Diagnosis, prognosis and treatment of severe falciparum malaria in African children
Hendriksen, I.C.E.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 6

Defining falciparum malaria-attributable severe febrile illness in moderate to high transmission settings based on plasma PfHRP2

Ilse C. E. Hendriksen,1,2 Lisa J. White,1,2 Jacobien Veenemans,3,4 George Mtove,5 Charles Woodrow,1,2 Ben Amos,6 Somporn Saiwaew,1 Samwel Gesase,7 Behzad Nadjm,6 Kamolrat Silamut,1 Sarah Joseph,9 Kesinee Chotivanich,1 Nicholas P. J. Day,1,2 Lorenz von Seidlein,10 Hans Verhoef,3,11 Hugh Reyburn,8 Nicholas J. White,1,2 Arjen M. Dondorp1,2

1. Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
2. Centre for Tropical Medicine, Churchill Hospital, University of Oxford, Oxford, United Kingdom
3. Wageningen University, Cell Biology and Immunology Group, Wageningen, The Netherlands
4. Laboratory for Microbiology and Infection Control, Amphia Hospital, Breda, The Netherlands
5. National Institute for Medical Research, Amani Centre, Tanga, Tanzania
6. Teule Hospital, Muheza, Tanzania
7. National Institute for Medical Research, Tanga Medical Research Centre, Tanga, Tanzania,
8. London School of Tropical Medicine and Hygiene, London, United Kingdom
9. Medical Research Council, London, United Kingdom
10. Menzies School of Health Research, Casuarina, Northern Territory, Australia
11. MRC International Nutrition Group, London School of Hygiene and Tropical Medicine, London, United Kingdom

Journal of Infectious Diseases 2012; accepted
Summary

Background In malaria-endemic settings, asymptomatic parasitaemia complicates the diagnosis of malaria. Histidine-rich protein-2 is produced by *Plasmodium falciparum* (PfHRP2), and its plasma concentration reflects the total body parasite burden. We aimed to define the malaria-attributable fraction of severe febrile illness using plasma PfHRP2 distributions from parasitaemic children with different clinical presentations.

Methods Plasma samples and peripheral blood slides were collected from 1435 children aged 6–60 months in communities and a nearby hospital in Northeastern Tanzania. The study population included children with severe or uncomplicated malaria, asymptomatic carriers and healthy RDT-negative controls. PfHRP2 distributions in the different groups were used to model severe malaria-attributable disease.

Results Plasma PfHRP2 showed a close correlation with the severity of infection. PfHRP2 concentrations above 1000 ng/ml denoted a malaria-attributable fraction of 99% (95% CI 96–100%) with a sensitivity of 74% (95% CI 72–77%), whereas a concentration below 200 ng/mL denoted a proportion of >10% (95% CI 3–27%) of patients with severe febrile illness of an alternative diagnosis. Bacteraemia was more common in patients within the lowest and highest PfHRP2 quintiles.

Conclusions Plasma PfHRP2 concentration defines malaria-attributable disease and distinguishes severe malaria from coincidental parasitaemia in African children in a moderate to high transmission setting.
**Introduction**

Children under 5 years carry the highest burden of malaria and malaria-associated mortality in sub-Saharan Africa. In these moderate to high transmission areas, the diagnosis of severe malaria is challenging. Parasitaemic children with severe febrile illness can suffer from severe malaria, but can also have coincidental parasitaemia with an alternative illness causing severe disease. Host partial immunity develops early in life in highly malaria-endemic regions, and malaria parasites can be tolerated without development of symptoms. Community-based cross sectional studies conducted in these settings typically show that over 10% of children under 5 years are parasitaemic by microscopy yet symptom-free, with prevalence varying by age, exposure to infection, transmission season, amongst other factors. Commonly used malaria case definitions rely on the presence of fever and malaria parasites on peripheral blood films and thus lack specificity. In addition, symptoms of severe malaria are non-specific and can have different etiologies. More accurate case definitions for clinical or severe malaria are required for clinical management and research purposes. Specificity of a malaria case definition can be improved by applying a parasite density threshold based on peripheral blood parasitaemia. This approach is useful as an epidemiological tool, but lacks accuracy for clinical management. Peripheral blood parasitaemia does not represent the sequestered parasite burden, which is pivotal to the pathophysiology of severe falciparum malaria. Asexual parasites in the second half of the erythrocytic life-cycle effectively adhere to the endothelial lining of the microcirculation, which prevents their detection in peripheral blood films.

*Plasmodium falciparum* histidine-rich protein-2 (*Pf*HRP2) is a parasite derived water-soluble protein and is released in discrete amounts into the plasma predominantly during schizont rupture. Therefore *Pf*HRP2 plasma concentrations reflect the total body parasite burden, including the sequestered parasites. Studies in Asian adults and African children show that in contrast with peripheral blood parasite density, plasma *Pf*HRP2 correlates strongly with disease severity and outcome.

We hypothesized that plasma *Pf*HRP2, as a measure of the total parasite burden determining disease severity, can be used to define malaria-attributable disease in endemic regions where coincidental peripheral blood parasitaemia is common.

In this study, we compared the distribution of peripheral blood parasitaemia versus plasma *Pf*HRP2 concentrations in healthy RDT-negative controls, asymptomatic carriers, uncomplicated and severe malaria patients and used this to estimate the malaria-attributable fraction of severe disease.
Methods

The study was conducted in the rural lowlands of Northeastern Tanzania. Peripheral blood slides and plasma PfHRP2 samples were collected in one community and two hospital studies in neighbouring Handeni and Muheza districts in Tanga region with similar malaria transmission intensity.9,22

Four clinical severity groups were defined: severe malaria, uncomplicated malaria, asymptomatic carriers and healthy RDT-negative controls. Severe malaria cases from the hospital studies were defined by modified clinical WHO criteria confirmed by a positive pLDH (OptiMAL-IT (DiaMed AG, Switzerland) and/or PfHRP2-based (Paracheck Orchid Biomedical, India) rapid diagnostic test (RDT). Severity criteria included decreased consciousness (coma or severe prostration), convulsions, respiratory distress or acidotic breathing, shock, severe symptomatic anaemia (haemoglobin concentration <5 g/dL) and hypoglycaemia (glucose concentration <2.5 mmol/L).23 Uncomplicated malaria cases, asymptomatic carriers and healthy controls were identified in the community study by pLDH-based RDT (CareStart, Access Bio, USA). Uncomplicated malaria was defined by fever, absence of severity criteria and a positive pLDH-based RDT.24 Asymptomatic carriers were defined as afebrile children (by history and axillary temp <37.5°C at presentation) with a positive pLDH-based RDT. Controls were afebrile children with a negative pLDH-based RDT. Children aged 6 to 60 months were included.

In the community, asymptomatic children were recruited in the context of the baseline screening (February-August 2008) for a randomised trial that assessed the effect of micronutrient supplementation on the incidence of uncomplicated malaria.25 In four villages in Handeni district, all resident children aged 6-60 months were invited for the screening, and those with height-for-age Z-scores ≤1.5 SD, weight-for-height Z score >-3 SD and haemoglobin concentrations >7 g/dL were eligible to participate. Those unlikely to comply with interventions, whose parents/guardians refused consent, or with signs of severe or chronic disease upon clinical examination were excluded.

In total, 246 of 612 children had a plasma sample and a positive RDT for Plasmodium falciparum. From these, 177 were afebrile upon examination and without reported fever within the last 48 hours. Slide results were available for 172 asymptomatic individuals, which were included in the present study (“group 2”). All parasitaemic children at baseline were treated with an effective antimalarial (artemether-lumefantrine). We selected the first 60 consecutively enrolled RDT negative children as controls (“group 1”), of whom 11 were subsequently excluded because of the presence or history of fever. Uncomplicated malaria cases were detected during the follow-up period of the trial. Parents were
requested to bring study children to the clinic if their child developed a fever or became unwell. From these, a total of 285 randomly selected febrile children with positive pLDH-based RDT ("group 3") were included in the analysis (Figure 1).

Severely ill parasitaemic patients originated from two consecutive studies conducted at Teule Hospital. The details of these studies have been published elsewhere. The first study assessed the causes of fever in 3639 febrile children ("group 4") admitted from June 2006 to June 2007. The dataset was selected for all patients with a pathogen isolated by blood culture in the presence of a positive RDT for falciparum malaria, complemented with a random sample of children with RDT-positive severe malaria, but a negative blood culture (n=226). The second severe malaria group ("group 5") was part of a severe malaria treatment trial (AQUAMAT) conducted from February 2007 till July 2010 (n=703). These subjects were also part of a separate paper describing the prognostic value of plasma PfHRP2 in 3826 children across all AQUAMAT study sites.

Figure 1

Study population
The studies were approved by the Tanzania Medical Research Coordinating Committee. The community study was also approved by the Ethical Review Committee of Wageningen University, The Netherlands. Hospital-based studies were also approved by the London School of Hygiene and Tropical Medicine and Oxford Tropical Research Ethics Committee (UK). In all studies, written individual informed consent was obtained from parents or guardians.

Malaria slide reading was conducted by experienced microscopists at the National Institute of Medical Research (NIMR) Tanga research laboratory in Korogwe, Teule Hospital (Joint Malaria Programme) research laboratory and the Mahidol-Oxford Tropical Medicine Research Unit, which was also responsible for quality control. Parasites/µl was calculated from the thick film per 200 white blood cells (WBC) and the actual WBC or, if missing, assuming 8000 WBC/µl (count/200 WBCx40). In the AQUAMAT study, parasites/µl was calculated from thin film per 1000 red blood cells (RBC; count/1000 RBCx125.6xHct).

Plasma PfHRP2 was assessed 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. Reference plasma with known PfHRP2 concentration was used to construct standard curves. Concentrations in diluted plasma dilutions were determined in duplicate according to the linear segment of the standard curve. “Positive” cases were defined as those where duplicate derived concentrations were in agreement (ratio 0.5–2) and the OD relative to background was more than 3 SDs of the average background based on all plates.

Statistical analysis
Data were analysed with STATA, version 12 (StataCorp, TX, USA). Parasite counts and PfHRP2 concentrations were normalized by log10 transformation. Normally distributed or log10-normalized variables were compared using a Student’s t test, the remainder by Wilcoxon rank-sum test. PfHRP2 concentrations between blood culture positive and blood culture negative patients were compared according to PfHRP2 quintiles in patients with severe malaria (group 4 and 5).

Modeling PfHRP2 distributions according to diagnostic group
Analysis of the observed PfHRP2 distributions suggested distinctive distributions according to severity of P. falciparum infection (Figure 2, middle column). In addition, the PfHRP2 distributions observed in patients with clinical severe malaria suggested contributions of underlying plasma PfHRP2 distributions as observed in RDT-negative
controls, asymptomatic carriers and patients with uncomplicated malaria (Figure 2, right column), all representing severe illness with alternative causes. It was assumed that each diagnostic group (k) had a distinctive Weibull distribution of plasma PfHRP2 and that the observed plasma PfHRP2 distribution in the different clinical groups (j) is a composite of these Weibull distributions. The diagnostic groups (k) comprised of healthy controls (k=1), asymptomatic carriers (k=2), and patients with uncomplicated (k=3), or severe malaria (k=4). The diagnostic groups of uncomplicated and severe malaria, in contrast with the clinically defined groups, exclude patients with coincidental parasitaemia. A mechanistic model was constructed to infer the most likely Weibull distributions in each diagnostic group (k), described by the coefficients α_k and β_k. The probability (P) that an individual (i) has a particular plasma PfHRP2 concentration P(h_{ij}) is then determined by the probability, denoted as m_{jk}, that this individual belongs to diagnostic group (k).

\[
P(h_{ij}) = \begin{cases} 
  m_{ji} & \text{for } h_{ij} = 0 \\
  \sum_{j=2}^{5} m_{jk} W(\alpha_k, \beta_k) & \text{for } h_{ij} > 0
\end{cases}
\]

Two different groups with clinical severe malaria were included in the model, of which one was partly selected on the presence concomitant bacteraemia (group 4, see above). The model was used to define the plasma PfHRP2-based malaria-attributable fraction in the unselected group of parasitaemic patients with a clinical diagnosis of severe malaria (group 5). It differentiates severe malaria from both the populations with asymptomatic parasitaemia and uncomplicated malaria. The proportion of malaria-attributable disease (y) according to PfHRP2 (h) is given by:

\[
y(h) = \begin{cases} 
  100 m_{s1} & \text{for } h = 0 \\
  \frac{100 m_{s4} W(\alpha_5, \beta_5)}{\sum_{j=2}^{5} m_{jk} W(\alpha_k, \beta_k)} & \text{for } h > 0
\end{cases}
\]

That is for each value of plasma PfHRP2 (h), the malaria-attributable fraction of severe disease, is \( m_{s4} W(\alpha_5, \beta_5) \) divided by the total number of individuals with the same PfHRP2 (h) predicted by the model as:

\[
\sum_{j=2}^{5} m_{jk} W(\alpha_k, \beta_k)
\]

The parameters were estimated by implementing a mixture model within WinBUGS. Three chains were run for a burn-in of 5000 iterations followed by a further 5000
iterations to obtain posterior distributions. The model parameters were estimated with 95% credible intervals. Sensitivity was calculated using the model derived number of patients with severe malaria as reference.

Results

Subject characteristics
We analyzed data of 49 healthy RDT-negative controls (group 1), 172 children with asymptomatic parasitaemia (group 2), 285 patients with uncomplicated malaria (group 3), and 226 (group 4) and 703 patients (group 5) with clinical severe malaria (Figure 1). Microscopy was negative in all RDT-negative controls, except for one case with parasitaemia of 145 parasites/µL. Baseline clinical and laboratory characteristics according to malaria clinical group are summarized in Table 1. Children with severe malaria were younger than children with uncomplicated malaria (p<0.0001) or asymptomatic parasitaemia (p<0.0001) and also had lower haemoglobin concentrations (p<0.0001). Admission characteristics and outcome of patients with severe malaria (clinical group 4 and 5) are summarized in Table 2.

\( PfHRP2 \) concentrations were detectable in 8/49 (16%) healthy controls, 156/172 (91%) asymptomatic cases, 269/285 (94%) uncomplicated and 222/226 (98%) and 698/703 (99%) severe malaria patients and are given in Table 1. The distributions of peripheral blood parasitaemia and \( PfHRP2 \) concentrations according to clinical groups are displayed in Figure 2 (left and middle columns). Plasma \( PfHRP2 \) concentrations were associated with the severity of \( P. falciparum \) infection, whereas peripheral blood parasitaemia was not.

Plasma \( PfHRP2 \)-based malaria-attributable disease in parasitaemic severe febrile illness
The observed \( PfHRP2 \) distributions in the clinical groups were modelled as a composite of the \( PfHRP2 \) distributions of the contributing diagnostic groups (Figure 2, right column). The model derived parameter estimates for \( m_{jk} \) denoting the probability that an individual from clinical group \( j = 1 \) to 5 belongs to diagnostic groups \( k = 1 \) to 4 are given in the supplement. From these parameter estimates the predicted distributions were fitted to the observed distributions and used to derive malaria-attributable proportions according to plasma \( \log_{10} PfHRP2 \) in the unselected clinical group of severely ill parasitaemic children (group 5, Figure 3). This shows that \( PfHRP2 \) levels above 1000 ng/mL correspond to a malaria-attributable fraction of 99% (95% CI 96–100%), with a sensitivity of 74%.
Table 1. Baseline characteristics of the study population according to malaria clinical group

<table>
<thead>
<tr>
<th></th>
<th>RDT-negative controls (1)</th>
<th>asymptomatic carriers (2)</th>
<th>uncomplicated malaria (3)</th>
<th>severe malaria (4)</th>
<th>severe malaria (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>49</td>
<td>172</td>
<td>285</td>
<td>226</td>
<td>703</td>
</tr>
<tr>
<td>Female (%)</td>
<td>20 (41%)</td>
<td>91 (53%)</td>
<td>141 (49%)</td>
<td>125 (55%)</td>
<td>339 (48%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>2.3 (1.5–3.6)</td>
<td>3.2 (2.3–4.1)</td>
<td>2.8 (1.9–4.0)</td>
<td>1.7 (1.1–2.6)</td>
<td>2.2 (1.2–3.1)</td>
</tr>
<tr>
<td>Weight-for-age Z-scoresa</td>
<td>-1.6 (0.7)</td>
<td>-1.6 (0.7)</td>
<td>NA</td>
<td>-1.5 (1.1)a</td>
<td>-1.1 (1.2)a</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>36.4 (0.4)</td>
<td>36.5 (0.4)</td>
<td>37.9 (1.3)</td>
<td>38.0 (1.1)</td>
<td>38.1 (1.0)</td>
</tr>
<tr>
<td>Haemoglobin (g/dL)b</td>
<td>11.3 (10.4–11.9)</td>
<td>10.3 (9.3–11.2)</td>
<td>9.8 (8.9–10.8)</td>
<td>4.8 (3.7–6.4)</td>
<td>6.5 (4.4–8.2)</td>
</tr>
<tr>
<td>Slide Pf positive</td>
<td>1 (2.0%)</td>
<td>118 (68.6%)</td>
<td>275 (96.5%)</td>
<td>208 (92.0%)</td>
<td>701 (99.7%)</td>
</tr>
<tr>
<td>Parasitaemia (parasites/µL)</td>
<td>145</td>
<td>1602</td>
<td>29 836</td>
<td>28 187</td>
<td>46 619</td>
</tr>
<tr>
<td>geometric mean (95% CI),</td>
<td></td>
<td></td>
<td>(1189–2157)</td>
<td>(22 312–35 607)</td>
<td>(39 476–55 054)</td>
</tr>
<tr>
<td>range</td>
<td>19–35 471</td>
<td>96–1 448 094</td>
<td>221–62 643</td>
<td>16–1 375 069</td>
<td></td>
</tr>
<tr>
<td>PfHRP2 (ng/mL)c,</td>
<td>4</td>
<td>19</td>
<td>163</td>
<td>1510</td>
<td>1746</td>
</tr>
<tr>
<td>geometric mean (95% CI),</td>
<td></td>
<td></td>
<td>(1–11)</td>
<td>(15–23)</td>
<td>(1577–1934)</td>
</tr>
<tr>
<td>range</td>
<td>1–29</td>
<td>1–546</td>
<td>3–4343</td>
<td>4–87 199</td>
<td>5–56 818</td>
</tr>
</tbody>
</table>

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

Abbreviations: NA, not available; PfHRP2, *Plasmodium falciparum* histidine-rich protein-2

a Missing weight-for-age Z scores in some children; n=219 in group 4 and n=702 in group 5.
b Missing haemoglobin in some children; n=48 in group 1, n=169 on group 2, n=701 in group 5.
c PfHRP2 concentrations shown for individuals with detectable concentrations, n=8, n=156, n=269, n=222 and n=698 in group 1 to 5, respectively.
(95% CI 72–77%). The proportion of malaria-attributable disease declined at lower PfHRP2 concentrations. Below 200 ng/mL, an alternative diagnosis than malaria was suggested in >10% (3–27%) of patients, whereas this proportion increased above 50% (95 CI 31–67%) at concentrations below 50 ng/ml.

### Table 2. Admission characteristics and outcome of children with severe malaria

<table>
<thead>
<tr>
<th>Feature</th>
<th>Severe malaria (4)</th>
<th>Severe malaria (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=226</td>
<td>n=703</td>
</tr>
<tr>
<td>Coma (BCS ≤2 or GGS ≤10)</td>
<td>30 (13%)</td>
<td>213 (30%)</td>
</tr>
<tr>
<td>Prostration (inability to sit)</td>
<td>106 (47%)</td>
<td>403 (57%)</td>
</tr>
<tr>
<td>Convulsions (≥2 within 24 h)</td>
<td>40 (18%)</td>
<td>268 (38%)</td>
</tr>
<tr>
<td>Severe anaemia (haemoglobin&lt;5g/dL)</td>
<td>128 (57%)</td>
<td>221 (32%)</td>
</tr>
<tr>
<td>Hypoglycaemia (glucose&lt;2.5 mmol/L)</td>
<td>27 (12%)</td>
<td>145 (21%)</td>
</tr>
<tr>
<td>Acidosis (lactate&gt;5 mmol/L or base excess&lt;-8 mmol/L)</td>
<td>97 (43%)</td>
<td>314 (49%)</td>
</tr>
<tr>
<td>Respiratory distress(^b)</td>
<td>74 (33%)</td>
<td>131 (19%)</td>
</tr>
<tr>
<td>Shock (^c)</td>
<td>21 (9%)</td>
<td>111 (16%)</td>
</tr>
<tr>
<td>Blood culture positive(^d)</td>
<td>47 (20.8%)</td>
<td>36 (5.1%)</td>
</tr>
<tr>
<td>Mortality</td>
<td>31 (13.7%)</td>
<td>99 (14.1%)</td>
</tr>
</tbody>
</table>

Data are No. (%) of patients.

Abbreviations: BCS, Blantyre coma scale; GCS, Glasgow coma scale.

\(^a\) Missing lactate or base excess in some children; n=197 in group 4 and n=642 in group 5, respectively.

\(^b\) Defined as nasal alar flaring, costal indrawing, use of accessory muscles or severe tachypnoea.

\(^c\) Compensated shock (capillary refill time ≥3 sec or presence of a temperature gradient with systolic blood pressure ≥70 mmHg) and decompensated shock (systolic blood pressure<70 mmHg) combined.

\(^d\) Missing blood culture in some children; n=700 in group 5.
Figure 2

Frequency distributions of peripheral blood parasitaemia, plasma *Pf*HRP2 and modelled fitted *Pf*HRP2 according to malaria clinical group (1=healthy RDT-negative controls, 2=asymptomatic carriers, 3=uncomplicated malaria, 4=severe malaria, 5=severe malaria). The fitted *Pf*HRP2 distributions (right column) show the modelled *Pf*HRP2 distributions with the underlying contributing *Pf*HRP2 distributions of different diagnostic groups (dotted lines), comprised of RDT-negative controls (light green), asymptomatic carriers (green) and patients with uncomplicated (blue turquoise) and severe malaria (bright blue and purple).
Malaria-attributable proportion (black/grey lines, left axis) and sensitivity (median, 95% CI, black/striped lines, right axis) for severe disease according to plasma log₁₀ *Pf*HRP₂ concentration. The malaria-attributable proportion was derived from the predicted *Pf*HRP₂ distributions from the median (95% CI) values of the m_ij distributions of individuals in each malaria diagnostic group (see Text S1).

**Blood cultures**

Blood cultures were positive in 83 patients with severe malaria (Table 2), and as expected the proportion was higher in the selected clinical severe malaria group 4. Patients with a positive blood culture were overrepresented in the lowest and highest plasma *Pf*HRP₂ quintiles (Figure 4). In patients with a *Pf*HRP₂ concentrations below the threshold of 200 ng/mL, 16/90 (18%) had positive blood cultures.
Blood culture positivity according to plasma PfHRP2 quintiles in patients with severe malaria.
Gram-negative bacteria included *Salmonella spp.*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella spp.*

**Discussion**

This study shows a clear stepwise increase in plasma PfHRP2 concentrations according to disease severity from asymptomatic parasitaemia, to uncomplicated, to severe malaria. There was substantially less overlap in plasma PfHRP2 distributions between groups compared to the distributions of peripheral blood parasitaemia. The distinct distributions between diagnostic groups enabled to model the proportion of malaria-attributable disease based on admission plasma PfHRP2, and distinguish this from patients with coincidental peripheral blood parasitaemia in whom severe disease is caused by an alternative disease. The PfHRP2-based model performed better than a previously described model based on peripheral blood parasitaemia. The proportion of malaria-attributable disease dropped
below 50% with a sensitivity >99% at plasma PfHRP2 concentrations below 50 ng/mL, in which case additional diagnostic tests are indicated to identify alternative diseases. The current model also accurately identified patients with a very high probability of severe malaria, with still acceptable sensitivity. A threshold of 1000 ng/mL defined a population of patients with severe malaria not diluted by patients with coincidental parasitaemia (<1%), which is mainly useful for defining a study population in a research setting, but also for the treating clinician. Low plasma PfHRP2 in a parasitaemic patient with severity signs should not withhold treatment with antimalarials, but should prompt the treating physician to look for other possible diseases, depending on the clinical presentation and resources (e.g. blood culture, lumbar puncture, chest X-ray, CT scan of the cerebrum). In African settings, were diagnostic facilities are scarce and treatment stock-outs occur, PfHRP2 can also help to prioritize these resources.

A previous study reported the prognostic significance of plasma PfHRP2 for death in a large cohort of African children with severe malaria. PfHRP2 was a better prognostic marker than peripheral blood parasitaemia or in combination with peripheral blood parasitaemia. The current study enabled a more accurate definition of the probability of non-malarial disease at low plasma PfHRP2 concentrations by incorporating individuals with asymptomatic parasitaemia and uncomplicated malaria. It is reassuring that the identified plasma PfHRP2 thresholds denoting a high or low probabilities of alternative disease were highly consistent between these studies, that used different modelling techniques.

Our findings are supported by two recent studies in African children. A small study in Tanzanian children showed a mean PfHRP2 value of 1008 ng/mL in cerebral malaria versus 443 ng/mL in uncomplicated malaria. The diagnostic potential of plasma PfHRP2 in pediatric cerebral malaria was also confirmed in a Malawian study, where presence of malarial retinopathy was used as the reference test. In contrast, two other studies in moderate to high transmission settings report that PfHRP2 concentration does not reflect severity in children. In Papuan children the median concentrations in uncomplicated and severe malaria were similar 584 versus 456 ng/mL. However, the case fatality in the severe malaria group was <1% suggesting moderately severe malaria in accordance with the low PfHRP2 concentrations reported. A small study in Kenyan children with severe malaria (n=22) reported low median PfHRP2 concentrations of 63 ng/mL with absence of decay over 48 hours, which could be related to problems in the PfHRP2 assay. The prognostic utility of plasma PfHRP2 is in line with previous reports in adult populations. A study in Thai adults showed a similar stepwise increase in plasma PfHRP2 according to disease severity. In Indonesian adults, the mean PfHRP2 value in severe
malaria was 1863 ng/mL versus 314 ng/mL in moderate severe malaria. In both studies plasma PfHRP2 was prognostic for mortality.

This is the first study assessing PfHRP2 concentrations in healthy asymptomatic children in a moderate to high transmission area. Parasite densities that can be tolerated without causing symptoms vary substantially between individuals of different age groups, transmission intensities and seasons. In moderate to high transmission settings, children under-five represent a heterogeneous group regarding levels of immunity. This is reflected by the younger age of children with severe malaria and the older age of asymptomatic children of whom 13/172 (8%) had parasite densities >10 000 parasites/µl. Similar high parasite densities have been reported in cross-sectional surveys in settings with moderate to high malaria transmission. The accuracy of PfHRP2 thresholds for defining malaria-attributable disease will vary with the level of acquired immunity in the population, because this will determine the relative population sizes of individuals with asymptomatic parasitaemia, versus uncomplicated or severe malaria and thus determines the corresponding overlap of plasma PfHRP2 distributions. The model prediction as a function of transmission intensity will be explored in a separate study. In addition, the prevalence of bacteraemia will also affect the size of the population with asymptomatic parasites or uncomplicated malaria but presenting with severe illness. Indeed, in the current study, selection of patients with a positive blood culture (group 4), resulted in a relatively higher proportion of parasitaemic patients with severe illness due to other diseases than malaria.

Detection of malarial retinopathy by fundoscopy is an alternative diagnostic tool evaluated for identification of cerebral malaria versus encephalopathic children with coincidental parasitaemia. In the African setting this has only been evaluated in comatose patients and requires considerable expertise, training and expensive equipment. In comparison, plasma PfHRP2 is positively associated with the entire clinical severity spectrum of P. falciparum infection. In this study plasma PfHRP2 was assessed by quantitative ELISA. Our findings call for the development of a low-cost semi-quantitative rapid test for the detection of plasma PfHRP2 with suitable thresholds.

Positive blood cultures particularly with gram-negative organisms were overrepresented in patients within the lowest and highest PfHRP2 quintiles. Blood cultures are known to have a limited sensitivity (around 40%) in detecting bacteraemia. The actual number of bacteraemic patients could thus be 2.5 fold higher than detected, implying an actual proportion of bacteraemic patients close to 50% in patients with plasma PfHRP2 below 200 ng/mL (2.5 times the observed proportion of 18%). This would be consistent with results from Malawian autopsy series, reporting invasive bacterial infection as the cause
of death in 4/7 (64%) parasitaemic patients with an alternative diagnosis.\textsuperscript{42} Positive blood cultures in patients with high \textit{P}fHRP2 concentrations indicate concomitant bacteraemia in severe malaria. There are several mechanisms that may explain this high rate of concomitant bacteraemia including a reduction in gut barrier function due to intense sequestration,\textsuperscript{43} facilitating translocation of gut bacteria, or a general immune suppression due to hemozoin and heme-oxygenase-1 induced macrophagocytic dysfunction.\textsuperscript{44–46} Particularly severe malarial anaemia is associated with invasive disease, mainly non-\textit{typhi} \textit{Salmonella} bacteraemia.\textsuperscript{47} \textit{P}. \textit{falciparum} infection predisposes to gram-negative bacteraemia and can account for more than half of invasive bacterial disease in malaria-endemic areas.\textsuperscript{48} Our data show that bacteraemia contributes to severe illness, but also occurs concomitantly in patients with severe malaria, warranting the use of broad spectrum antibiotics in addition to prompt antimalarial treatment, preferably with parenteral artesunate.

The current study has several limitations. This is a retrospective analysis of pooled datasets. Patients with severe malaria in group 5 were also included in a previous publication on the prognostic value of \textit{P}fHRP2. Patients with severe malaria in group 4 were partly selected on blood culture positivity. However, patients were selected on clinical criteria and RDT results and not on the basis of \textit{P}fHRP2 concentrations, and the \textit{P}fHRP2 distributions in both severe malaria groups were similar. In patients with low parasitaemia, the sensitivities of the peripheral blood slide and the RDT are relatively low which could have affected the composition of the clinical groups.

In conclusion, our study shows that plasma \textit{P}fHRP2 can be used to estimate the proportion of malaria-attributable disease in African children in moderate to high transmission settings and can distinguish severe malaria from severe febrile illness with coincidental peripheral blood parasitaemia. Bacteraemia is prominent in patients with severe illness and low plasma \textit{P}fHRP2 concentrations, suggesting that malaria may not be their primary diagnosis. Bacteraemia is also more frequent in patients with high plasma \textit{P}fHRP2, denoting concomitant sepsis with severe malaria, which implies that administration of antibiotics is warranted in all patients with a clinical diagnosis of severe malaria.
Acknowledgements

We thank Tedson Lukindo from Joint Malaria Programme Tanzania for assistance with the ELISA assay; Benjamas Intharabut, Ketsanee Srinamon, Forradee Nuchsongsin, from the Mahidol-Oxford Tropical Medicine Research Unit for malaria slide reading, Tharisara Sakulthaew for coordinating the sample shipments, and Montri Rijaibun and Nuttapol Panachuenwongsakul for data management. Permission to publish this work was given by the Director General, National Institute for Medical Research, Tanzania.

This work was supported by grants 076908 and 082541 from the Wellcome Trust, and was coordinated as part of the Wellcome Trust Mahidol University Oxford Tropical Medicine Research Programme funded by the Wellcome Trust of Great Britain. The community study was supported by the Netherlands Organisation of Scientific Research, Foundation for the Advancement of Tropical Research (grants W 93–413, WAO 93–441); Cornelis Visser Foundation. HV is supported by the INSTAPA project, which receives funding from the European Union’s Seventh Framework Programme (FP7/2007–2013) under grant agreement No. 211484. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
References


Supporting information

**Text S1. The mechanistic model parameter estimates for \( m_{jk} \) with 95% confidence interval**

The observed PfHRP2 distributions in the clinically defined groups (\( j \)) healthy RDT-negative controls (\( j=1 \)), asymptomatic carriers (\( j=2 \)), uncomplicated malaria (\( j=3 \)), or severe malaria (\( j=4 \) or \( j=5 \)) were modelled as a composite of the PfHRP2 distributions of the contributing diagnostic groups (\( k \)) (see Figure 2, right column). The diagnostic groups of uncomplicated and severe malaria, in contrast with the clinically defined groups, exclude patients with coincidental parasitaemia. The model derived parameter estimate \( m_{jk} \) denotes the probability that an individual from clinical group \( j=1 \) to 5 belongs to diagnostic group (\( k \)): RDT-negative control (\( k=1 \)), asymptomatic carrier (\( k=2 \)), uncomplicated malaria (\( k=3 \)), or severe malaria (\( k=4 \)).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2.5%</th>
<th>median</th>
<th>97.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>( m_{11} )</td>
<td>0.7321</td>
<td>0.8475</td>
<td>0.9285</td>
</tr>
<tr>
<td>( m_{12} )</td>
<td>0.07153</td>
<td>0.1525</td>
<td>0.268</td>
</tr>
<tr>
<td>( m_{21} )</td>
<td>0.06734</td>
<td>0.1077</td>
<td>0.1596</td>
</tr>
<tr>
<td>( m_{22} )</td>
<td>0.8404</td>
<td>0.8923</td>
<td>0.9327</td>
</tr>
<tr>
<td>( m_{31} )</td>
<td>0.03531</td>
<td>0.05819</td>
<td>0.08908</td>
</tr>
<tr>
<td>( m_{32} )</td>
<td>0.07897</td>
<td>0.1367</td>
<td>0.2031</td>
</tr>
<tr>
<td>( m_{33} )</td>
<td>0.7341</td>
<td>0.804</td>
<td>0.8665</td>
</tr>
<tr>
<td>( m_{41} )</td>
<td>0.006988</td>
<td>0.02048</td>
<td>0.04407</td>
</tr>
<tr>
<td>( m_{42} )</td>
<td>0.03614</td>
<td>0.07437</td>
<td>0.1248</td>
</tr>
<tr>
<td>( m_{43} )</td>
<td>0.004961</td>
<td>0.06056</td>
<td>0.1468</td>
</tr>
<tr>
<td>( m_{44} )</td>
<td>0.7577</td>
<td>0.8406</td>
<td>0.9039</td>
</tr>
<tr>
<td>( m_{51} )</td>
<td>0.006988</td>
<td>0.02048</td>
<td>0.04407</td>
</tr>
<tr>
<td>( m_{52} )</td>
<td>0.03614</td>
<td>0.07437</td>
<td>0.1248</td>
</tr>
<tr>
<td>( m_{53} )</td>
<td>0.004961</td>
<td>0.06056</td>
<td>0.1468</td>
</tr>
<tr>
<td>( m_{54} )</td>
<td>0.7577</td>
<td>0.8406</td>
<td>0.9039</td>
</tr>
</tbody>
</table>