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MAJOR ARTICLE

Differential Effects of Antimalarial Drugs on Parasite Clearance Rates Are Reflected by *Plasmodium falciparum* Ring Ratio

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Background. The location of *Plasmodium falciparum* within the body is determined by the life cycle of the parasite; young rings are in the peripheral blood, whereas mature parasites are sequestered in deep tissues. We can calculate a "ring ratio," the proportion of parasites in the periphery to the total number of parasites in the body. Artesunate acts on all parasite life stages, whereas quinine is effective only on sequestered parasites. Children with cerebral malaria (CM) treated with artesunate clear parasites faster than those treated with quinine. In this study, we established the relationship between ring ratio and parasite clearance rate and used the ring ratio to determine if the benefit derived from artesunate treatment could be attributed to its broader effect on life cycle stages.

Methods. Ring ratios were calculated for 400 hospitalized children with CM in Blantyre, Malawi between 2010 and 2019 (quinine: 2010–2013, artesunate: 2014–2019).

Results. In both treatment groups, parasite clearance rates were positively associated with the ring ratios, with a stronger association in the artesunate era than the quinine era. In the quinine era, an increase of 1-unit $\log_{10}$ difference between parasitemia and plasma *P falciparum* histidine-rich protein 2 (a proxy for ring ratio) resulted in a 0.27-unit increase in the parasite clearance rate, whereas in the artesunate era an equal increase resulted in a 0.41-unit increase ($P = .04$ for the difference).

Conclusions. This analysis provides in vivo evidence supporting the hypothesis that more rapid parasite clearance rates in artesunate recipients are due to its superiority over quinine in killing ring-stage parasites.

Keywords. artesunate; cerebral malaria; *Plasmodium falciparum* histidine-rich protein 2; quinine; ring ratio.

Malaria is one of the most prevalent parasitic diseases affecting humans in endemic areas. Despite extensive control efforts, there were still 593 000 deaths recorded in 2021, an increase from the 544 000 cases seen in 2019. The African region still accounts for 95% of cases and 96% of deaths worldwide [1].

Severe malaria is common in children <5 years of age; however, the average age of patients admitted to hospital for treatment of malaria is now increasing [2]. Along with severe malarial anemia, cerebral malaria (CM) is the main clinical syndrome responsible for malarial deaths. The primary virulence process in CM is the sequestration or "hiding" of late-stage trophozoite- and schizont-infected red blood cells in the microvasculature [3, 4]. This can complicate diagnosis as these parasites are not visible by the gold-standard malaria diagnostic procedure, microscopy performed on a smear of peripheral blood. The early ring-stage *Plasmodium falciparum* parasites circulating in peripheral blood, which are easily quantified by microscopy, do not accurately reflect the total body load of parasites as they do not account for this sequestered parasitic load [3]. Toward the last half of the *P falciparum* life cycle, especially during schizont rupture, parasites release DNA, *P falciparum* histidine-rich protein 2 (*Pf*HRP2), and *P falciparum* lactate dehydrogenase (*Pf*LDH), among other parasite markers [3]. Although not ideal, given that the majority of the protein is released at the time of schizont rupture, modeling based on plasma *Pf*HRP2 levels can provide an estimate of total body parasite biomass, a measure that is not accounted for when analyzing peripheral parasitemia [3, 5].

It has been shown in large clinical trials that artesunate is superior to quinine for the treatment of severe malaria in both children and adults [6, 7]. Based on these findings and World Health Organization (WHO) recommendations, the Blantyre

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Malaria Project at the Paediatric Research Ward at Queen Elizabeth Central Hospital (QECH) changed first-line therapy for CM in 2014. Using historical data, we recently confirmed that participants treated with artesunate cleared parasites more rapidly than those treated with quinine [8]. We have now measured plasma *Pf*HRP2 and peripheral parasitemia in these patients to characterize the compartmentalization of their parasites. These data can be combined to generate a ring ratio (RR), the ratio of parasites in the peripheral circulation (measured by peripheral smear) as a portion of total body parasites (measured by plasma *Pf*HRP2 levels). Artesunate acts on malaria parasites at all life cycle stages. It is effective against the ring stage (in the periphery) as well as the sequestered trophozoite stage, whereas quinine is effective only on sequestered parasites [9]. If the more rapid parasite clearance rates observed in artesunate recipients are related to increased artesunate efficacy at the ring stages, we would expect to see artesunate providing an increased benefit at higher RRs compared to quinine.

METHODS

Patient Consent Statement

Permission to carry out this study was granted by the University of Malawi College of Medicine Research Ethics Committee under authorization number P.11/20/3207. Participants in this study were part of an ongoing study of malaria pathogenesis. Consent to participate in the main study was obtained in the local language by a study-specific nurse and was granted by either of the child's parents or their guardians, who also agreed to secondary analyses of de-identified data. Patients were aged between 6 months and 14 years and were admitted with the diagnosis of CM as defined by the WHO (Blantyre coma score of ≤2, *P falciparum* parasitemia on peripheral blood smear, and no other known cause of coma) [5] between the years 2010 and 2019. We included only participants with complete measurements of age, sex, weight, multiple timed parasite counts, plasma *Pf*HRP2 levels, and hematocrit.

Samples

Blood collected at admission was used to measure admission hematocrit and then processed to extract plasma that was stored at −80°C for *Pf*HRP2 analysis after discharge. Smears were prepared at admission and every 6 hours until no parasites were seen after searching 100 high-power fields in 2 consecutive samples. All blood films and plasma samples for *Pf*HRP2 were prepared and analyzed according to methods previously described [8].

Statistical Analysis

Clinical data were extracted from case report forms and entered into the REDCap data management system. Sample size was determined by the number of children admitted to the Paediatric Research Ward with the diagnosis of CM since the change to artesunate as a first-line therapy in 2014, and an equal number of cases treated with quinine was identified by working backwards from 2014.

Peripheral parasite concentration ($P_{peripheral}$) was calculated by counting parasites present in 500 erythrocytes in a thin smear and multiplying by the red cell concentration from the full blood count (Coulter A^C.T5diff AL, Beckman Coulter

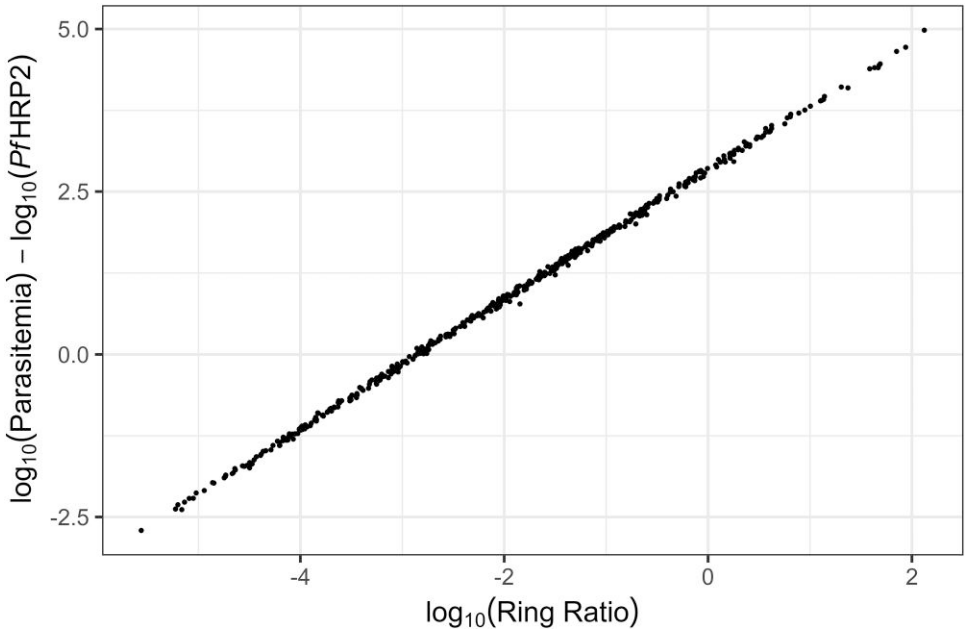

Figure 1. The $\log_{10}$-scale ring ratio shows near perfect correlation with $\log_{10}$(parasitemia) − $\log_{10}$(*Pf*HRP2) (Spearman rank correlation coefficient >0.99). Abbreviation: *Pf*HRP2, *Plasmodium falciparum* histidine-rich protein 2.

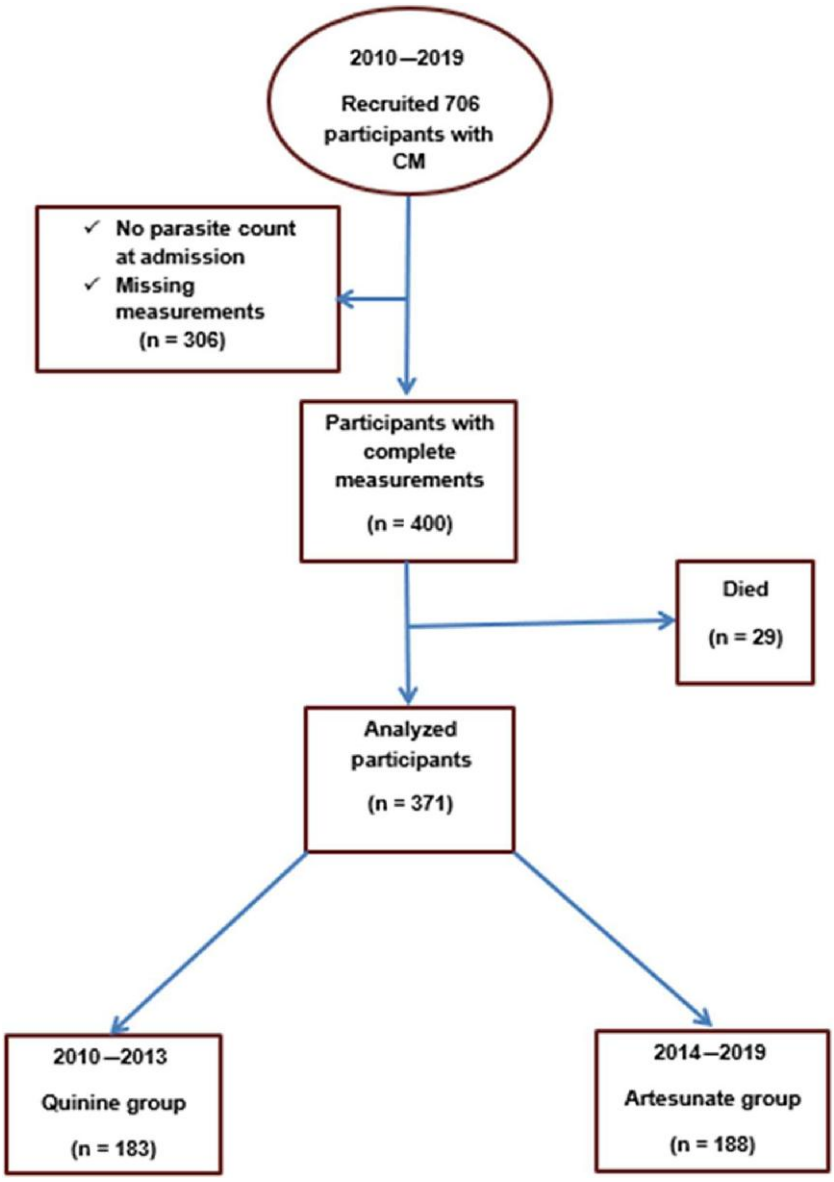

Figure 2. Patient recruitment and analysis. Abbreviation: CM, cerebral malaria.

Life Sciences, Indianapolis, Indiana). For participants with low parasitemias, parasites were counted on a thick smear in the same number of high-power fields as 200 white blood cells. A peripheral parasitemia was then calculated based on the white cell count from the full blood count. Total peripheral parasitemia was estimated using the following formula: parasites/μL × 0.08 × patient weight (kg) × 10^6 [4, 10]. Total parasite concentration (P_{tot}) was estimated based on the previously published formula: $P_{tot} = 7.3 \times$ *Pf*HRP2 × (1 − Hematocrit) × body weight × 10^{13}, when *Pf*HRP2 is measured in grams per liter [4]. The RR was then calculated using the formula $RR = P_{peripheral} / P_{tot}$ where parasitemia was measured in parasites per microliter (p/μL). Algebraically, the $\log_{10}$-scale RR, $\log_{10}(RR)$, equals $\log_{10}(P_{peripheral}) - \log_{10}(P_{tot})$, and it is nearly perfectly correlated with a simple difference between the $\log_{10}$-scale parasitemia and $\log_{10}$-scale *Pf*HRP2 (Spearman rank correlation coefficient >0.99; Figure 1). Therefore, in the analysis, we have used $\log_{10}$(parasitemia) − $\log_{10}$(*Pf*HRP2) as a proxy for the exposure variable $\log_{10}(RR)$.

Clearance rates were calculated for participants who had at least 3 parasitemia measurements whose admission parasitemia were >40 p/μL but not exceeding 3 000 000 p/μL using the Parasite Clearance Estimator (PCE) tool version 2 (see wwarn.org/PCE) [11]. Output is included as Supplementary Files 1 and 2.

Our primary analysis consists of participants who did not die during the study period and for whom a clearance rate estimate could be obtained from the PCE. We estimated the association

Table 1. Clinical, Demographic, and Outcome Data on Admission

Demographic or Clinical Measure	Quinine-Treated (n = 183)	Artesunate-Treated (n = 188)	*P* Value for Difference
Age, mo			.465
Mean (SD)	51.30 (26.69)	55.09 (30.79)	
Median (IQR)	49 (31–68)	47 (31–77)	
Parasites/μL			<.001
Mean (SD)	206 841 (276 981)	147 140 (279 765)	
Median (IQR)	71 600 (18 180–284 520)	27 760 (1298–186 150)	
Log(parasites)			<.001
Mean (SD)	4.65 (1.07)	4.25 (1.14)	
Median (IQR)	4.85 (4.26–5.45)	4.44 (3.11–5.27)	
*Pf*HRP2, ng/mL			<.001
Mean (SD)	8730.96 (11 236.20)	4973.97 (7495.42)	
Median (IQR)	4208 (978–11 035)	1623 (374–6380)	
Log(*Pf*HRP2)			<.001
Mean (SD)	3.43 (0.92)	3.10 (0.92)	
Median (IQR)	3.62 (2.99–4.04)	3.21 (2.57–3.80)	
Participants who received antimalarial treatment before hospital admission, No. (%)	108 (59.0)	180 (95.7)	<.001

P values are based on the Wilcoxon rank-sum test for continuous variables and Fisher exact test for categorical variables.

Abbreviations: IQR, interquartile range; *Pf*HRP2, *Plasmodium falciparum* histidine-rich protein 2; SD, standard deviation.

between the parasite clearance rate and $\log_{10}$(RR), approximated using the formula $\log_{10}$(parasitemia) − $\log_{10}$(*Pf*HRP2), in a regression analysis. Specifically, we consider the following regression model:

$$\text{Clearance rate} = \beta_0 + \beta_1 \times \text{Treatment} + \beta_2 \times \log_{10}(\text{RR}) + \beta_3 \times \log_{10}(\text{RR}) \times \text{Treatment},$$

where the coefficient β_2 quantifies how the clearance rate changes with the $\log_{10}$(RR) and the coefficient β_3 quantifies the differential impact of the RR during the quinine period and the artesunate period. In a sensitivity analysis, we repeat the same analysis but on a cohort that further includes patients who died during the study period but nevertheless had a clearance rate estimate from the PCE. The code used for analysis is included as Supplementary File 3.

RESULTS

Of the 706 participants admitted between 2010 and 2019, there were 400 participants with complete measurements including parasitemia at admission, plasma *Pf*HRP2 measurement, and clearance rate derived from the PCE. Among these 400 participants, 29 died during the study period, so our primary analysis cohort consists of 371 participants. Among these participants, 183 participants received quinine in the years 2010–2013 and the other 188 participants received artesunate between 2014 and 2019 (Figure 2).

The basic clinical, laboratory, and outcome data for the 2 groups are shown in Table 1. Participants in the quinine era had statistically significantly higher peripheral parasitemias at admission, were younger, and had higher *Pf*HRP2 levels at admission than artesunate recipients. A higher percentage of patients in the artesunate era received antimalarial treatment prior to referral to our hospital, which likely contributed to the lower admission peripheral parasitemia.

Figure 3 shows the $\log_{10}$-scale RR, as approximated by $\log_{10}$(parasitemia) − $\log_{10}$(*Pf*HRP2), among 183 participants in the quinine period and 188 participants in the artesunate period. There was no significant difference among participants (1.22 vs 1.15; Wilcoxon rank-sum test, $P = .47$).

Table 2 summarizes the estimated coefficients and their associated standard errors in the multivariate regression analysis. The multivariate analysis revealed that the $\log_{10}$-scale RR, as approximated by $\log_{10}$(parasitemia) − $\log_{10}$(*Pf*HRP2), was significantly associated with the clearance rate in both periods. In the quinine period, 1-unit increase in $\log_{10}$(parasitemia) − $\log_{10}$(*Pf*HRP2) is associated with a 0.027-unit increase in the clearance rate. On the other hand, in the artesunate period, 1-unit increase in $\log_{10}$(parasitemia) − $\log_{10}$(*Pf*HRP2) is associated with a 0.041-unit increase in the clearance rate, an approximately 50% increase compared to the quinine period. In a sensitivity analysis, we repeated the analysis on a cohort that further included 29 participants who died but with clearance rate estimates. The results were near-identical to the primary analysis.

DISCUSSION

Plasmodium falciparum has a complicated life cycle in the human host. It spends the first portion of the life cycle (12–18 hours) in the peripheral circulation where it is accessible to diagnosis by microscopy on a sample collected by a finger prick. It then sequesters for the remainder of the life cycle in

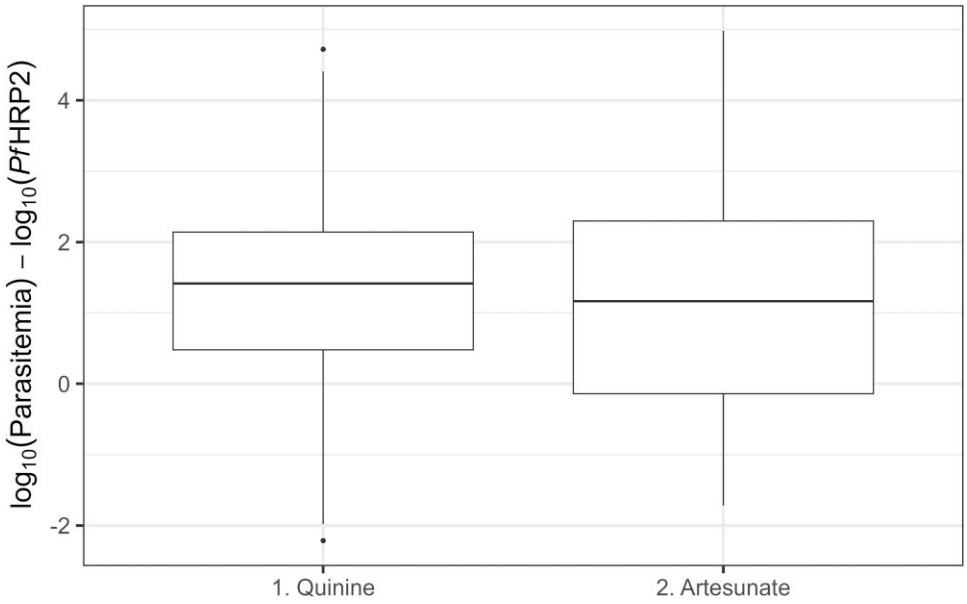

Figure 3. Log_{10}-scale ring ratio, as approximated by log_{10}(parasitemia) – log_{10}(*Pf*HRP2), for patients treated with quinine (n = 183) or artesunate (n = 188) at admission. Abbreviation: *Pf*HRP2, *Plasmodium falciparum* histidine-rich protein 2.

the deep vasculature where parasitized red cells bind to vascular endothelial cells. The parasitized erythrocyte is significantly altered at this point and, among other physiologic changes, now has decreased deformability, which would compromise its passage through the spleen. In addition to avoiding the spleen, these sequestered parasites also avoid standard diagnostic procedures based on peripheral blood [3, 12].

The use of plasma *Pf*HRP2 levels moves beyond the measurement of only peripheral parasites. This is a soluble protein that is released into the plasma of *P falciparum*–infected individuals by both peripheral parasites as well as sequestered parasites [13]. Quantification of this protein in the bloodstream can lead to an estimation of the total body load of parasites and, in combination with the peripheral parasitemia, estimate the proportion of parasites that are sequestered [4].

First-line therapy for severe malaria underwent a shift after large trials proved the superiority of intravenous artesunate over the previously preferred quinine [5, 6]. Parasite clearance rates were more rapid in artesunate recipients, compared to quinine recipients, and this is thought to be due to its broader range of action across the parasite life cycle [14]. Whereas quinine is active mostly against late-stage (sequestered) parasites, in vitro studies show that artesunate is active against the early-stage ring parasites in the periphery as well as the later, sequestered stages. We have recently confirmed the more rapid parasite clearance times in children with CM who were treated with artesunate compared to those receiving quinine [8]. The current study was aimed at determining the extent to which the more rapid parasite clearance rates were associated with an in vivo increase in life cycle coverage, similar to that seen in vitro.

Table 2. Estimated Coefficients From the Multivariate Regression Analysis

Variable	Estimate	Standard Error	*P* Value
Treatment (quinine)		Reference	
Treatment (artesunate)	−0.01	0.01	.439
Ring ratio (log_{10} scale)	0.027	0.005	<.001
Ring ratio (log_{10} scale), artesunate vs quinine	0.014	0.007	.041

If the increased in vivo clearance rates seen are due to an increased drug efficacy on the early-stage parasites, we would expect to see a greater artesunate advantage in those children with a higher peripheral load or "ring ratio" at time of presentation, and this was what we observed (Table 2, Figure 4).

Approximately 85% of our participants received antimalarial therapy prior to admission to QECH. Although our blood samples were collected immediately upon arrival at the hospital, this would have been at variable time points from initial disease onset and from initial antimalarial therapy. This initial therapy will have altered the distribution of parasites between the 2 body compartments (peripheral and sequestered) prior to assessment at the hospital.

One of the limitations of the study is the inaccuracies of formulas used to estimate sequestered and peripheral parasites, which are necessary to calculate parasite compartmentalization. These formulas have several assumptions, including equal and known parasite replication within each child as well as first-degree elimination of the *Pf*HRP2 protein from the body. Future work includes refining these formulas using

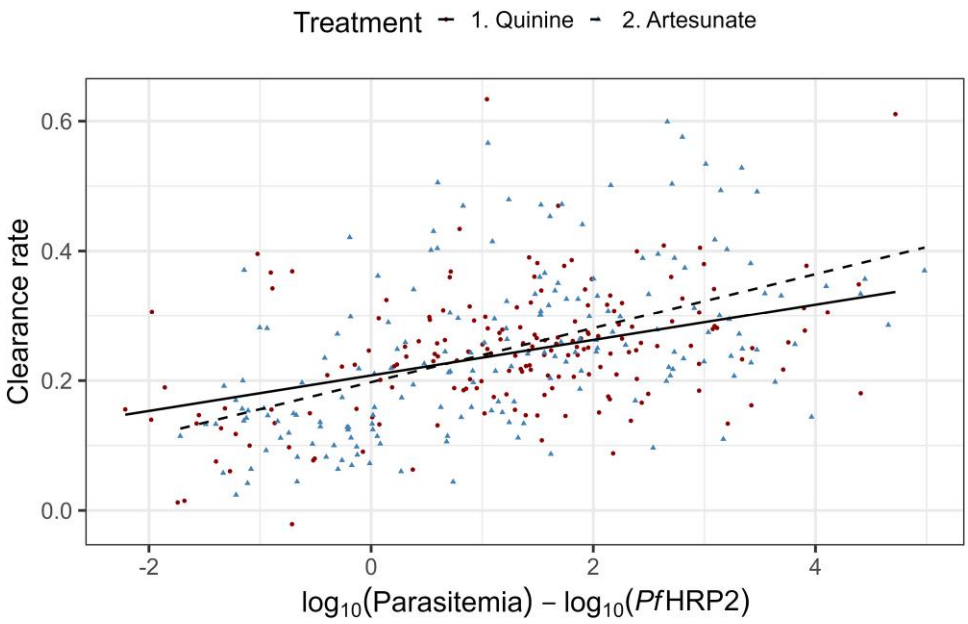

Figure 4. Comparison of clearance rate in artesunate- and quinine-treated participants in relation to ring ratio. Abbreviation: *Pf*HRP2, *Plasmodium falciparum* histidine-rich protein 2.

more appropriate proteins, such as *Pf*LDH. The shorter in vivo half-life of this protein would make it more appropriate for measuring total body parasite load. Using this approach to estimate parasite life cycle stage distribution in vivo strongly suggests that the more rapid parasite clearance rates in artesunate recipients are due to its effects across the entire *P falciparum* life cycle.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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