Measuring the *Plasmodium falciparum* HRP2 protein in blood from artesunate-treated malaria patients predicts post-artesunate delayed hemolysis


Artesunate, the recommended drug for severe malaria, rapidly clears the malaria parasite from infected patients but frequently induces anemia—called post-artesunate delayed hemolysis (PADH)—for which a simple predictive test is urgently needed. The underlying event in PADH is the expulsion of artesunate-exposed parasites from their host erythrocytes by pitting. We show that the histidine-rich protein 2 (HRP2) of the malaria parasite *Plasmodium falciparum* persists in the circulation of artesunate-treated malaria patients in Bangladesh and in French travelers who became infected with malaria in Africa. HRP2 persisted in whole blood (not plasma) of artesunate-treated patients with malaria at higher levels compared to quinine-treated patients. Using an optimized membrane permeabilization method, HRP2 was observed by immunofluorescence, Western blotting, and electron microscopy to persist in once-infected red blood cells from artesunate-treated malaria patients. HRP2 was deposited at the membrane of once-infected red blood cells in a pattern similar to that for ring erythrocyte surface antigen (RESA), a parasite invasion marker. On the basis of these observations, we developed a semiquantitative titration method using a widely available artesunate-based rapid diagnostic dipstick test. Positivity on this test using a 1:500 dilution of whole blood from artesunate-treated patients with malaria collected shortly after parasite clearance predicted subsequent PADH with 89% sensitivity and 73% specificity. These results suggest that adapting an existing HRP2-based rapid diagnostic dipstick test may enable prediction of PADH several days before it occurs in artesunate-treated patients with malaria.

**INTRODUCTION**

The artemisinin drug artemesunate is a first-line treatment for severe *Plasmodium falciparum* infection worldwide (1, 2), saving more patients with severe malaria than the previous reference drug quinine (3–5). Use of artemesunate is associated with fatality rates consistently lower than 5% (6–8) in travelers with severe malaria, but a substantial proportion is affected by hemolytic episodes occurring after parasitological cure (7–11). Such episodes are called post-artesunate delayed hemolysis (PADH) (6, 12). In France, where artemesunate was introduced for the treatment of severe malaria in 2011, anemia accounted for 82 and 85% of adverse events graded 3 and 4, respectively (7). PADH can have a marked clinical impact, as illustrated by the 14% rate of severe anemia observed in this cohort (7) and by a recent report of severe renal insufficiency triggered by a PADH episode (13). PADH also occurs after parenteral artesunate treatment in children in malaria-endemic countries (14), where it may also follow oral treatment of uncomplicated malaria with artemisinn-based combination therapy (15). PADH-type episodes have not been described in patients treated with quinine alone (16). Thus, although artesunate is superior to quinine for the treatment of severe malaria worldwide, weekly follow-up of hematological and renal parameters for 1 month after treatment is generally recommended to ensure a sustained positive outcome (10). World Health Organization (WHO) has also called for the urgent setup and validation of a simple test that can predict PADH in artesunate-treated patients with malaria who have been cleared of their infection (2).

PADH is linked to the destruction of circulating red blood cells (RBCs) that were once infected but from which parasites have been removed by “pitting” in the spleen after artesunate treatment (6). In travelers treated with artesunate, PADH correlates with the peak concentration of these once-infected RBCs measured with flow cytometry based on the ring erythrocyte surface antigen (RESA) (6), a method potentially available only in well-equipped hospitals. Here, we validated a predictive test for PADH for use in endemic settings using the parasite protein histidine-rich protein 2 (HRP2), which persists in the blood of artesunate-treated patients with malaria.

**RESULTS**

*P. falciparum* HRP2 persists in circulating erythrocytes of artesunate-treated malaria patients

In the course of kinetic studies of diagnostic antigens in the blood of 95 patients with *P. falciparum* malaria in Bangladesh (Table 1),
we determined that about half of the initial HRP2 detected in whole blood before artesunate treatment at day 0 remained in the circulation 3 days after treatment started. The median concentration was 2693 ng/ml at day 3 versus 6139 ng/ml at day 0 (Fig. 1A). Although significant ($P < 0.0001$), the fall in whole-blood HRP2 was less than that in parasitemia, with complete microscopic clearance of parasites observed in 75 of 89 patients (84.3%) at day 3 after treatment started (Table 1). Analogous studies on samples from 53 French travelers (all infected in Africa) treated for severe malaria with artesunate (Table 1) also showed marked HRP2 persistence in whole blood: The median concentration was 8460 ng/ml at day 3 versus 10,575 ng/ml at day 0 ($P = 0.1614$) (Fig. 1B) despite rapid parasite clearance (94.6% of patients were microscopically clear of asexual parasites at day 3). The persisting HRP2 concentration at day 3 in whole blood was higher than that in plasma (median concentration, 8460 ng/ml versus 119.3 ng/ml; $P < 0.0001$) (Fig. 1B). HRP2 persistence in whole blood of artesunate-treated patients at day 3 was 77% of the day 0 concentration (equivalent to a fraction drop of 23% from day 0 to day 3), whereas persistence in plasma was 36% (fraction drop of 64%). Only two patients had optically detectable gametocytes in the circulation at day 3 after artesunate treatment (positive on thick smear and negative on thin smear).

In contrast, in 49 French travelers treated with intravenous quinine over the same period (Table 1), the concentration of whole-blood HRP2 at day 3 was only 18.4% of baseline (median concentration, 2892 ng/ml at day 3 versus 15,725 ng/ml at day 0; $P = 0.0006$) (Fig. 1B), a lower proportion than for artesunate-treated patients ($P = 0.0081$). In these quinine-treated patients, HRP2 persistence at day 3 was only 20% of the day 0 concentration in whole blood and only 32% of the day 0 concentration in plasma. These findings indicated that after treatment of adults with intravenous artesunate (but not with quinine), HRP2 persisted in the blood, potentially in the subpopulation of once-infected erythrocytes (6, 17, 18).

**HRP2 is present in the erythrocyte cytosol soon after parasite invasion**

HRP2 synthesis begins in ring-stage parasites (19), although HRP2 is produced predominantly by mature parasite stages (20, 21). To determine more precisely when and where HRP2 is expressed in *P. falciparum* parasites (19), we grew two *P. falciparum* parasite lines, FUP/CB (resa 1-WT) and an isogenic A3F8 resa 1 knockout strain (resa 1-KO) (22), in asynchronous cultures and visualized using the reference erythrocyte membrane immunofluorescence method (23). Using either a monoclonal (RESA=mAb) or a polyclonal anti-RESA antibody (RESA-Ab), a typical staining pattern, predominantly on the erythrocyte plasma membrane, was observed during all red blood cell parasite stages (Fig. 2, A and B). No signal was observed in parasites using either monoclonal or polyclonal anti-RESA antibodies. Under the same experimental conditions, no (or weak) staining was observed when using a monoclonal anti-HRP2 antibody in RBCs infected with wild-type *P. falciparum* parasites (Fig. 2A). However, when immunofluorescence was performed using a previously optimized membrane permeation method (24), HRP2 was readily detected during all matura-
HRP2 is expressed at the erythrocyte membrane

Cellular localization of both RESA and HRP2 proteins was also investigated by examining extracts from different cellular compartments (cytosolic soluble cell fraction,ghost fraction, and membrane insoluble cell fraction) of P. falciparum-infected erythrocytes at the ring stage in the two cultured parasite lines (resa 1-WT and resa 1-KO). Western blot analysis showed that, as previously reported (25), RESA was exclusively detected in association with the membrane-cytoskeleton complex obtained by lysing P. falciparum-infected erythrocytes with hypotonic phosphate-buffered saline (PBS) or buffer containing SDS. HRP2 was also present in these membrane-based extracts and in the cytosolic extract (Fig. 2C).

HRP2 persists in once-infected erythrocytes from artesunate-treated malaria patients

The presence of HRP2 in ring-infected erythrocytes suggested that, like RESA, HRP2 may persist in once-infected RBCs after expulsion of the parasite during the process of pitting in the spleen. Because pitting occurs exclusively in vivo in patients with a functional spleen (26), we tested this hypothesis using samples from artesunate-treated patients with malaria. Blood samples from patients with severe malaria were collected before and at various time points after artesunate treatment. Using the optimized immunofluorescence method (6, 24), HRP2 was detected both in infected erythrocytes on day 0 and in once-infected erythrocytes on day 3 after the treatment started (Fig. 3A), but not in uninfected RBCs. To more precisely determine the deposition of HRP2 in once-infected RBCs, we performed transmission electron microscopy on paired day 0 and day 3 blood samples in a series of five prospectively studied patients with malaria. The smaller gold particles indicating HRP2 were observed at or immediately under the erythrocyte membrane, as well as in small vesicles in the erythrocyte cytosol (Fig. 3B). There was no binding of gold-labeled antibody in uninfected RBCs. RESA was found to be associated only with the membrane-cytoskeletal complex of RBCs, consistent with its known interaction with spectrins (27) and its exclusive detection in insoluble cell fractions.

HRP2 can be used to quantify once-infected erythrocytes

Venous blood samples from artesunate-treated malaria patients that contained infected RBCs (at day 0) or once-infected RBCs (at day 3 to day 11) were analyzed by Western blotting. This analysis confirmed the persistence of HRP2 in both the soluble (Triton extract) and insoluble cell fraction (hypotonic buffer extracts) (Fig. 3C); under the same conditions, RESA was detected only in the insoluble cell fraction. On day 11, bands on Western blots corresponding to both HRP2 and RESA were less intense than on day 3, consistent with the declining concentration of once-infected erythrocytes in the circulation (Fig. 3C). To determine whether the presence of HRP2 in once-infected RBCs...
allowed the accurate quantification of this erythrocyte subpopulation, day 3 and day 7 samples from 11 patients infected with *P. falciparum* were labeled with antibodies to either HRP2 or RESA and analyzed by flow cytometry, as previously described (24). Quantification of day 3 once-infected erythrocytes using HRP2 correlated with that performed using RESA as a reference ($r^2 = 0.757; P = 0.0001; \text{Spearman } r = 0.86$), confirming that HRP2 could be used to quantify once-infected RBCs (Fig. 4A). A small subset of erythrocytes was negative for HRP2 but positive for RESA (Fig. 4A, iii).

Measuring HRP2 using a rapid diagnostic dipstick test predicts PADH

We then determined whether measurement of HRP2 after artesunate treatment using a rapid diagnostic test could predict subsequent PADH in blood samples collected from patients with severe malaria after artesunate treatment (see the STARD diagram in Fig. S1). We used the BinaxNOW malaria kit, an HRP2-based rapid diagnostic test widely used for qualitative diagnosis of a *P. falciparum* infection. The HRP2 titer was defined as the highest dilution remaining positive after a twofold serial dilution; the high sensitivity of the rapid diagnostic test required dilution of blood samples by several hundred-fold in PBS. Receiver operating characteristic (ROC) curves confirmed that predictive performance of PADH was greater at day 3 after the start of treatment [area under the curve (AUC), 0.901; 95% confidence interval (CI), 0.8159 to 0.9854] than at day 7 (AUC, 0.759; 95% CI, 0.6163 to 0.8884) (Fig. 4B). In terms of accuracy for predicting PADH, a day 3 titer of 1:500 predicted subsequent PADH with an 89% sensitivity and 73% specificity, whereas a titer of 1:1000 had 78% sensitivity and 95% specificity (Fig. 4, B and C). At day 7, the sensitivity and specificity of the dipstick-based prediction of PADH were 64 and 80%, respectively, using a 1:500 dilution of blood and 41 and 100%, respectively, using a 1:1000 dilution of blood (Fig. 4B). Notably, at day 0, all patient blood samples showed high titers (reflecting high parasitemias); consistent with previous studies (28), there was no significant association between day 0 titer and subsequent occurrence of PADH (Fig. 4B). As expected, 49 quinine-treated patients, none of whom showed the

Fig. 3. HRP2 persists at the plasma membrane and around the cytoplasmic vesicles of once-infected RBCs. (A) RESA and HRP2 parasite proteins were present in both ring-infected and once-infected (pitted) RBCs. Uninfected RBCs (uRBC) from healthy donors, samples containing ring-infected RBCs from patients at admission on day 0 before treatment with artesunate, and samples containing once-infected RBCs collected 3 days after initiation of artesunate treatment are shown. Day 0 and day 3 samples were stained with Hoechst (for DNA) and HRP2 antibody or RESA polyclonal antibody and then were analyzed by fluorescence microscopy. Data were generated using samples from five patients. (B) HRP2 was localized at the plasma membrane and around the intracytoplasmic vesicles of once-infected RBCs. Transmission electron microscopy revealed RESA (25-mum beads) and *P. falciparum* HRP2 (10-mum beads) proteins in ring-infected RBCs (i) and once-infected RBCs (ii to vi). HRP2 and RESA proteins were observed at the membrane-cytoskeletal complex of once-infected RBCs (ii to v), whereas only HRP2 was observed around small vesicles in the cytosol (iv to vi). Data were generated using samples from five patients. (C) *P. falciparum* HRP2 was present in membranous and cytosolic extracts of once-infected RBCs. Immunoblots of blood samples collected during follow-up of artesunate-treated patients with *P. falciparum* malaria are shown. RBCs were lysed sequentially either with Triton and SDS buffer to analyze the cytosolic or membrane-associated proteins or with a hypotonic 5 mM sodium phosphate buffer (pH 7.4) to analyze proteins associated with RBC membranes or ghosts. Proteins were detected using anti-HRP2 or anti-RESA monoclonal antibodies. Bands were quantified using the ImageJ software. Corresponding RBC intensity (set at 1) was used as a reference panel within each experiment. Data were generated using samples from five patients.
PADH pattern, had low titers of HRP2 at days 3 and 7 after the start of treatment, consistent with minimal pitting in the spleen.

DISCUSSION

We show that a simple method based on a rapid diagnostic test may predict PADH. We demonstrated the presence of the parasite antigen HRP2 in once-infected RBCs, which previously could only be detected using RESA and flow cytometry (24). Localization studies in cultured parasite lines and treated patient samples confirmed that HRP2 is retained in once-infected RBCs even after the parasite has been expelled by the pitting process in the spleen.

Artesunate reduces mortality from severe malaria and remains the drug of choice in malaria-endemic settings and in travelers returning from those regions. A key advantage of artemisinin derivatives over quinine is their ability to kill ring-stage parasites, preventing their maturation and inhibiting sequestration of infected erythrocytes in small vessels of vital organs such as the brain. Many nonviable ring-stage parasites are cleared in the spleen by the process of pitting during which the parasite is expelled and the erythrocyte becomes resealed and then

returns to the circulation as a once-infected RBC (6, 18, 24, 29–32). Such cells, although spared from immediate destruction, have a reduced life-span (1 to 4 weeks) (6, 33). This time course corresponds to the onset of PADH seen clinically (6). Once-infected RBCs look normal and remain undetected on conventional blood smears. After the introduction of artemisinins for malaria treatment, it was a long time for pitting to be observed (18, 24, 26, 29, 30) and the phenomenon to be linked to PADH (6, 17). Once-infected RBCs have been visualized by labeling with RESA. RESA is a 155-kDa protein exported by \textit{P. falciparum} into the cytosol of the host erythrocyte immediately after invasion (34, 35), which rapidly associates with the cytoplasmic layer of the erythrocyte membrane (18, 22, 29) through interactions with β-spectrins in the cytoskeleton (27, 36–38). Flow cytometric quantification of once-infected RBCs by labeling with RESA has provided accurate prediction of PADH (6, 24), but practical application at scale requires a rapid diagnostic test. Antigen-based rapid diagnostic tests provide robust, scalable diagnosis of malaria in a range of health care settings and have been important for achieving parasite-based diagnosis for all suspected cases of malaria, a core commitment of the WHO (39). More than 300 million malaria rapid diagnostic tests are used globally each year, the unit cost being well below $1. Most of the rapid diagnostic tests for malaria detect either HRP2, a protein specific to \textit{P. falciparum}, or the glycolytic enzyme lactate dehydrogenase (LDH), which has orthologs in all eukaryotic cells. The different behavior of the two parasite antigens after antimalarial treatment is well established: HRP2-based tests remain positive for several days or weeks after successful treatment with artemisinin-based combination therapies, whereas LDH-based tests become negative around the same time as microscopic detection of circulating parasites (40–42). Although the short persistence of LDH was attributed to parasite clearance, the reason for the long persistence of HRP2 in the circulation remained elusive. In patients with \textit{P. falciparum} infection in Bangladesh, we noted that, after parasite clearance, HRP2 was still at half of the baseline concentration, with most of the HRP2 in the cellular compartment. Using an optimized immunofluorescence protocol and electron microscopy, we confirmed that HRP2 was tightly associated with the membrane-cytoskeletal complex in ring-infected RBCs (19). We then showed that HRP2 was associated with membranous structures in the cytosol of once-infected RBCs, probably corresponding to export structures (19) that remained after the pitting process. Consistent with previous observations, artesunate treatment did not appear to result in the loss of HRP2 (43–47). We observed that HRP2 appeared as small aggregates in the cytoplasm of ring-infected RBCs but that these aggregates were absent in RBCs infected with \textit{P. vivax} K1 parasites. RESA may play a role in HRP2 transport in the host RBCs. There was a close correlation between the number of once-infected RBCs measured by RESA and HRP2, with only a small population of cells negative for HRP2 but positive for RESA, presumably indicating very early ring stages into which HRP2 had not yet been exported in sufficient quantities for labeling. We finally showed that postclearance HRP2 (measured using a rapid diagnostic test) could replace RESA as a marker of once-infected RBCs.

There are a number of limitations to our study. First, the central role of once-infected RBCs in the pathogenesis of PADH does not definitively rule out additional mechanisms of erythrocyte loss during typical hemolytic episodes. Hemoglobin loss is often greater than what would be expected from the initial parasitemia or early concentration of circulating once-infected RBCs. Erythrocyte loss during PADH may be amplified by mechanisms distinct from the delayed loss of once-infected RBCs, such as through opsonization of uninfected RBCs with anti-phosphatidylserine antibodies (48). A second limitation is the presence of \textit{P. falciparum} HRP2 in gametocytes, which may potentially contribute to the presence of HRP2 in the circulation, thereby interfering with PADH prediction and thus limiting the practical impact of our approach. However, although mature gametocytes persist in the circulation for several days after treatment, their concentration is generally several logs below that of asexual stage \textit{P. falciparum}–infected and once-infected RBCs (6), making interference with prediction of PADH unlikely. A third potential limitation is the variability in the HRP2 gene sequence between \textit{P. falciparum} isolates. Although an insertion-deletion polymorphism is common in the gene encoding HRP2, this is not thought to influence quantitation of the HRP2 protein (49). However, some parasite isolates have a genomic deletion in HRP2 (as well as the less abundant protein HRP3), suggesting that in these cases, HRP2-based tests would not work. This situation occurs predominantly in Latin America (50), although possible HRP genomic deletions may also have been described in India and several African countries (51, 52). There are no reports of a negative HRP2 rapid diagnostic test in patients with severe or hyperparasitemic malaria in patients from Africa (including Senegal, Mali, and Ghana); hence, our predictive strategy is likely to work in these countries.

The fact that blood concentrations of HRP2 can be semiquantitatively assessed using a rapid diagnostic test has important consequences for PADH prediction. Unlike flow cytometry–based quantification of once-infected RBCs, which requires fresh cells, HRP2 measurements with a rapid diagnostic test can be performed retrospectively on frozen whole-blood samples. This should allow a wide range of research groups to study PADH in more detail. The same rapid diagnostic test can be used both on day 0 blood samples for diagnosis of malaria and on day 3 blood samples for the prediction of PADH. Diagnosis is qualitative (positive/negative), whereas PADH prediction involves a quantitative element that assesses whether once-infected RBCs have reached a concentration associated with a high risk of subsequent hemolysis. Such an approach could be applied with any currently available HRP2-based rapid diagnostic test (6). However, routine assessment of HRP2 to predict PADH would be more efficient if the whole (rather than diluted) blood could be used directly. Current HRP2-based rapid diagnostic tests are too sensitive for the presence of a band to define those at risk of PADH, hence the need for the dilution step. This issue could be addressed by a test containing both a high-sensitivity band (for diagnosis) and a lower-sensitivity band (to predict PADH). For the test to accurately define the number of circulating once-infected RBCs, it must be carried out after parasite clearance (generally 2 to 3 days after the start of treatment), given that high HRP2 titers obtained before parasite clearance reflect high parasitemia itself. Patients with persistently high HRP2 titers are at risk of PADH in the next few days or weeks. The test performed better at day 3 than at day 7 (Fig. 4), suggesting that small numbers of infected RBCs persisting in the circulation beyond day 2 after the start of treatment (a frequent occurrence in severe malaria) do not alter predictive performance and that clearance of once-infected RBCs may sometimes begin before day 7. Future large prospective studies will more precisely determine the robustness of prediction in different geographic and logistical settings. The test should be assessed for its ability to detect PADH as well as the subsequent risk of severe hemolysis or anemia and any organ damage these conditions can induce (for example, renal failure). For a direct estimate of medical impact, outcome evaluation should measure how adequately transfusion of RBCs was performed in parallel randomized groups of patients with \textit{P. falciparum} malaria in whom the test was administered. If its accuracy is confirmed, this test may be preferable...
to routine follow-up of all patients for 28 days and will bring two potential advantages for patient management. First, there can be greater focus on the subset of patients who are at high risk of developing PADH, with closer follow-up of clinical, hematological, and renal measures, enabling an optimized preparation for blood transfusion. Second, the approach should allow earlier identification of distinct causes of clinical deterioration in the follow-up period that might be confused with PADH. Predicting PADH with a currently available cheap, simple rapid diagnostic test using a dipstick should ensure a sustained positive outcome in patients with severe malaria treated with artesunate.

**MATERIALS AND METHODS**

**Study design**

Here, we aimed to explore if the observed persistence of the parasite protein HRP2 after treatment of malaria patients with artesunate could be explained by the persistence of HRP2 in once-infected RBCs that contribute to PADH. We also devised an HRP2 titration method that could be used as a simple test to predict PADH. We assessed the persistence of HRP2 at day 3 after artesunate treatment started in patients with *P. falciparum* malaria in an endemic area of Bangladesh and in French travelers who became infected in Africa. Persistence of HRP2 was detected by ELISA alone in the Bangladesh patient cohort and by both ELISA and HRP2-based dipstick quantification in the French traveler cohort. Blood samples were centrifuged to compare the concentration of HRP2 in the RBC compartment to that in plasma. The cellular localization of HRP2 in once-infected RBCs was assessed in blood samples from malaria patients by Western blotting, immunoﬂuorescence, flow cytometry, and electron microscopy. RBCs infected with different stages of *P. falciparum* in culture were also analyzed. The accuracy of PADH prediction was evaluated with the malaria BinaxNOW dipstick, a rapid diagnosis test that detects the HRP2 parasite antigen. For this part of the study, blood samples were collected from French travelers with severe *P. falciparum* malaria.

**Bangladesh cohort**

In Bangladesh, patients were nonpregnant adults (≥16 years old) enrolled in prospective studies who required admission to the hospital with slide-confirmed or rapid diagnostic test-confirmed *P. falciparum* malaria at Chittagong Medical College Hospital or Ramu Upazila Health Complex, Chittagong Division, Bangladesh. All were treated with parenteral artesunate. Ethical approval for these studies was obtained from the Oxford Tropical Research Ethical Committee and the Bangladesh Medical Research Council Ethical Committee.

**Patients with severe malaria in France**

Severe *P. falciparum* malaria was defined by a positive blood smear with asexual parasite forms of *P. falciparum* associated with at least one clinical or biological criterion of severity: neurological impairment (lethargy, confusion, multiple convulsions, and/or coma), that is, with Glasgow coma scale of <11), respiratory distress, cardio-circulatory impairment, spontaneous bleeding, macroscopic hemoglobinuria, jaundice and/or plasma bilirubin (>50 μM), anemia (hemoglobin, <7 g/dl; hematocrit, <20%), hypoglycemia (glucose, <2.2 mM), acidosis (plasma bicarbonate, <15 mM or pH <7.35), hyperlactatemia (arterial lactate, >1.8 mM), renal impairment, and hyperparasitemia (>4% Pf-RBC). Anemia was defined as blood hemoglobin below 12 and 13 g/dl for females and males, respectively. Hemolysis was defined as plasma LDH above 390 IU/liter and/or plasma haptoglobin below 0.1 g/liter. All French patients with imported severe malaria involved in the national surveillance program provided consent according to a procedure common to all National Reference Centers (CNR; www.invs.sante.fr/Espace-professionnels/Centrenationaux-de-reference/Textesreglementaires) on behalf of the National Agency of Medicine and Health Product Safety (Agence Nationale de Sécurité du Médicaments). The Ile de France II Institutional Review Board has approved this approach as a nonresearch process (Article L.1121-1 of the French Code for Public Health) embedded in the surveillance missions of the CNR, officially empowered to collect information and biological samples (Article L.1413-5 of the French Law no. 2004-806, 9 August 2004; www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000000810056). Forms and data from all patients were collected as part of an observational program implemented by the CNR for Malaria. Intravenous artesunate (Gulin Pharmaceuticals) has been available in France since May 2011 through a named-patient program for imported severe malaria cases. All French patients were treated initially with intravenous artesunate (2.4 mg/kg) at 0, 12, 24, and 48 hours and then either maintained under intravenous artesunate for 7 days (artesunate monotherapy) or switched to a 3-day recommended oral treatment with either atovaquone-proguanil, artenether-lumezantrine, or dihydroartemisinin-piperaquine. Patients treated with quinine received the recommended three daily infusions of 8 mg/kg on day 1 and then 4 mg/kg per day until either 7 days of quinine monotherapy had been administered or switch to oral treatment had been initiated. Choice of the treatment option was at the discretion of the attending physician and depended mostly on the local availability of intravenous artesunate. Consistent with the kinetics of action of quinine and artesunate (53), patients who were switched from quinine to artesunate the same day after starting quinine were those who were considered as treated with artesunate, and patients who were switched from quinine to artesunate after more than 24 hours of treatment with quinine were those who were considered as treated with quinine. Blood sample collection was scheduled at the time of admission and on days 3, 7, 14, 21, and 28, as recommended by the French High Committee for Public Health. A minimum of four samples, two collected until day 7 and two after day 7, were required for a patient to be included in the analysis of dipstick-based prediction of PADH.

**Rapid diagnostic dipstick test for predicting PADH**

The PADH pattern was defined as per Jauréguibury et al. (6) and Arguin (54) by a greater than 10% drop in hemoglobin concentration or a greater than 10% rise in LDH concentration occurring any time between day 8 and the end of follow-up. The non-PADH pattern corresponded to a rising pattern or a persistent pattern. The “rising” pattern was defined by a nadir of hemoglobin and a peak of hemolysis occurring before day 8 without positive markers of hemolysis from day 8 to the end of follow-up. The “persistent” pattern was defined by the presence of anemia and positive hemolysis markers both before and after day 8 and a pattern that did not fit the definition of PADH. Patients for whom information was lacking or could not be unequivocally allocated to one of the three previously defined patterns were classified as indeterminate and not included in the analysis. The determination of the PADH/non-PADH patterns was performed by members of the team unaware of the result of the index test. Eligible patients had severe malaria treated with either artesunate or quinine and had blood samples collected at day 0 and day 3 ± 1 sent to the CNR team (fig. S1). The reference standard was the published case definition of PADH based on clinical information and hematological
parameters collected during posttreatment follow-up, as defined above (6, 54). The index test was based on HRP2 titration using whole-blood samples prospectively collected in severe malaria patients from January 2012 to December 2014 and then kept frozen at −20°C until use. The test was considered positive when a P. falciparum band was observed with a blood sample diluted by 1:500 or more and negative when no band was observed with a blood dilution of 1:500. All tests performed throughout the study showed a positive control band. The HRP2-based rapid diagnostic test used was the Malaria BinaxNOW dipstick (55), a commercial rapid diagnostic test used in our hospital over the past 10 years. Readers were unaware of the PADH status of the patients.

Enzyme-linked immunosorbent assay
Quantification of HRP2 from patients was undertaken by sandwich ELISA using established methods and reference standards (56). HRP2 assays were performed with the Malaria Antigen Celisa commercial kit (Cellabs), as recommended by the manufacturer, on day 0 and day 3 ± 1 when available for plasma and whole-blood samples. Samples were diluted in RPMI 1640 (Life Technologies), according to the initial parasite density. HRP2 was quantified with the calibration curve obtained from three dilutions of a plasma pool–derived patient samples (from each sites: Bangladesh or France) calibrated itself against internal positive kit control. Samples with optical density values outside the range of the calibration were retested at an adapted dilution. In each series, diluted positive HRP2 plasma and whole-blood samples and negative HRP2 plasma of known patients were tested as internal control.

Parasite cell lines and cultures
Resa 1-WT (FUP/CB) and resa 1-KO (22) P. falciparum lines were cultured in RPMI 1640 containing 25 mM Hepes, 25 mM NaHCO3, glutamine (0.3 g/liter), gentamicin (10 mg/liter), and 10% human serum at 2.4 to 5% hematocrit in an atmosphere of 5% CO2, 10% O2, and 85% N2 and a temperature of 37°C. Cultures were exposed to sequential sorbitol lysis (Sigma-Aldrich) and two cycles of differential gelatin ﬂotation to obtain synchronized populations.

Flow cytometry
RESA and/or HRP2 were analyzed as previously described (24) by flow cytometry (Accuri C6, BD Biosciences). For each sample, three labeling approaches were used: (i) labeling of RESA with an African polyclonal puriﬁed from hyperimmune seraums (RESA-Ab) (P. H. David, Institut Pasteur, France) at 1:40 in a suspension of PBS/1% AlbuMax II (Life Technologies) or a monoclonal murine RESA antibody (RESA-mAb provided by R. Anders, Latrobe University, Melbourne, Australia) at 1:40, (ii) labeling of HRP2 with a monoclonal murine antibody (HRP2-mAb; M0071029, MyBioSource) at 1:100 in a suspension of PBS/1% AlbuMax II (Life Technologies), and (iii) double labeling of both RESA at 1:40 and HRP2 at 1:100. Samples were then washed twice with PBS and then incubated with secondary antibody. The secondary antibody for RESA was a goat anti-human IgG or a goat anti-mouse IgG coupled with Alexa Fluor 568 (Life Technologies) at 1:2000, and the secondary antibody for HRP2 was a goat anti-mouse IgG coupled with Alexa Fluor 488 (Life Technologies). Finally, samples were washed twice with PBS and analyzed with BD Accuri C6 flow cytometer (BD Biosciences).

Immunofluorescence microscopy
Two methods were performed for immunofluorescence microscopy: indirect immunofluorescence labeling of suspension erythrocytes (IFSE) and erythrocyte membrane immunofluorescence on slide (EMIF). For IFSE, samples prepared for flow cytometry with double labeling against HRP2 and RESA were retrieved the same day and were incubated with Hoescht 33342 dye (Life Technologies) at 37°C during 15 min for parasite DNA detection. Samples were centrifuged, and the pellet was examined between slide and cover slip. For EMIF, immunofluorescence assay slides were prepared as previously described by Ndour et al. (24) adapted from Perlmann (57). Images were acquired on a Leica DMi3000 microscope with DIC, using a Leica DFC310FX camera controlled by LAS Superposition Images software (Leica Mikrosystemes SAS).

Transmission electron microscopy
Cellular localization of HRP2 and RESA was studied on patient samples by transmission electron microscopy after immunogold labeling. For primary antibodies, packed RBCs were processed for monolabeling or double labeling with RESA and HRP2, as indicated for immunofluorescence, and then washed three times with PBS before incubation with secondary immunogold antibodies (Aurion, Biovalley S.A.), corresponding to F(ab’2) fragment of goat anti-mouse coupled to 10-nm gold particle for the detection of mouse anti-HRP2 and goat anti-human IgG coupled to 25-nm gold particle for the detection of human anti-RESA, each of them being diluted 1:40 in PBS. Samples were washed three times and then postfixed with 1% osmium tetroxide (EMS) in phosphate buffer (0.1 M; pH 7.4) for 30 min at 4°C. After an extensive wash (3 × 10 min) with distilled water, samples were dehydrated in a graded series of ethanol solutions (2 × 5 min each): 50, 70, 80, 90, and 100%. A final dehydration step (2 × 10 min) was performed with pure acetone. Blood cell pellets were then progressively infiltrated with an epoxy resin Epon 812 (EMS): one night in 50% resin and 50% acetone at 4°C and 2 × 2 hours in pure fresh resin at room temperature. They were finally embedded in the bottom of capsules (Beem size 3; Oxford Instruments), and the resin was cured at 56°C for 48 hours in a dry oven. Blocks were cut with an UC7 ultramicrotome (Leica, Leica Mikrosystemes SAS). Semithin sections (0.5 µm thick) were stained with 1% toluidine blue in 1% borax. Ultrathin sections (70 nm thick) were recovered on either copper (conventional morphology) or nickel (immunoelectron microscopy) grids and contrasted with 2% uranyl acetate and Reynolds’s lead citrate (58). Ultrathin sections were observed with a Hitachi HT7700 electron microscope (Flexience) operating at 70 kV. Pictures (2048 × 2048 pixels) were taken with an AMT41B camera (pixel size, 7.4 µm × 7.4 µm). Pictures were processed with open-source ImageJ (National Institutes of Health), when needed.

Protein extraction
Packed RBC samples were adjusted as an equivalent to a parasitemia of 10% for day 0 samples or to a concentration of once-infected RBCs of 10% for day 3 or later samples. After storage at −20°C, they were thawed at room temperature and then resuspended in PBS/1% Triton X-100 in the presence of a cocktail of protease inhibitors (Complete, Roche Diagnostics). For the sequential extraction, samples were incubated at +4°C for 30 min and then centrifuged at 14,000g for 30 min. The supernatant was defined as Triton extract (cytosolic cell fraction). The pellet was then resuspended in two volumes of PBS/2% SDS, incubated at room temperature for 30 min, and centrifuged at 14,000g for 30 min. This last supernatant was resuspended in two volumes of PBS/1% Triton X-100 and was defined as SDS extract (insoluble proteins from membrane complex). For hypotonic extraction, Pf-RBC (and uninfected) were washed twice in PBS at room temperature and lysed with 20 volumes of ice-cold hypotonic buffer.
5 mM sodium phosphate buffer (pH 7.4) containing protease inhibitor cocktail (cOmplete EDTA-free and pepstatin A, Roche). Lysate was centrifuged at 15,000g for 20 min, and the white membranous pellet was washed several times with ice-cold phosphate buffer until the ghosts became white.

**Western blot analysis**

Protein extracts were mixed with Laemmli buffer and then heated at 100°C for 5 min. They were loaded on an SDS–polyacrylamide gel electrophoresis gel (4 to 15% Criterion TGX, Bio-Rad) and run for 2 to 3 hours at 90 V. Proteins were then transferred to a 0.45-trohoresis gel (4 to 15% Criterion TGX, Bio-Rad) and run for 2 to 3 hours. After washing in TTBS, the membrane was incubated with a goat anti-mouse antibody coupled to an alkaline phosphatase (Bio-Rad) at 1:3000. Finally, the membrane was revealed with a colorimetric kit (Immuno-Blot AP Colorimetric kit, Bio-Rad) in 5 to 10 min.

**HRP2-based dipstick test for predicting PADH**

The BinaxNOW Malaria test is an in vitro immunochromatographic assay for the qualitative detection of *Plasmodium* antigens circulating in human venous and capillary EDTA-treated whole blood. The BinaxNOW Malaria test meets all of the criteria of thermal stability and performance defined by the WHO malaria rapid diagnostic test product testing program. The test targets HRP2 specific to *P. falciparum* and a pan-malarial antigen, aldolase, which is common to all malaria species that infect humans. A subgroup of fresh blood samples was tested and compared to the same sample frozen at −20°C to make sure that the freezing process did not interfere with the results. All of the samples for the study were stored at −20°C before being tested simultaneously. Whole-blood samples collected at days 0, 3, and 7 were diluted at 1:500, 1:1000, 1:2000, 1:4000, and 1:8000 in PBS. Fifteen microliters of each diluted sample was deposited in the sample well, and the rapid diagnostic test was then performed according to the manufacturer’s recommendations. A positive result was defined by the presence of both an HRP2 band and control band after 15 min. A negative result was defined by the presence of a control band and the absence of an HRP2 band. The test was not valid if the control band was not present. The result of each test was noted by a reader who did not know the anemia status of the patients (reader 1), and then the rapid diagnostic test dipstick was scanned. The maximum positive dilution was defined as the strongest dilution with a positive result. Scanned pictures of the rapid diagnostic test were then analyzed again by two other readers (readers 2 and 3), who determined the maximum positive dilution for each sample. Missing samples and indeterminate index tests were not included in the analysis. A global consistency of 81% concordance was obtained between three readers (Kendall’s test). For the analysis, we used the results from the most experienced reader in the team.

**Statistical methods**

Statistical analyses were performed using GraphPad PRISM 6 software. Differences with $P < 0.05$ were considered significant. Sensitivity and specificity of PADH predictions were determined for each dilution, each day of follow-up (days 3 ± 1 and 7 ± 2), and each reader. Results are shown as ROC curves. Correlations were performed using the Spearman method. AUC was determined for each day of follow-up (days 3 and 7). Inter-reader agreement was analyzed with the Kendall’s concordance test.

**SUPPLEMENTARY MATERIALS**

www.sciencetranslationalmedicine.org/cgi/content/full/9/397/eaaf9377/DC1

**REFERENCES AND NOTES**


Acknowledgments: We thank the CNR network and collaborators for their input. We thank all patients and health care workers for their contributions to the findings shown in this work. We also acknowledge R. Anders for the RESA antibody and D. Mazier, E. Caumes, C. Abul Hassan, P. Sujat, B. Anupam, and F. Md Abul for their help.

Funding: This study was funded by a grant from the Bill & Melinda Gates Foundation (#OPP1123683, C15/0872a01). P.A.N. was supported by a Laboratory of Excellence GR-Ex fellowship (#ANR-11-LABX-0051), which was funded by the program Investissements d’avenir of the French National Research Agency (reference ANR-11-IDEX-0005-02). Mahidol-Oxford Tropical Medicine Research Unit was funded by the Wellcome Trust of Great Britain through grant #089275/Z/09/Z. P.A.N., N.C., P.A.B. received grants from Fast-Track North Potomac and Guillin Pharmaceutical to work on intravenous artesunate. A.M.D. was a principal investigator on a grant from Guillin Pharmaceutical for the study of delayed anemia after artesunate treatment. P.A.B. and S.J. served on the scientific advisory board of Sigma Tau Laboratories. All other authors declare that they have no competing interests. Data and materials availability: Materials are available upon request from P.A.B., including parasite lines and monoclonal antibodies.

Submitted 26 June 2016
Resubmitted 2 November 2016
Accepted 28 March 2017
Published 5 July 2017
10.1126/scitranslmed.aaf9377

Measuring the *Plasmodium falciparum* HRP2 protein in blood from artesunate-treated malaria patients predicts post-артесунате delayed hemolysis


Sci Transl Med 9, eaaf9377.
DOI: 10.1126/scitranslmed.aaf9377

A quick test to predict artesunate-induced anemia

Anemia frequently affects patients treated for severe malaria with artemisinin drugs. Artemisinin kills malaria parasites, which are then expelled from infected red blood cells. These "deparasitized" red blood cells persist in the blood but are later destroyed, resulting in anemia. Ndour et al. now show that the deparasitized red blood cells retain the parasite protein HRP2, which explains why HRP2 can still be detected in patients after the malaria infection has been cleared. The amount of HRP2 in the blood immediately after treatment with artemisinin correlates with the number of deparasitized red blood cells in the circulation. HRP2 can be measured with a rapid diagnostic dipstick test that then can be used to predict the risk for delayed hemolysis and anemia in malaria patients treated with artemisinin.

ARTICLE TOOLS
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SUPPLEMENTARY MATERIALS
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