Plasma *Plasmodium falciparum* Histidine-Rich Protein-2 Concentrations Do Not Reflect Severity of Malaria in Papua New Guinean Children

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Background. In areas of unstable malaria transmission, plasma *Plasmodium falciparum* histidine-rich protein 2 (PfHRP-2) concentrations parallel total parasite biomass and thus infection severity. However, where transmission is more intense, plasma PfHRP-2 might not reliably predict complications and mortality.

Methods. As part of a prospective case-control study of severe pediatric illness in Madang, Papua New Guinea, we recruited 220 children aged 6 months to 10 years with severe falciparum malaria, 48 with uncomplicated malaria, and 139 healthy controls. Groups were matched by age, sex, and province of parental birth. Plasma PfHRP-2 levels were quantified by validated immunoassay.

Results. Detectable plasma PfHRP-2 concentrations were present in 21 healthy controls (15.1%). Although plasma PfHRP-2 levels were higher in the children with clinical malaria (*P* < .001), there was no difference between those with uncomplicated and severe infections (median, 584 and 456 ng/mL, respectively [interquartile range, 77–1114 and 113–1113 ng/mL, respectively]; *P* = .43). Log parasitemia, hemoglobin, log plasma bilirubin, and plasma creatinine levels were independently associated with plasma PfHRP-2 levels in multiple regression analysis (*P* < .014), but coma, blood lactate level, and plasma bicarbonate level were not. The 1 severely ill child who died had a plasma PfHRP-2 concentration of 483 ng/mL, close to the group median.

Conclusions. The clinical and prognostic utility of plasma PfHRP-2 concentrations depends on the epidemiologic circumstances. In areas of intense malaria transmission, plasma PfHRP-2 reflects recent as well as present infections.

*Plasmodium falciparum* histidine-rich protein-2 (PfHRP-2) is a water-soluble protein that is synthesized by both asexual and early sexual stages of the parasite. Its production increases as the asexual parasite matures such that most (~90%) is released at schizogony [1, 2]. Circulating PfHRP-2 may be unbound in plasma, antibody-bound in plasma, inside infected erythrocytes, bound to uninfected erythrocytes as part of immune complexes, and/or bound to other cells such as leukocytes [3]. Its presence in plasma has allowed the development of inexpensive point-of-care rapid detection tests (RDTs) [4]. These have a primary diagnostic role where reliable microscopy is unavailable or in situations in which the densities of circulating young non-cytoadherent parasite forms are below those detectable by microscopy, such as in highly synchronous infections [5] or pregnancy [6].

PfHRP-2 can be more accurately quantified in plasma using conventional techniques including enzyme-linked immunosorbent assay (ELISA) [1, 3, 7]. Because of the stage-dependency of its release into the circulation, plasma PfHRP-2 concentrations have been suggested as a clinically useful marker of the sequestered biomass of cytoadherent mature parasites and thus the presence of
complications such as coma and renal failure. In studies of Asian adults, plasma PfHRP-2 concentrations at presentation have been associated with severe falciparum malaria [1, 7] and subsequent mortality [7, 8]. However, in another relatively small study involving 22 African children, no such association was found [9].

The role of quantitative plasma PfHRP-2 as a marker of the severity and outcome may depend on the epidemiologic context. PfHRP-2 can be detected in blood for up to 4 weeks after successful treatment of falciparum malaria [10, 11] including in children [12]. In areas of unstable transmission with no or limited immunity to malaria, in adults with no recent infection and a relatively early symptomatic presentation, plasma PfHRP-2 concentrations at admission may parallel prior parasite replication and thus reflect the probability of complications and mortality [1, 7]. However, in children from areas with hyperendemic transmission who are likely to have experienced recent asymptomatic or symptomatic infections, who have a degree of immunity including to PfHRP-2 itself [13], and who may thus present relatively late, plasma PfHRP-2 quantified by immunoassay may not reliably identify those with severe infections and those at risk of death [9].

We hypothesized, therefore, that plasma PfHRP-2 concentrations in Melanesian children from a hyperendemic area of Papua New Guinea (PNG) would not reliably distinguish those with uncomplicated from those with severe falciparum malaria.

PATIENTS AND METHODS

Study Site and Participants
 Madang Province on the north coast of PNG has an estimated population of ~450,000, 54% of whom are <20 years old [14]. Transmission of Plasmodium falciparum and P. vivax is hyperendemic [15] with an estimated entomologic inoculation rate of 50–150 infective bites per year [16]. Modilon Hospital is the provincial hospital and the health care facility to which the majority children with severe illness are referred.

The children with severe malaria in the present substudy comprised all those aged 6 months to 10 years admitted to Modilon Hospital between October 2006 and August 2009 for whom stored frozen plasma was available. The healthy controls (children without acute illness or a history of malaria within the previous 2 weeks) were recruited from community immunization clinics surrounding Madang Town and were matched with severe cases by age, sex, and province of parental birth. The children with uncomplicated malaria were recruited from the immunization clinics, the Modilon Hospital Pediatric Outpatient Clinic, and the nearby Alexishafen Health Centre and were also matched with the severe cases by age, sex, and province of parents’ birth. The healthy controls and uncomplicated malaria cases were selected to be of an age that was within 12 months of the index severe malaria case. Written informed consent was obtained from parent/guardian(s). The present substudy was approved by the PNG Institute of Medical Research Institutional Review Board and the Medical Research Advisory Committee of the PNG Health Department and conducted in accordance with the Declaration of Helsinki.

Clinical Assessment and Management
 After recruitment, a standardized case report form was completed that included demographic information, medical history, and details of the current illness. Venous blood was taken for preparation of Giemsa-stained blood smears, rapid antigen testing (ICT Malaria Combo Cassette Test M102; ICT Diagnostics), and measurement of hemoglobin and glucose levels using Glucose 201+ and Hb 201+ analyzers, respectively. Blood lactate level was measured enzymatically using Lactate Pro (Arkray). Remaining plasma was stored at −80°C for subsequent assay. Blood cultures were performed as part of the standard assessment to exclude concomitant bacteremia. Lumbar punctures were performed when there was clinical suspicion of bacterial meningitis, in accordance with national management protocols [17].

Children were classified as having severe malaria if they had >1000 asexual forms of P. falciparum per microliter of whole blood in the presence of 1 or more of the following: (1) impaired consciousness or coma (Blantyre Coma Score of <5 [18]), (2) prostration (an inability to sit or stand unaided), (3) multiple seizures, (4) hyperlactatemia (blood lactate level of >5 mmol/L), (5) severe anemia (hemoglobin level of <50 g/L), (6) dark urine, (7) hypoglycemia (blood glucose level of <2.2 mmol/L), (8) jaundice, or (9) respiratory distress. These criteria are consistent with the World Health Organization definition [19]. Severely ill children in whom P. vivax was subsequently identified by polymerase chain reaction (PCR) with or without P. falciparum were excluded. Children with a P. falciparum density of >1000 parasites per microliter and no signs of severity were classified as having uncomplicated malaria. The healthy controls had no current or recent history of malaria, irrespective of the presence or absence of P. falciparum on microscopy. Antimalarial therapy was administered and complications managed by hospital staff according to national protocols [17].

Laboratory Methods
 Microscopy was performed independently by at least 2 experienced microscopists. The P. falciparum parasitemia was determined from counting the number of parasites per 200 leucocytes and an assumed white cell count of 8000 cells/μL. Plasmodium species was confirmed by PCR. Biochemical tests including plasma bilirubin, alanine aminotransferase, and creatinine were performed on stored frozen plasma using a Cobas Integra 800 analyzer (Roche Diagnostics). Diagnostic
RESULTS

Subject Characteristics
We recruited 139 healthy controls, 48 children with uncomplicated malaria, and 220 children with severe malaria. The baseline clinical and laboratory characteristics of subjects in these three groups are summarized in Table 1. There were no significant differences between groups in age, sex, or body weight. Children with either severe or uncomplicated malaria had higher axillary temperatures, parasite densities, and plasma creatinine concentrations and lower hemoglobin concentrations than the healthy controls, and higher proportions had a palpable spleen. The respiratory rate was higher in children with severe malaria when compared with those children with uncomplicated malaria. Concomitant bacteremia or acute bacterial meningitis was not identified in any child with severe malaria.

Plasma PfHRP-2 Concentrations
Plasma PfHRP-2 concentrations in the 3 groups of subjects are summarized in Figure 1. The median concentrations in the uncomplicated and severe malaria groups (584 and 456 ng/mL, respectively [IQR, 77–1,114 and 113–1,113 ng/mL; P < .001]). Twenty-one of the healthy children (15.1%), including 16 (11.5%) with a positive blood smear for malaria, had detectable plasma PfHRP-2 concentrations that ranged between 2 and 121 ng/mL. The remaining 118 had an undetectable plasma PfHRP-2 concentration and were aperasitic. There were 5 children in the other 2 groups who were parasitic at presentation but had a negative PfHRP-2 assay (see Figure 1).

When the 268 children in the uncomplicated and severe groups were considered together, there were significant bivariate correlations between the log plasma PfHRP-2 concentration and log parasite density, hemoglobin level, and log plasma bilirubin level (P < .001 in each case) (Figure 2). No significant correlation was seen for other variables associated with severity of malaria in children, including Blantyre Coma Score (Figure 3) and, for the severe cases only, blood lactate level (P > .08 in each case). The 3 significant variables were entered into a multiple linear regression model that included plasma creatinine and alanine aminotransferase level in view of the possibility that PfHRP-2 is renally excreted and/or hepatically metabolized even though neither of these variables was bivariately associated with plasma PfHRP-2 concentration (P > .37). The final multivariate model is shown in Table 2. The log parasite density, hemoglobin level, log plasma bilirubin level, and plasma creatinine level were each independently associated with plasma PfHRP-2 concentration (P ≤ .014). The adjusted r² value was .25, with the greatest changes in this parameter (Δr² = .16) seen with the addition of log plasma bilirubin level to the model.

One child in the severe malaria group who presented with multiple convulsions and prostration died. The parasite density at presentation was 154,750 per microliter, and the plasma PfHRP-2 concentration was 483 ng/mL, a value close to the group median. A postmortem brain smear revealed numerous late-stage parasites sequestered within the microvasculature. All other children responded to treatment and were discharged well.

DISCUSSION
The present study provides strong evidence that plasma PfHRP-2 concentrations are of limited use in assessing complications and prognosis in pediatric malaria in PNG. Our severely ill
children did not have significantly higher plasma PfHRP-2 concentrations than those with uncomplicated infections at presentation, with individual values in a wide range from 1.5 to 27,338 ng/mL. In addition, there was no association between plasma PfHRP-2 concentration and the recognized phenotypic expression of severe malaria, including altered consciousness, raised blood lactate level, and low plasma bicarbonate level. Multivariate analysis of the present data suggests that peripheral parasitemia and associated acute hemolysis are predictors of plasma PfHRP-2 concentrations in PNG children, but significant associations with hemoglobin and plasma creatinine levels could also mean that prior episodes of malaria and renal clearance are also important determinants in this epidemiologic setting.

There have been 3 previous studies of PfHRP-2 in severe malaria. In the first [7], a sample of 337 Thai adults with malaria had a mean plasma PfHRP-2 concentration of 840 ng/mL. Although separate PfHRP-2 data for the 170 patients with uncomplicated malaria and 167 patients with severe malaria were not provided, the implications of a mathematical model of parasite biomass based on PfHRP-2 concentrations suggest that they were substantially higher in the severe group and highest in fatal cases [7]. PfHRP-2-based estimates of parasite burden were also associated with coma, renal failure, hyperlactatemia, and hyperbilirubinemia. Hematocrit level was not associated with estimated biomass but was a key variable in its derivation [7].

In the second study [8], plasma PfHRP-2 concentrations in 51 Indonesian adults with severe malaria (mean, 1,863 ng/mL) were higher than in 77 with moderately severe malaria (mean, 314 ng/mL), with an implied association with mortality [8]. In the only study to have assessed the utility of quantitative PfHRP-2 measurements in pediatric malaria [9], 22 Kenyan
children with severe infections had a median plasma PfHRP-2 concentration of only 63 ng/mL. Plasma PfHRP-2 concentration was significantly associated with the peripheral parasitemia but not with sequestered parasite load derived mathematically from serial measurements of parasitemia after treatment. The median plasma PfHRP-2 concentrations in our 2 groups of children with malaria appear similar to those in Thai and Indonesian adults with uncomplicated or moderately severe falciparum malaria [7, 8], lower than those in Asian adults with severe malaria [7, 8], and higher than those in African children with severe malaria [9]. These discrepancies may reflect differences in assay methodology, demography, and/or malaria epidemiology.

Although the present study and 2 previously published adult studies [7, 8] have used the same ELISA system, no laboratory methods were provided for the Kenyan study [9], and it is therefore possible that its relatively low median value was a function of the assay used. An alternative explanation of the discrepancy between the present and Kenyan studies is that the production of PfHRP-2-specific blocking antibodies that form part of the development of functional immunity to malaria in areas of high transmission could interfere with the PfHRP-2 assay. High-titer antibodies can lead to negative PfHRP-2-based RDTs despite a high parasite density [13], and it is possible that the low quantitative plasma PfHRP-2 concentrations in African children result from this form of assay interference [9]. Nevertheless, the intensity of transmission in coastal PNG parallels

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**Figure 2.** Scatterplots of plasma PfHRP-2 concentration against parasite density, haemoglobin level, and plasma bilirubin level for the 268 children with either uncomplicated or severe malaria. The filled circles represent children with uncomplicated malaria and the open circles those with severe malaria.

**Figure 3.** Box plot showing the median, interquartile range, maximum, and minimum for plasma PfHRP-2 concentrations in the children with uncomplicated malaria and, by Blantyre Coma Score, for those with severe infections. The threshold for assay positivity (15 ng/mL) is shown as a horizontal dashed line. The numbers in parentheses are the total in each group. There were no significant between-group differences.
that of sub-Saharan Africa. In the same way, the lack of a difference in plasma PfHRP-2 concentration between uncomplicated and severe malaria in our children could reflect antibody effects, but it is likely that the children with severe malaria had less established immunity than those with uncomplicated infections. Nevertheless, 11 of our children with severe malaria had low plasma PfHRP-2 concentrations (<5.0 ng/mL), including 4 with undetectable levels, despite relatively high parasite densities. All these 11 children had positive RDTs. This apparent discrepancy may result from cross-reactivity between other antigens such as PfHRP-3 and the monoclonal antibody embedded in the RDT [21].

In studies of PfHRP-2-based RDTs, false negative results have also been attributed to an excess of antigen, as well as antibodies that block the detection antibody target site. This prozone phenomenon can be mitigated by sample dilution [22]. Because all our samples were assayed in multiple dilutions from neat to 1:256, this effect is unlikely to have influenced our plasma concentration data, but whether it might have had an effect in the Kenyan study [9] is unknown. Geographic strain variation in PfHRP-2 could also account for apparent discrepancies between the present study and published data. Variation in the number of children with malaria and the presence of detectable PfHRP-2 in the PNG Institute of Medical Research for clinical and logistic assistance, research nurses, microscopists, data management team, and support staff of the PNG Institute of Medical Research for clinical and logistic assistance,

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<tr>
<td>Log plasma bilirubin level*</td>
<td>.752 (.149)</td>
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<td>Hemoglobin level, increase of 10 g/L</td>
<td>−.089 (.018)</td>
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<td>Log parasite density*</td>
<td>.274 (.073)</td>
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<td>Plasma creatinine level, increase of 10 µmmol/L</td>
<td>.086 (.035)</td>
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* An increase of 1 parasite per microliter in log parasite density or 1 mmol/L in log plasma bilirubin level corresponds to a 10-fold increase in these parameters.

change during the first 2 d after treatment. These data suggest that PfHRP-2 clearance is slower and more dependent on renal function in children than in adults. In any case, persistence of PfHRP-2 for up to 4 weeks (almost 8 half-lives based on the estimate provided by Dondorp et al [7]) has been documented in adults using RDTs [10, 11], implying significant between-subject variability in elimination half-life. It would be interesting to know whether this was also observed in the initial kinetic study carried out by Dondorp et al [7]. Slow PfHRP-2 clearance during a single episode of malaria could increase plasma concentrations at presentation but could also contribute when the time between recurrences of malaria is short, as is a characteristic of intense transmission in areas such as coastal PNG and sub-Saharan Africa. The association between plasma creatinine and PfHRP-2 levels we observed might simply suggest that both are measures of parasite biomass, but the lack of association between PfHRP-2 level and other complications makes this unlikely.

The combination of independent associations between plasma PfHRP-2 concentration and plasma bilirubin level, parasite density, and hemoglobin level we observed is consistent with the obligatory hemolysis of parasitized red cells with liberation of PfHRP-2, metabolism of free hemoglobin, and an acute decrease in hemoglobin level. A positive association with hyperbiliruinemia has also been observed in adults [7]. However, the degree of anemia in our severely ill children and the presence of intense transmission of both *P. falciparum* and *P. vivax* suggest that the association with hemoglobin may also reflect, in part, other recent episodes of malaria and subsequent persistence of PfHRP-2 [10, 11]. This would help explain the similarity between plasma PfHRP-2 concentrations in the 2 groups of children with malaria and the presence of detectable PfHRP-2 in 15% of our healthy controls. Other potential biomarkers of parasite burden such as quantitative parasite DNA or plasmodium lactate dehydrogenase may be more reliable than PfHRP-2 because they do not persist for as long after treatment but were not measured in the present study.

Although modeling of baseline plasma PfHRP-2 data to generate estimates of parasite biomass may be valid in areas of low and unstable transmission [7], the present data suggest that this is inappropriate in hyperendemic and holoendemic areas such as in Africa and PNG. In addition, the clinical value of PfHRP-2 as an index of severity and a guide to prognosis, whether available as a simple plasma concentration or as part of a mathematically derived parasite load, appears limited in countries such as PNG.

**Acknowledgments**

We thank the children and their parents/guardians for their participation. We are also grateful to the medical and nursing staff of the Pediatric Ward and Children’s Outpatient Department at Modilon Hospital and the research nurses, microscopists, data management team, and support staff of the PNG Institute of Medical Research for clinical and logistic assistance.
and to the laboratory and research staff of the Fremantle Hospital Biochemistry Department for biochemical testing. Cellabs provided the recombinant PFHRP-2 and ELISA kits at a discounted rate. We also acknowledge infrastructure support from the MalariaGen Genomic Epidemiology Network.

Funding support. This work was supported by the National Health and Medical Research Council of Australia (513782, scholarship to L.M., and Practitioner Fellowship to T.M.E.D.); the Fogarty Foundation (scholarship to M.L.); the Royal Australasian College of Physicians (Basser scholarship to L.M.).

Potential conflicts of interest. All authors: no conflicts.

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