circulating peripheral blood parasite burden did not differ significantly between survivors and fatal cases (p=0.66). The geometric mean (95% CI)
calculated sequestration index, the ratio of total parasitaemia to circulating parasitaemia was 17 (15–18) in survivors, versus 30 (23-40) in fatal cases (p=0.0001). The sequestered parasite burden, calculated by subtracting the circulating parasite burden from the total parasite burden, gave a negative result in 296/3397 (8.7%) patients. Excluding these patients, the geometric mean (95% CI) total sequestered parasite burden was $7.7 \times 10^{11}$ parasites/body ($7.3 \times 10^{11}$-8.2x10$^{11}$, n=3101). A sensitivity analysis varying the multiplication factor and PfHRP2 plasma half-life is shown in Text S3.

**Figure 1**

![Comparison of circulating parasite burden and total parasite burden between surviving (circles, n=3070) and fatal (squares, n=327) cases. Circulating parasite burden was calculated from the peripheral blood parasitaemia and the total parasite burden was estimated from plasma PfHRP2, including 3397 patients with both detectable PfHRP2 and malaria parasites on the peripheral blood smear.](image)

**Plasma PfHRP2 and risk of death**

There was a U-shaped association between plasma PfHRP2 and risk of death with a nadir in case fatality rate at a logPfHRP2 of 2.24 (=174 ng/mL; Figure 2A). In an adjusted logistic regression model, stratified by study site, plasma PfHRP2 was a strong independent predictor of death. Odds for death were 20% higher per unit increase in logPfHRP2 (adjusted odds ratio [AOR] 1.21, 95% CI 1.05-1.39; p=0.009) above
a threshold log$_{10}$P$f$HRP2 value of 2.24 (=174 ng/ml). Below this concentration, risk of death increased with decreasing plasma log$_{10}$P$f$HRP2 (AOR 2.3, 95% CI 1.1-5.0; p=0.03). The final model was adjusted for plasma BE, BUN, coma, convulsions, hypoglycaemia, peripheral blood parasitaemia and antimalarial treatment (Hosmer-Lemeshow ρ-value for goodness-of-fit=0.35).

Figure 2

2A

2B
Figure 2. Continued

Observed and modelled malaria-attributable mortality and morbidity according to plasma PfHRP2 concentrations

2A. Observed number of patients (grey bars, n=3826) and observed probability of death (squares with 95% CI error bars, n=381) according to PfHRP2 half-log_{10} strata. The statistical polynomial regression model (dashed line) and the mechanistic model (black line) show the probability of death according to PfHRP2 half-log_{10} strata. For a detailed description of the mechanistic model see Text S2.

2B. Malaria-attributable mortality and morbidity according to plasma PfHRP2 concentrations. The curve derived from the mechanistic model (Figure 2A) describing the relationship between log_{10} plasma PfHRP2 concentration and probability of death has been deconvoluted in two separate functions: (1) Non-malaria-attributable probability of death (dotted line, left axis), which describes the negative exponential probability of dying from non-malaria illness with increasing plasma PfHRP2 concentrations, at a constant PfHRP2 independent case fatality rate of 30%. (2) Malaria-attributable probability of death (thin solid line, left axis), which describes the exponential increase in the probability of death with increasing plasma PfHRP2 concentration, a measure of total parasite burden, in the patient population with “true” severe malaria. From these deconvoluted functions the proportion of the total number of deaths attributable to “true” severe malaria was derived according to PfHRP2 half-log_{10} strata (diamonds and heavy solid line, malaria-attributable deaths, right axis). Using the “true” severe malaria case fatality rates per PfHRP2 half-log_{10} strata, the proportion of “true” severe malaria-attributable cases according to PfHRP2 half-log_{10} strata was derived (circles and dashed line, malaria-attributable cases).

Distinguishing death attributable to severe malaria from death attributable to other causes

High mortality rates were associated with either low or very high values of plasma PfHRP2 (Figure 2A), with the former presumably resulting from a disease other than malaria (including sepsis). The observed case fatalities in the lowest PfHRP2 half log stratum and the higher PfHRP2 strata of ≥3.5-4.0 were both over 15%. A mechanistic model describing the U-shaped correlation between log PfHRP2 stratum and risk of death showed a good fit with the observed data and the statistical model (Figure 2A). This model was deconvoluted into 2 separate functions corresponding to non-malaria- and malaria-attributable case fatality rates (Figure 2B). The model showed that below a plasma logPfHRP2 value of 2.24 (=174 ng/mL) (derived from the nadir in the polynomial logistic regression model), the probability that death resulted from malaria fell below
50%, corresponding to overall proportions of malaria-attributable severe disease <90% (Figure 2B). In the logPfHRP2 stratum of 3 to 3.5 (1000–3162 ng/mL) and above, the absolute risk of death due to malaria exceeded 8% with a probability of “true” severe malaria >95% and a probability that a death was caused by severe malaria >85% (Figure 2B). For a sensitivity analysis of the mechanistic model see Text S2.

In patients within the highest PfHRP2 tertile, corresponding to logPfHRP2 ≥3.4 (2300 ng/ml), the odds ratio (OR) for death in patients treated with artesunate versus quinine was 0.61 (95% CI 0.44–0.83; p=0.0018). In patients in the lowest PfHRP2 tertile, there was no difference in mortalities with an odds ratio for death of 1.05 (95% CI 0.69–1.61; p=0.82; Figure 3). The geometric mean (95% CI) sequestration index, the ratio of total to circulating parasite numbers was 69.8 (60.8–80.1) in patients in the highest and 4.6 (4.0–5.3) in the lowest PfHRP2 tertile (Table 3).

**Figure 3**

Treatment effect, as odds ratio for death, of artesunate versus quinine according to plasma PfHRP2 tertiles and compared to the overall treatment effect observed in the AQUAMAT trial\textsuperscript{17} in 5425 African children and in the similar SEAQUAMAT trial\textsuperscript{32} in 1461 (predominantly) adults in Asia.
Table 3. Parasite density, sequestration index and treatment effect of artesunate versus quinine according to PfHRP2 tertiles

<table>
<thead>
<tr>
<th>PfHRP2 tertilesa</th>
<th>Low (n=1115)</th>
<th>Middle (n=1154)</th>
<th>High (n=1128)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma PfHRP2 (n=3397) (range, geometric mean, 95% CI)</td>
<td>0–829 (201–236)</td>
<td>830–2298 (1379–1424)</td>
<td>2299–78 848 (4598–4932)</td>
</tr>
<tr>
<td>Parasitaemia (n=3397) (geometric mean, 95% CI)</td>
<td>32 934 (28 993–37 410)</td>
<td>60 864 (53 924–68 698)</td>
<td>50 597 (44 463–57 577)</td>
</tr>
<tr>
<td>p&lt;0.0001</td>
<td>p=0.041</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequestration Index (n=3397) (geometric mean, 95% CI)</td>
<td>4.6 (4.0–5.3)</td>
<td>16.9 (15.0–19.2)</td>
<td>69.8 (60.8–80.1)</td>
</tr>
<tr>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OR (95% CI) for fatal outcome artesunate versus quinine (n=3826)</td>
<td>1.05 (0.69–1.61)</td>
<td>0.81 (0.54–1.22)</td>
<td>0.61 (0.44–0.83)</td>
</tr>
<tr>
<td>p=0.82</td>
<td>p=0.32</td>
<td>p=0.0018</td>
<td></td>
</tr>
</tbody>
</table>

a Tertiles derived from complete PfHRP2 data set (n=3826)

Discussion

This very large prospective study in African children with severe falciparum malaria shows the strong and independent prognostic value of admission plasma PfHRP2 concentration, but not the conventional peripheral blood malaria parasite count. In addition, plasma PfHRP2 was found to be the best immediate measure available to distinguish severe disease caused by malaria from severe febrile illness resulting from another disease with incidental P. falciparum parasitaemia. Since PfHRP2 is a measure of total parasite burden, this suggests a critical pathophysiological role played by sequestered parasites in severe falciparum malaria. This is supported by studies which have correlated obstruction of microcirculatory flow in the rectal and retinal circulations to disease severity and outcome, the strong prognostic value of metabolic acidosis in severe malaria, and autopsy studies showing intense sequestration in vital organs.8,25,28-31 These results suggest that in areas of moderate or high malaria transmission where a high proportion of children are parasitaemic, admission plasma PfHRP2 can differentiate children at highest risk of death of severe falciparum malaria from those with likely alternative causes of severe febrile illness. These findings are supported by several observations.

Firstly, plasma PfHRP2 derived total parasite numbers (geometric mean 7.5x10^11/body) are biologically plausible, and were significantly higher in fatal cases. In contrast, less pathogenic circulating peripheral blood parasite numbers were not correlated with a fatal
outcome. The calculated sequestration index was 17 in surviving patients and 30 in non-survivors, which is similar to the median (IQR) sequestration index of 40 (9.9–273.8) calculated directly from post-mortem blood vessel counts in 50 Thai and Vietnamese adults who died from cerebral malaria.25

Second, the U-shaped curve with a nadir at 174 ng/mL describing the relationship between PfHRP2 and risk of death fits with the assumption that with low PfHRP2 death is caused by non-malarial febrile illness (including sepsis) which are independent of the low parasite burden, whereas in patients with plasma PfHRP2 above this nadir the probability of death increases with PfHRP2, representing “true” severe malaria with increasing sequestered parasite burdens. The mechanistic model based on these assumptions had a close fit with the observed data. An alternative explanation could be the presence of highly virulent parasite strains causing severe disease independent of a high total parasite burden. However, this would result in a PfHRP2-independent mortality at the left side of the curve and cannot explain the U-shape that was actually observed. Assumptions in constructing the mechanistic model included an alternative cause of death in 20% of patients and a risk of death in non-malaria disease of 30%, based on published autopsy and clinical microbiology data.4,27 The conclusions were robust to the plausible ranges of values defined for the sensitivity analysis.

Third, the treatment benefit of artesunate over quinine was absent in patients in the lowest PfHRP2 tertile, and strongest in the highest tertile (OR 0.61, 95% CI 0.44–0.83; p=0.0018). Since injectable artesunate can benefit only patients with “true” severe malaria, this provides strong supportive evidence that patients with high PfHRP2 do represent this group, and patients with low PfHRP2 do not. The OR of 0.61 in the highest PfHRP2 tertile is remarkably close to the OR of 0.60 (95% CI 0.45–0.79) reported in the large SEAQUAMAT trial comparing artesunate with quinine in the treatment of severe falciparum malaria in 1461 patients in low-transmission settings in Asia.32 In these epidemiological settings incidental peripheral blood malaria parasitaemia is rare. The diagnosis of severe malaria based on a peripheral blood slide is therefore highly specific, and so the treatment effect of artesunate over quinine is undiluted by non-malarial disease.

Identification of children with slide-positive severe febrile illness but who do not have severe malaria is very important for patient management, since overdiagnosis of severe malaria is associated with increased mortality.6 A low plasma PfHRP2 should prompt investigation of alternative diagnoses including septicaemia, early administration of parenteral broad spectrum antibiotics (if not already routine), and intensive monitoring. Often antibiotics are given only after a disappointing clinical response to antimalarials,
which may be too late. High plasma PfHRP2 concentrations should not discourage antibiotic treatment combined with antimalarial treatment, because of the high proportion of concomitant invasive bacterial disease.\textsuperscript{2} Patients with high plasma PfHRP2, which indicates “true” severe malaria with a poor prognosis, should be monitored closely, preferentially in a high-dependency or intensive care unit. As a tool in the design of clinical trials, plasma PfHRP2 is substantially better than peripheral blood parasitaemia in assessing the malaria-attributable fractions and defining the group of patients with “true” severe malaria and a high risk of death (Figure S3 in Text S4 and \textsuperscript{33}).

An alternative tool is the presence of malaria retinopathy, which has been shown to be highly specific for cerebral malaria as confirmed by post-mortem autopsy,\textsuperscript{4} although this tool does require training and skilled ophthalmoscopy.\textsuperscript{34,35} It has been evaluated for cerebral malaria,\textsuperscript{36,37} whereas many patients with severe falciparum malaria presents with other syndromes.\textsuperscript{17} PfHRP2 can be used in both cerebral and severe non-cerebral malaria. A direct comparison between the two methods is currently underway. Development of a semi-quantitative rapid test for the detection of plasma PfHRP2 with carefully chosen thresholds could be a valuable tool in high transmission settings to distinguish “true” severe malaria from severe non-malarial febrile illness. For example, a plasma PfHRP2 concentration $>1000$ ng/mL (62.1\% of cases in our cohort) denotes a probability $>95\%$ of “true” severe malaria with an overall case fatality rate of 11.6\% (95\% CI, 10.3–12.9). Defining populations with “true” severe malaria and high mortality is thus critical information for clinicians as well as researchers. In contrast, a plasma PfHRP2 concentration $<100$ ng/mL (8.1\% of cases in our cohort) denotes a probability $>15\%$ that severe non-malarial illness is the cause of illness, warranting additional investigations.

Limitations of this study include the inherent dependency of the models on certain assumptions. Estimating the total parasite burden from PfHRP2 is sensitive to the assumed parasite multiplication factor. In the current study the multiplication rate was assumed to be 3, based on in vitro data comparing multiplication rates and multiplication potency of parasites obtained from African children compared to Asian adults. The multiplication rate of 8 used in the original model in Asian adults was based on non-immune adult patient data from the era of malaria therapy of neurosyphilis, and comparable information is obviously not available for our patient group. Applying this higher multiplication rate in this study results in an implausibly high total parasite burden. In addition to differences in parasite multiplication rates, the calculated total parasite burden is dependent on the assumed half-life of plasma PfHRP2 which can vary between patients, and on the amount of PfHRP2 released per parasite per cycle, which can vary between strains.\textsuperscript{15,20} A sensitivity analysis of these parameters is shown
in Figure S2 in Text S3. The half-life of plasma \( PfHRP2 \) in the current study was shorter than observed in adult patients in Southeast Asia (mean 1.1 versus 3.7 days).\(^{15} \) This is presumably related to the African setting where malaria transmission is high and immunological factors including high \( PfHRP2 \) antibody titers could increase plasma clearance of \( PfHRP2 \).\(^{38,39} \) Since variations in the model parameter estimates are applied to the entire patient group, the model renders either pathophysiological implausible upper (more parasites than the number of circulating red cells) or lower limits (less total parasites than the calculated circulating parasitaemia). Actual total parasite numbers can thus be slightly different from the model estimates. However, differences in the calculated total parasite burdens between subgroups do not depend on the choice of these variables, since these variables will affect this value by the same factor in all subgroups. A recent study in Papuan children with falciparum malaria did not show a correlation between \( PfHRP2 \) and disease severity.\(^{40} \) However, children (\( n=220 \)) in this study diagnosed with severe malaria appeared to be only moderately ill as reflected by the <1% case fatality rate and low plasma \( PfHRP2 \) values (median 456 ng/mL), whereas patients in that study considered to have uncomplicated malaria had lower plasma bicarbonate concentrations as a measure of acidosis than those with severe malaria. In the present study, <1% cases had undetectable plasma \( PfHRP2 \) concentrations, despite presence of \( P. falciparum \) on the blood slide. This could have been caused by genetic variation in \( PfHRP2 \),\(^{41} \) although this polymorphism is thought not to affect the ELISA assay.\(^{42,43} \) Deletions of the \( PfHRP2 \) gene have been reported in field isolates from the Amazon region and in a single report from sub-Saharan Africa.\(^{44,45} \) However, the incidence of this genotype is thought to be low in parasites causing severe malaria related to reduced parasite fitness.\(^{46,47} \) A study sequencing the \( PfHRP2 \) gene in parasites from all patients in the current study who had low plasma \( PfHRP2 \) concentrations is underway.

In conclusion, admission plasma \( PfHRP2 \) provides a tool in areas of moderate and high malaria transmission to distinguish “true” severe falciparum malaria from severe febrile illness with incidental malaria parasitaemia. Plasma \( PfHRP2 \) concentrations are a valuable prognosticator in African children with severe falciparum malaria.
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References


Supporting information

**Text S1. Description of enrolment criteria for severe falciparum malaria**

Patients needed to fulfill at least one severity criterion of malaria:

**Coma**
- Blantyre coma scale ≤2 for preverbal children, or Glasgow coma scale ≤10 for older children.

**Prostration**
- Inability to sit unsupported (for children over 6 months of age) or the inability to drink or breast-feed in younger children.

**Convulsions**
- A duration >30 minutes or a ≥2 in the 24 h preceding admission.

**Compensated shock**
- Peripheral capillary refill time ≥3 sec and/or the presence of a temperature gradient with a systolic blood pressure ≥70mmHg.

** Decompensated shock**
- Systolic blood pressure <70 mmHg.

**Severe respiratory distress**
- Nasal alar flaring, costal indrawing/recession, use of accessory muscles, or severe tachypnoea.

**Severe acidosis**
- Presence of deep breathing.

**Hypoglycaemia**
- Blood glucose <3 mmol/L, or clinical improvement in the level of consciousness immediately after administration of 10% dextrose.

**Anaemia**
- Severe pallor combined with respiratory distress.

**Blackwater fever**
- By carer’s history or observation of dark or black urine.

**Jaundice**
- Yellow discoloration of the sclera and skin.

**Hyperparasitaemia**
- Asexual parasitaemia above 10%.
Text S2. The mechanistic model and sensitivity analysis

Description of the mechanistic model
The mechanistic model of *PfHRP2* describing the relationship between plasma *PfHRP2* and probability of death caused by “true” severe malaria and probability of “true” severe malaria in patients diagnosed with severe malaria.

The observed data show a U-shaped relationship between the probability of death and plasma *PfHRP2* strata (Figure 2A, nadir log$_{10}$ 2.24 (=174 ng/mL). It was assumed that this U-shaped relationship is a composite of two intersecting curves:

1. The right end of the curve at high *PfHRP2* concentrations represents cases with “true” severe malaria with an exponential increase of the risk of death with increasing plasma *PfHRP2* concentrations. Two functional forms for this curve were evaluated: a simple exponential function (exp($k_1 h$)-1) and an exponential of a power of $h$ (exp($k_1 h^{k_2}$)-1). The latter choice provided a better fit with the observed data and was included in the model, given as:

$$ Pr_{death|malaria} = 1 + \exp(k_1 \log PfHRP2^{k_2}) \quad (eq. 1), $$

with the total number of “true” malaria cases ($d_m$) defined as:

$$ d_m = \left[ 1 + \exp(k_1 \log PfHRP2^{k_2}) \right] \times S, $$

with $S$ the total number of cases (eq. 2).

2. The left end of the curve represents cases dying from non-malaria illnesses, which is composed of a probability of non-malaria illness decreasing exponentially with increasing plasma *PfHRP2* concentration and a fixed probability of death in cases with non-malaria disease. The exponential form was the most simple decay function that provided the closest fit with the observed data, given as:

$$ Pr_{non-malaria} = \exp(-k_4 \log PfHRP2) \quad (eq. 3), $$

with a total number of non-malaria cases ($d_0$) defined as:

$$ d_0 = \exp(-k_4 \log PfHRP2) \times S, $$

with $S$ the total number of cases (eq. 4),

The probability to die given that the patient has a non-malaria illness is independent of plasma *PfHRP2*:

$$ Pr_{death|non-malaria} = k_3 \quad (eq. 5) $$

From eq. 3 and 5 it follows that:

$$ d_0 = \exp(-k_4 \log PfHRP2) \times k_3 \times S, \quad (eq. 6) $$
This explains why the left side of the curve in Figure 2B declines with increasing PfHRP2, because the proportion of patients with a different disease than severe malaria (who have a PfHRP2 independent risk of death) declines with increasing PfHRP2 levels, so that the risk of death declines too.

Since the total number of deaths $D = d_0 + d_m = S \times [-1 + \exp(k_1 \log PfHRP2^{k2}) + \exp(-k_4 \log PfHRP2) \times k_3]$, this represents the overall number of deaths and can be used to fit the relationship between PfHRP2 and observed number of deaths. These equations have 4 parameters to estimate ($k1, k2, k3, k4$).

To reduce the number of possible estimates, it was assumed for the model that $k_3 = 0.3$ and that the total number of deaths caused by non-malaria disease is 20% of the total number of deaths in all PfHRP2 strata (eq. 7). These proportions are based on published literature, but were further explored in the sensitivity analysis within a plausible range according to the consensus of the investigators.

\[
\text{Death}_{\text{non-malaria}} / \text{Death}_{\text{total}} = k_5 = 0.2 \quad \text{(eq. 7)}
\]

Fitting the parameters $k1$-$k5$ is done by maximizing the log-likelihood (LL) defined as:

\[
LL = \sum_{D=1}^{D} \left( D \frac{d_0 + d_m}{S} + n N \left( \frac{\sum d_i}{\sum D}, \nu, \sigma \right) \right)
\]

With $n$=the number of PfHRP2 strata and $N(x, \mu, s)$ being the probability density function of the normal distribution, with mean $\mu$ and standard deviation $s$, evaluated at $x$.

The fit of the mechanistic model with the statistical model (adjusted logistical regression model, stratified by study site, see methods section) was confirmed by comparing the predicted probability of death from both models. The mean difference in the predicted probabilities (for $n=3024$, due to missing values from variables in the statistical model) was 0.96% (95% CI 0.49 – 1.43).

In the sensitivity analysis, $k3$ was fixed to be in the set $\{0.3, 0.4\}$ and the proportion of all deaths cases that are not malaria, $k5$, was fixed to be in the set $\{0.15, 0.2, 0.25\}$. The six possible combinations were used in the sensitivity analysis and the model was then refit for every pair of $k3$ and $k5$.

**The sensitivity analysis**

A sensitivity analysis including the main model assumptions was conducted for the mechanistic model describing the relationship between PfHRP2 stratum and malaria-attributable disease and mortality. The non-PfHRP2 dependent risk of death in non-malarial illness was tested for the values of 0.3 and 0.4. In addition, the total proportion
of death caused by non-malarial disease was varied from 0.15 to 0.30. Overall the conclusions derived from the model regarding malaria-attributable disease and mortality were robust within the chosen range of values. The variation in the nadir, describing the PfHRP2 value where the risk of death caused by malaria and non-malaria is equal, is within half a log\(_{10}\) value of PfHRP2 (Figure S1-A). Taking the most conservative approximation, the risk of death due to malaria falls below 50% with PfHRP2 <100 ng/ml. In the log PfHRP2 stratum of 3 to 3.5 (1000-3162 ng/ml) and above, the probability of “true” severe malaria varied between 93% and 97% and the probability that death was caused by severe malaria varied between 83% and 93% (Figure S1-B).
Figure S1-B

Figure S1-A

Assumptions
Risk of death of non-malarial illness
Proportion of deaths caused by non-malarial illness

0.15

0.3

0.4

0.2

0.3
Text S3. Sensitivity analysis of the estimated total parasite burden as a function of parasite multiplication factor and PfHRP2 half-life

The partial rank correlation coefficient between each parameter and the calculated total parasite burden according to the model were 0.57, -0.27 and -0.44 for the parasite multiplication factor, the PfHRP2 half-life and the amount of PfHRP2 secreted per erythrocytic cycle. This indicated that the multiplication factor was the most influential factor affecting the total parasite burden estimate, followed by the amount of PfHRP2 secreted per cycle and the variations in PfHRP2 half-life respectively.

The figures below show the impact of the multiplication factor and the PfHRP2 half-life on the total parasite burden for a patient with a plasma PfHRP2 concentration of 1000 ng/mL, a (population median) haematocrit of 19% and a (population median) bodyweight of 11.2 kg.

Figure S2-A and S2-B. Estimated total parasite burden as a function of the parasite multiplication factor or the PfHRP2 half-life using the model as described in the methods. Values chosen for the model parameters were PfHRP2 concentration of 1000 ng/mL, Haematocrit of 19% and bodyweight of 11.2 kg.
Text S4. Plasma PfHRP2 and parasitaemia by outcome

Figure S3. Scatter plot of parasitaemia and plasma PfHRP2 in surviving (blue dots, n=3070) and fatal (red squares, n=327) cases in patients with both detectable plasma PfHRP2 and malaria parasites seen on the peripheral blood smear.