Plasma *Plasmodium falciparum* Histidine-Rich Protein-2 concentrations in children with malaria infections of differing severity in Kilifi, Kenya

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Summary:

Through a clinical surveillance study, we show that plasma levels of PfHRP2, a marker of total body parasite load, are strongly correlated with the severity of Plasmodium falciparum infections among children in Kilifi, Kenya.
Abstract

Background

Most previous studies support a direct link between total parasite load and the clinical severity of Plasmodium falciparum malaria infections.

Methods

We estimated P. falciparum parasite loads in three groups of children with malaria infections of differing severity: (1) children with WHO-defined severe malaria (n=1,544); (2) children admitted with malaria but without features of severity (n=200) and; (3) children in the community with asymptomatic parasitemia (n=33).

Results

Peripheral parasitemias were highest in those with uncomplicated malaria (geometric mean 111,064; 95%CI 86,798-141,819 parasites/µl), being almost three times higher than those with severe malaria (39,588; 34,990-44,791 parasites/µl) and >100 times higher than in those with asymptomatic malaria (1,092; 523-2,280 parasites/µl). However, geometric mean PfHRP2 values (95% CI) increased with severity, being 7 (4-12) ng/ml in asymptomatic malaria, 843 (655-1,084) ng/ml in uncomplicated malaria and 1,369 (1,244-1,506) ng/ml in severe malaria. PfHRP2 concentrations were markedly lower in the sub-group of severe malaria patients with concomitant invasive bacterial infections (IBIs) of blood or CSF (GM 312 ng/ml; 95%CI 175-557; p<0.0001) than in those without IBIs (GM 1,439 ng/ml; 1,307-1,584; P<0.001).
Conclusions

The clinical severity of malaria infections related strongly to the total burden of *P. falciparum* parasites. A quantitative test for plasma concentrations of PfHRP2 could be useful in identifying children at the greatest clinical risk and to identify critically ill children in whom malaria is not the primary cause.

Key words: Malaria, *Plasmodium falciparum* Histidine-Rich Protein-2, PfHRP2, parasite biomass, sequestration.
Introduction

*Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) is a 30 kd molecule that is produced by most strains of *P. falciparum* malaria parasites [1] and is involved in the conversion of the toxic molecule heme to the more neutral malaria pigment hemozoin [2]. Around 5 fg of PfHRP2 is released from the cytoplasm of *P. falciparum*-infected red blood cells during schizont rupture [3, 4] and PfHRP2 can therefore be detected in the plasma of individuals with *P. falciparum* infection by ELISA or rapid diagnostic tests [5, 6]. Previous studies have shown that plasma PfHRP2 provides a more accurate reflection of the total *P. falciparum* load in individuals than parasite counts in peripheral blood because, unlike the latter, they provide information about the burden of mature-phase parasites that are sequestered in the deep vasculature[7].

Positive associations have been reported between both plasma PfHRP2 and disease severity in a number of previous studies [7-12]. However, this has not been seen universally [13, 14], prompting some to question the causal link between sequestration and severe malaria [15]. We have estimated the *P. falciparum* parasite-loads in children with malaria infections of differing severity in Kilifi, Kenya with the aim of contributing to this debate.
Methods

Study design and participants

Our study involved three groups of children <14 years of age who were residents of Kilifi County, Kenya: (1) admissions to the High Dependency Unit (HDU) at Kilifi County Hospital (KCH) with one or more features of severe falciparum malaria; (2) admissions to the general paediatric ward with uncomplicated malaria; and (3) children from the surrounding area with asymptomatic malaria infections.

Clinical and laboratory data

Data and samples from children in Groups 1 and 2 were captured through a routine surveillance system that has operated at KCH since 1989, as described in detail previously [16]. Severe malaria was defined as *P. falciparum* blood-film positivity with one or more of the following clinical or laboratory features: cerebral malaria (CM; Blantyre Coma Score of <3), respiratory distress (RD; abnormal deep breathing), severe malaria anemia (SMA; Hb <5 g/dL) or other features of severity as previously described [17, 18]. Children in Group 1 were admitted between 1998 and 2010 and in Group 2 between 2004 and 2005 while Group 3 were recruited during cross-sectional community surveys conducted between 2010-2011 [19], based on the availability of archived samples.

All laboratory tests were conducted at the KEMRI-Wellcome Trust Research laboratories that are GCLP accredited under assessment by Qualogy Ltd. Parasitemia was determined in real-time from blood films using standard methods [20], while PfHRP2 was batch-analysed by ELISA in plasma samples archived at -80°C since the time of collection [10]. HbS and α-thalassemia were genotyped by PCR as previously described [21, 22].
**Parasite burden**

The total number of circulating parasites ($P_{circ}$), the whole-body total parasite load ($P_{tot}$), and the sequestration index (SI), an indication of the proportion of parasites that are hidden from detection through sequestration in deep vascular beds, were estimated for each participant individually using the published formulae [7, 10]:

1) $P_{circ} = \text{parasites/µL} \times 10^6 \times \text{circulating blood volume [0.08 L/kg]} \times \text{body weight [kg]}$

2) $P_{tot} = 7.3 \times \text{PfHRP2 [ng/ml]} \times (1-\text{Hematocrit}) \times \text{body weight [kg]} \times 10^{13}$

3) $SI = \frac{P_{tot}}{P_{circ}}$

**Statistical analysis**

Continuous data were compared using Student’s $t$-tests or Mann-Whitney tests as appropriate while proportions were compared using the $\chi^2$ test. Non-normal data were log-transformed prior to analysis. Linear regression was performed both with and without adjustment for ethnicity, HbS phenotype and $\alpha$-thalassemia genotype. Children displaying multiple features of severe malaria appeared in multiple categories. All data were analysed using Stata v15.1 (Stata Corp, Timberlake).
Ethics

Written informed consent was provided by the parents of all participants. Ethical approval was granted by the Kenya Medical Research Institute/National Ethical Review Committee in Nairobi, Kenya and the Oxford Tropical Research Ethics Committee in Oxford, UK.

Results

The study flow is summarised in Supplementary Figure 1. Clinical and laboratory data and plasma samples from a total of 1,544 children with severe malaria, 200 with uncomplicated malaria and 33 with asymptomatic malaria were retrieved for this study. PfHRP2 was undetectable in 30 (1.7%) of these samples (n=23 severe and n=2 uncomplicated and n=5 asymptomatic malaria), leaving 1,747 contributing data to the final analysis.

Demographic and clinical features

The demographic and clinical characteristics of participants are summarised in Table 1. Children with severe malaria were significantly younger than those with uncomplicated malaria while children with asymptomatic malaria were significantly older. Overall, in-hospital mortality was 11.7% in children admitted with severe malaria but zero in those admitted with uncomplicated malaria. Many children with severe malaria presented with more than one severity feature (Figure 1). Mortality was similar (9.5-11.1%) across the three main groups (CM, RD and SMA) but was higher in those with two features and higher still (20.5%) in children with all three (P<0.001). Conversely, mortality was comparatively lower (4.8%) in those with none of the main features but who instead displayed other features such as prostration, hypoglycemia and hyperparasitemia (Table 1).
Markers of parasite biomass

Overall, parasite densities in peripheral blood were lowest in children with asymptomatic malaria, highest in those with uncomplicated malaria and intermediate in those with severe malaria (Table 2). By contrast, a stepwise increase in plasma PfHRP2 levels was seen between these three severity classes, the highest (GM 1,369 ng/ml; 95%CI 1,244-1,506) being seen in those with severe malaria (Table 2).

One child with uncomplicated malaria and 50 with severe malaria had concomitant invasive bacterial infections (IBIs) diagnosed by culture of blood or CSF (see Table 1, footnote for organisms and origins). Among severe malaria patients, PfHRP2 values were markedly lower in those with IBIs (GM 312 ng/ml; 95%CI 175-557) than in those without (GM 1,439 ng/ml; 1,307-1,584; P<0.001). This difference was not seen in the subset of 11 infections with non-typhoidal Salmonella species (GM 1,883 ng/ml; 95%CI 811-4,371; p>0.99), being confined to the 39 other Gram-negative and Gram-positive infections (GM 188 ng/ml; 101-352; p<0.0001) (Figure 2 and Table 1). Twenty-three of these 39 cases with non-Salmonella IBIs had a plasma PfHRP2 of <200 ng/ml (a previously proposed cut-off for identifying cases at high risk of an alternative diagnosis), along with 240 cases without IBIs. Hence 23/263 (8.7%) patients with a plasma PfHRP2 of <200 ng/ml had an IBI compared to only 16/1,258 (1.3%) patients with levels above this threshold (an approximately 7-fold difference).

Plasma PfHRP2 exceeded 1,000 ng/ml in 1,007/1,521 (66.2%) children with severe malaria and 104/198 (52.5%) children with uncomplicated malaria but was <1,000 ng/ml (range 0.9–91.3 ng/ml) in all those with asymptomatic malaria. The parameter that most markedly differed between groups was the sequestration index. The geometric mean sequestration index was only 4.0 (1.8-8.8) and 4.9 (3.5-6.8) in those with asymptomatic and uncomplicated malaria respectively but was 40.6 (95%CI 35.7-46.0) in those with severe malaria (p<0.001).
Overall, after adjusting for HbS, α-thalassemia and ethnicity, the sequestration index was 8.3 (95%CI 5.6-12.3) times higher in children admitted with severe malaria than in those admitted with uncomplicated malaria. Within those with severe malaria only, parasite densities were significantly lower in the sub-group who died in hospital, although a trend was seen towards an increased sequestration index (Table 2).

**Markers of parasite biomass in children with different phenotypes of severe malaria**

Finally, we calculated the various measures of parasite biomass in children with specific severe malaria phenotypes and compared them to values from children in the uncomplicated group (Table 3). Among the children with severe malaria, peripheral parasite densities were lowest in those with none of the three major features and highest in those with respiratory distress. The most notable differences in PfHRP2-based values related to children with severe malaria anemia, in whom geometric mean plasma concentrations were 3.2 (2.3-4.5; P<0.001) times higher and sequestration index 18.2 (11.8-28.2; P<0.001) times higher than in children with uncomplicated malaria.

**Discussion**

We have estimated both the peripheral and sequestered burdens of *P. falciparum* parasites in children with malaria of differing severity in Kilifi County, Kenya. We found no significant differences in the sequestration index between those with asymptomatic and uncomplicated *P. falciparum* malaria infections but the index was approximately eight times higher in those with severe malaria, being particularly high in severe malaria anemia. Our study supports the hypothesis that malaria severity is proportionate to total parasite load, an observation that could be helpful in directing care to those at greatest need [5, 10].
A direct relationship between parasite load and malaria severity was first suggested in the late 19th century [23] and most studies that have investigated this question more recently through PfHRP2-based methods have supported this general conclusion. In the first study of that kind, a strong relationship was seen with total parasite load in adults admitted to hospital on the Thai-Burmese border [7]. Loads were approximately six-times higher in severe than non-severe patients and were especially high in those who went on to die. Similar results were found in a second study, in Indonesian adults [8].

Children presenting in coma with a positive malaria blood film in Africa are generally assumed to have cerebral malaria. However, asymptomatic parasitemia is common during childhood and one post-mortem study showed that many such children have an alternative diagnosis [24]. Subsequently, Seydel and colleagues demonstrated the potential utility of plasma PfHRP2 concentration as a marker of intracerebral parasite sequestration [25], identifying children with cerebral malaria confirmed either by autopsy or malaria retinopathy. Later studies have confirmed the strong relationship between total parasite burden and malaria severity in African children [10, 26]. First, within the multinational AQUAMAT trial, high PfHRP2 concentrations were found in the majority of children while levels were significantly higher in those who died than in those who survived. Of particular interest, a U-shaped association was seen between PfHRP2 concentration and death, potentially explained by the misclassification to malaria of the sub-group with the lowest concentrations [10].

In a subsequent study conducted in Tanzanian children, plasma PfHRP2 was 19 ng/ml (15-23) in asymptomatic carriers, 163 ng/ml (137-194) in children with uncomplicated malaria, detected in the community, and 1,510 ng/ml (1,180–1,933) and 1,746 ng/ml (1,577–1,934) among children with severe malaria admitted during two different time periods [26]. A
A concentration of <200 ng/mL was found to indicate severe febrile illness caused by an alternative diagnosis in >10% of patients. Our data relating to invasive bacterial infections (IBIs) extend these findings and exemplify the potential for plasma levels to contribute to clinical diagnosis. PfHRP2 was substantially lower in the sub-group of children with severe malaria who also had a concomitant IBI. As expected, this was not the case for the subset whose cultures were positive for non-typhoidal Salmonella species, since previous studies have shown that IBIs due to Salmonella can complicate malaria via a mechanism involving gut barrier dysfunction [27]. Compared to children without IBIs, plasma PfHRP2 levels were approximately 7.5 times lower in children with other Gram-negative as well as Gram-positive bloodstream infections, indicating that these IBIs were likely to have been the main agents of severe illness in many of these children. In our study, plasma PfHRP2 levels were <200 ng/ml in only one-sixth of severe cases but identified 23 of the 39 children with non-Salmonella IBI's: i.e. approximately 9% of children with these low PfHRP2 levels had an IBI. The 16 other children with such IBIs were among 1,258 with a PfHRP2 level above this cut-off (1.3%). Hence, the 200 ng/ml threshold enriched for children with non-malaria-associated IBI's by approximately 7-fold. These findings are similar to those of Hendriksen and colleagues, although in that study the relationship between plasma PfHRP2 concentration and distinct categories of IBI was less clear-cut than in the current study. PfHRP2 levels have also been correlated with childhood cerebral malaria in a number of smaller studies conducted in Tanzania [9], Malawi [11] and Uganda [12], and with various forms of adult malaria among imported cases in France [28]. At present, there are no point-of-care methods for quantifying PfHRP2. The development of such tests in the future would be of major potential benefit.

The pathogenesis of severe falciparum malaria is complex, but a compromised microcirculation in vital organs caused by the sequestration of cytoadhered parasitized red blood cells to the vascular endothelium is central, compounded by endothelial dysfunction,
reduced red blood cell deformability [29], and rosetting [30]. Other complications of the disease might relate to a disordered inflammatory response or oxidative damage caused by plasma free hemoglobin [31]. Moreover, the likelihood that any particular *P. falciparum* infection will progress to become severe or fatal probably depends on a wide range of genetic, immunological, physiological and behavioural characteristics of both the host and the infecting parasite [30]. With specific reference to our current study, the central role played by sequestration is a question that has been long debated. Although florid sequestration of parasites in the small vessels of multiple organs is a consistent feature of studies conducted post-mortem and is supported by observations of microvascular blockage in the retina and rectal mucosa in living patients with severe falciparum malaria [32], it is likely that other pathophysiological processes also play a major role. However, the results of our current study, which mirror those from the majority of previous studies, support the conclusion that the most common and dangerous clinical complications of *P. falciparum* are directly related to the sequestered parasite load.

Most previous studies of severe malaria have been too small to investigate differences in sequestration between children with different clinical phenotypes. However, the relatively large sample size allowed this in our current study. We found that the sequestration index was high across a range of different phenotypes but particularly high in severe malaria anemia. As with most complications, the aetiology of severe anemia is multifactorial, involving both hemolysis and an inappropriately low erythropoietic response [33]. Nevertheless, our study suggests that such processes may also be related to parasite load.

The children with “ uncomplicated” malaria who we recruited to our current study were significantly sicker than those in many previous studies, including the Tanzanian study described above [26], because we enrolled children from a hospital as opposed to an
outpatient setting. This may explain why a surprising proportion had a PfHRP2 value of >1,000 ng/ml, a value that has been proposed as a threshold for identifying children with “true” severe malaria [6, 26]. The study by Rubach [9] also found that a substantial proportion of children with uncomplicated malaria had a plasma PfHRP2 of >1000 ng/ml, again perhaps reflecting a sicker population, although the median level was still less than half that in children with severe malaria.

In our current study there was considerable overlap in plasma PfHRP2 concentrations between the uncomplicated and severe groups but the sequestration index was more discriminatory, being approximately eight-fold higher in the severe than in the uncomplicated group in which the sequestration index was similar to that in that in the group with asymptomatic malaria. The sequestration index of approximately 40 is close to estimates based on the post-mortem examination of brain tissue from Southeast Asian patients with cerebral malaria [34, 35]. The low predictive value of peripheral parasite densities is a consistent finding in previous studies [10, 26]. Also of relevance is the proportion of mature-stage parasites, consistent with the hypothesis that this also reflects the total parasite biomass and thus the severity of the disease [36]. Unfortunately, peripheral blood parasites were not staged in the current study.

Although a positive correlation between plasma PfHRP2 and malaria severity has been found in the majority of studies, this has not been universal. In one study from Papua New Guinea, no significant difference in PfHRP2 was found between children with severe versus uncomplicated malaria [13]. In agreement with observations from elsewhere in the Pacific [37], case fatality was very low in that study, and PfHRP2 concentrations in children defined as having severe malaria were considerably lower than in other studies. The same profile of low mortality and low PfHRP2 concentrations was reported in a second study, conducted in
the Gambia [14], in which the authors also found no correlation between plasma PfHRP2 and severity. The lack of agreement between these studies and our own might thus be explained by different definitions for severe malaria.

There are two potential caveats to the use of PfHRP2 levels for predicting prognosis and directing care to those at greatest risk. First is the recent recognition that deletions in the genes encoding *pfhrp2* (and its homolog *pfhrp3*) mean that some clones of *P. falciparum* parasites do not express PfHRP2 [38]. While the existence of such parasites is now well established, at present they have only been found in high proportions in Latin America and the horn of Africa [39, 40]; rates of more than 40% have been detected in a number of studies in the Peruvian Amazon. Rates exceeding 2% are rare among studies from sub-Saharan Africa [38] e.g. Ghana [41] and Rwanda [42]. This increasing trend towards the presence of HRP deletions could undermine the utility of quantitative PfHRP2 levels going forwards. Furthermore, our observations cannot be extended to non-falciparum infections, that are common in parts of Ethiopia and Eritrea [40].

The second caveat relates to the recognition that some individuals produce antibodies to PfHRP2 that could potentially reduce the levels measurable in plasma [43]. Although such antibodies are common in some settings, the degree to which they suppress plasma levels is yet to be determined [43]. In the current study, PfHRP2 was undetectable despite the presence of *P. falciparum* parasites in peripheral blood in only 1.7% of the children, suggesting that despite the above caveats, PfHRP2-based risk-assessment methods remain currently useful.
In summary, we have found that *P. falciparum* parasite load, as estimated through measurement of plasma PfHRP2, is strongly related to the severity of clinical malaria in children on the Kenyan coast, and that a low plasma PfHRP2 suggests an alternative pathology. This does not mean that severely ill children with a positive blood film but a low PfHRP2 should be denied appropriate antimalarial treatment nor that antibiotics should be withheld from those with a high PfHRP2 level. Both should be treated empirically as currently recommended in the WHO guidelines [18] but clinicians should be alert to the potential for an alternative diagnosis. Our observation adds weight to the hypothesis that treatments that reduce sequestration, such as the heparin-like molecule sevuparin [44], might be useful in mitigating or reversing disease severity in patients infected with *P. falciparum* parasites.
NOTES

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None of the authors have any conflicts of interest to declare.
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Titles and footnotes to Figures

Figure 1.

**Title** - The distribution of clinical syndromes among severe malaria cases.

**Footnote** - CM, cerebral malaria; RD respiratory distress; SMA severe malaria anemia. Figures show the number of children in each group while the in-hospital mortality rates are shown in parentheses.

Figure 2.

**Title** - Plasma PfHRP2 levels in severe malaria patients according to category of invasive bacterial infection. Error bars show median and interquartile range.

**Footnote** - Categories: NTS (non-typhoidal *Salmonella* species) (11); Other Gram-negatives = *Enterobacter cloacae* (1), *Escherichia coli* (4), *Haemophilus influenzae* (3), *Neisseria species* (1), *Pseudomonas species* (4) *Shigella sonnei* (1); Gram-positive = *Staphylococcus aureus* (4), Group A *Streptococcus* (4), *Streptococcus pneumoniae* (16) and *Streptococcus viridans* (1).
Table 1. Demographic and clinical characteristics of children with malaria, stratified by severity grouping.

| Characteristics                        | Severe malaria | Uncomplicated malaria | Asymptomatic malaria | P-value<sup>a</sup> | P-value<sup>a</sup> | P-value<sup>a</sup> |
|----------------------------------------|----------------|-----------------------|----------------------|---------------------|---------------------|---------------------|
|                                        | All CM SMA RD Other SM | All CM SMA RD Other SM | All CM SMA RD Other SM |
| n (%):                                 | 1,521 (100) 881 (58.0) 335 (22.0) 477 (31.4) 330 (21.7) 198 (100) N/A 28 (100) N/A | 1,521 (100) 881 (58.0) 335 (22.0) 477 (31.4) 330 (21.7) 198 (100) N/A 28 (100) N/A |
| Female, n (%):                         | 753 (49.5) 442 (50.2) 171 (51.0) 232 (48.6) 154 (46.7) 92 (46.5) N/A 7 (35.0) N/A | 753 (49.5) 442 (50.2) 171 (51.0) 232 (48.6) 154 (46.7) 92 (46.5) N/A 7 (35.0) N/A |
| Age, years (median, IQR):              | 2.4 (1.4, 3.7) 2.3 (1.4, 3.6) 1.7 (0.8, 2.5) 2.2 (1.3, 3.3) 2.8 (1.8, 4.7) 3.1 (1.9, 4.3) N/A 7.6 (6.5, 9.5) N/A | 2.4 (1.4, 3.7) 2.3 (1.4, 3.6) 1.7 (0.8, 2.5) 2.2 (1.3, 3.3) 2.8 (1.8, 4.7) 3.1 (1.9, 4.3) N/A 7.6 (6.5, 9.5) N/A |
| WAZ, mean (SD):                        | -1.9 (1.3) -1.9 (1.3) -2.1 (1.2) -1.9 (1.4) -1.7 (1.2) -1.7 (0.9) 0.122 N/A N/A | -1.9 (1.3) -1.9 (1.3) -2.1 (1.2) -1.9 (1.4) -1.7 (1.2) -1.7 (0.9) 0.122 N/A N/A |
| HAZ, mean (SD):                        | -1.4 (1.8) -1.3 (1.7) -1.5 (1.8) -1.4 (1.9) -1.4 (1.7) -1.5 (1.2) 0.530 N/A N/A | -1.4 (1.8) -1.3 (1.7) -1.5 (1.8) -1.4 (1.9) -1.4 (1.7) -1.5 (1.2) 0.530 N/A N/A |
| Prostration (BCS 3 or 4), n (%):       | 271/1,484 (18.3) 0/881 (0) 36/317 (11.4) 89/472 (18.9) 169/315 (53.7) 0 (0) <0.001 N/A N/A | 271/1,484 (18.3) 0/881 (0) 36/317 (11.4) 89/472 (18.9) 169/315 (53.7) 0 (0) <0.001 N/A N/A |
| Coma (BCS <3), n (%):                  | 881/1,484 (59.4) 881/881 (100) 151/317 (47.6) 317/472 (67.2) 0/315 (0) 0 (0) <0.001 N/A N/A | 881/1,484 (59.4) 881/881 (100) 151/317 (47.6) 317/472 (67.2) 0/315 (0) 0 (0) <0.001 N/A N/A |
| Hemoglobin, g/dL, mean (SD):           | 7.0 (2.5) 7.2 (2.4) 3.7 (0.9) 6.8 (2.6) 7.8 (2.0) 8.9 (1.6) <0.001 11.3 (1.2) <0.001 | 7.0 (2.5) 7.2 (2.4) 3.7 (0.9) 6.8 (2.6) 7.8 (2.0) 8.9 (1.6) <0.001 11.3 (1.2) <0.001 |
| Hematocrit %, mean (SD):               | 22.1 (7.8) 23.0 (7.5) 11.8 (2.8) 21.4 (8.0) 24.6 (6.3) 27.5 (4.7) <0.001 N/A N/A | 22.1 (7.8) 23.0 (7.5) 11.8 (2.8) 21.4 (8.0) 24.6 (6.3) 27.5 (4.7) <0.001 N/A N/A |
| Severe anemia (Hb <5 g/dL), n (%):     | 335 (22.0) 151 (17.1) 335 (100) 122 (25.3) 0 (0) 0 (0) <0.001 N/A N/A | 335 (22.0) 151 (17.1) 335 (100) 122 (25.3) 0 (0) 0 (0) <0.001 N/A N/A |
| Respiratory distress, n (%):           | 477 (31.5) 317 (36.2) 122 (36.4) 477 (100) 0 (0) 50 (25.1) <0.001 N/A N/A | 477 (31.5) 317 (36.2) 122 (36.4) 477 (100) 0 (0) 50 (25.1) <0.001 N/A N/A |
| Hypoglycemia, n (%):                   | 187 (12.3) 126 (14.3) 59 (17.6) 95 (19.9) 13 (3.9) 8 (4.0) <0.001 N/A N/A | 187 (12.3) 126 (14.3) 59 (17.6) 95 (19.9) 13 (3.9) 8 (4.0) <0.001 N/A N/A |
| Base deficit, mmol/L, mean (SD):       | -10.5 (6.2) -10.6 (5.9) -13.1 (7.0) -13.4 (6.4) -7.5 (5.4) -4.1 (4.1) <0.001 N/A N/A | -10.5 (6.2) -10.6 (5.9) -13.1 (7.0) -13.4 (6.4) -7.5 (5.4) -4.1 (4.1) <0.001 N/A N/A |
| Severe acidosis (BD <-8 mmol/L), n (%): | 766/1,238 (61.8) 471/720 (65.4) 196/260 (75.3) 319/400 (79.7) 111/275 (40.3) 24/176 (13.6) <0.001 N/A N/A | 766/1,238 (61.8) 471/720 (65.4) 196/260 (75.3) 319/400 (79.7) 111/275 (40.3) 24/176 (13.6) <0.001 N/A N/A |
| Invasive bacterial infections, n (%):  | 50 (3.3) 26 (2.9) 15 (4.5) 10 (2.1) 12 (3.6) 1 (0.5) 0.020 N/A N/A | 50 (3.3) 26 (2.9) 15 (4.5) 10 (2.1) 12 (3.6) 1 (0.5) 0.020 N/A N/A |
Hyperparasitemia n (%) 454 (29.8) 266 (30.0) 80 (23.8) 169 (35.4) 93 (28.1) 79 (39.9) 0.004 N/A N/A

In-hospital mortality n (%) 178 (11.7) 128 (14.5) 47 (14.0) 79 (16.5) 16 (4.8) 0 (0) <0.001 N/A N/A

SM severe malaria; CM cerebral malaria; SMA severe malaria anemia; RD respiratory distress. Some children with severe malaria manifest more than one clinical feature of severity; N/A not available or not applicable; a between Uncomplicated and all severe malaria; b between asymptomatic and all severe malaria. The organisms grown were: severe malaria patients: Enterobacter cloacae (1), Escherichia coli (4), Haemophilus influenzae (3), Neisseria species (1), Pseudomonas species (4), Shigella sonnei (1), non-typhoidal Salmonella species (11), Staphylococcus aureus (4), Group A Streptococcus (4), Streptococcus pneumoniae (16) and Streptococcus viridans (1). Uncomplicated malaria patients: Streptococcus viridans (1). All IBI organisms were grown from blood except for a single S. pneumoniae infection that was detected in CSF only.
Table 2. Quantitative markers of parasite load stratified by severity groupings.

|                      | Parasite density (parasites/µL) | PFHPR2 (ng/ml) | Total parasite biomass (parasites/child) | Circulating biomass (parasites/child) | Sequestered biomass (parasites/child) | Sequestration index |
|----------------------|---------------------------------|----------------|-------------------------------------------|--------------------------------------|---------------------------------------|-------------------|
| **Group 1:** All severe malaria | 39,588 (34,990-44,791)           | 1,369          | 1.2x10^{12}                               | 3.1x10^{12}                          | 1.2x10^{12}                          | 40.6 (35.7-46.0)  |
|                      | (1,244-1,506)                    | 1.4x10^{12}    | (2.8x10^{12})                             | (1.1x10^{12})                        | (1.4x10^{15})                        | 6.0x10^{11}       |
|                      |                                  | 6.8x10^{11}    | 1.3x10^{13}                               | 8.4x10^{11}                          | 4.9 (3.5-6.8)                        |                   |
| **Group 2:** Uncomplicated malaria | 111,064 (86,798-141,819)         | 5.2x10^{11}    | 1.0x10^{11}                               | 6.0x10^{11}                          | 4.0 (1.8-8.8)                        |                   |
|                      | (655-1,084)                      | (4.0x10^{11})  | (8.2x10^{11})                             | (4.3x10^{11})                        |                                     |                   |
| **Group 3:** Asymptomatic malaria | 1,092 (523-2,280)                | 6.4x10^{10}    | 1.5x10^{10}                               | 4.3x10^{10}                          | 9.4x10^{10}                          |                   |
|                      | (7-412)                          | (7.4x10^{10})  | (3.4x10^{10})                             | (1.9x10^{10})                        |                                     |                   |
|                      |                                  | 1.0x10^{10}    | 3.4x10^{10}                               |                                     |                                     |                   |
|                      |                                  | 6.4x10^{9}     | 1.0x10^{10}                               |                                     |                                     |                   |
|                      | P                                | <0.001         | <0.001                                    | <0.001                               | <0.001                               | <0.001            |
| **Severe malaria survived** | 43,840 (38,463-49,967)           | 1,401          | 1.3x10^{12}                               | 3.5x10^{15}                          | 1.3x10^{12}                          | 38.8 (33.9-44.4)  |
|                      | (1,268-1,547)                    | (9.2x10^{12})  | (3.0x10^{15})                             | (1.1x10^{12})                        | (1.4x10^{17})                        |                   |
| **Severe malaria died** | 25,737 (17,495-37,843)           | 9.4x10^{11}    | 1.7x10^{12}                               | 1.0x10^{12}                          |                                     |                   |
|                      | (833-1,577)                      | (6.7x10^{11})  | (1.1x10^{12})                             | (7.2x10^{11})                        |                                     |                   |
|                      |                                  | 1.3x10^{10}    | 2.5x10^{10}                               |                                     |                                     |                   |
|                      |                                  | 1.0x10^{10}    | 2.5x10^{10}                               |                                     |                                     |                   |
|                      | P                                | 0.012          | 0.052                                     | 0.001                                | 0.105                                | 0.012             |

Figures show geometric mean values with 95%CIs in parentheses of values calculated from within individual participants. The numbers within each group were: severe malaria n=1,521; uncomplicated malaria n=198; asymptomatic malaria n=28; severe malaria survived n=1,343; severe malaria died n=178.
Table 3. Linear regression analysis of markers of parasite biomass among patients with severe malaria.

|                         | N  | Geometric mean (95% CI) | Adjusted multivariate linear regression $^*$ |
|-------------------------|----|-------------------------|---------------------------------------------|
|                         |    |                         | exp Coef*  | 95% CI  | P     |
| Parasite density (per µl) |    |                         |            |         |       |
| UM                      | 198| 111,064 (86,798-141,819)| Ref        |         |       |
| CM                      | 871| 39,046 (33,121-46,030)  | 0.38       | 0.26-0.56| <0.001|
| SMA                     | 331| 40,901 (32,193-51,964)  | 0.35       | 0.24-0.52| <0.001|
| RD                      | 473| 57,158 (46,172-70,757)  | 0.54       | 0.36-0.78| 0.001 |
| Others                  | 320| 32,587 (24,756-42,896)  | 0.28       | 0.19-0.43| <0.001|
| PHRP2 (ng/ml)           |    |                         |            |         |       |
| UM                      | 198| 842 (655-1,084)         | Ref        |         |       |
| CM                      | 881| 1,297 (1,143-1,472)     | 1.5        | 1.1-2.0 | 0.006 |
| SMA                     | 335| 2,565 (2,137-3,078)     | 3.2        | 2.3-4.5 | <0.001|
| RD                      | 477| 1,728 (1,466-2,037)     | 2.1        | 1.5-2.8 | <0.001|
| Others                  | 330| 1,026 (829-1,270)       | 1.1        | 0.9-1.6 | 0.462 |
| Total biomass (parasites/child) |    |                         |            |         |       |
| UM                      | 191| 5.2x10$^{11}$ (4.0x10$^{11}$-6.8x10$^{11}$) | Ref        |         |       |
| CM                      | 876| 1.2x10$^{12}$ (1.0x10$^{12}$-1.4x10$^{12}$) | 2.2        | 1.6-3.0 | <0.001|
| SMA                     | 335| 2.3x10$^{12}$ (1.9x10$^{12}$-2.8x10$^{12}$) | 4.7        | 3.3-6.5 | <0.001|
| RD                      | 473| 1.5x10$^{12}$ (1.3x10$^{12}$-1.8x10$^{12}$) | 2.9        | 2.1-4.1 | <0.001|
| Others                  | 328| 1.1x10$^{12}$ (8.5x10$^{12}$-1.3x10$^{12}$) | 1.9        | 1.3-2.7 | 0.001 |
| Sequestration index     |    |                         |            |         |       |
| UM                      | 191| 4.9 (3.5-6.8)           | Ref        |         |       |
| CM                      | 875| 38.7 (32.6-46.0)        | 7.2        | 4.8-11.0| <0.001|
| SMA                     | 335| 82.4 (64.5-105)        | 18.2       | 11.8-28.2| <0.001|
| RD                      | 473| 36.1 (28.6-45.5)        | 7.4        | 4.8-11.2| <0.001|
| Others                  | 327| 35.8 (27.4-46.7)        | 7.6        | 4.9-12.0| <0.001|

Linear regression analysis was adjusted for HbS, α-thalassemia genotype and ethnicity. * values show the exponentiated coefficients of the log$_{10}$transformed data.
Figure 1

- Central malaria n=881
  - 591 (11.9%)
  - 88 (20.4%)
  - 126 (5.5%)

- Other severe malaria
  - 63 (11.1%)
  - 24 (17.6%)
  - 330 (4.4%)

- Respiratory distress n=477
  - 229 (18.7%)

Severe maternal anemia n=335

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Figure 2

Log plasma HRP2 (ng/ml)

Culture-negative  NTS  Other Gram-negative  Gram-positive

Category

200ng/ml