MAJOR ARTICLE

In Vivo Parasitological Measures of Artemisinin Susceptibility

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Parasite clearance data from 18,699 patients with falciparum malaria treated with an artemisinin derivative in areas of low ($n = 14{,}539$), moderate ($n = 2077$), and high ($n = 2083$) levels of malaria transmission across the world were analyzed to determine the factors that affect clearance rates and identify a simple in vivo screening measure for artemisinin resistance. The main factor affecting parasite clearance time was parasite density on admission. Clearance rates were faster in high-transmission settings and with more effective partner drugs in artemisinin-based combination treatments (ACTs). The result of the malaria blood smear on day 3 (72 h) was a good predictor of subsequent treatment failure and provides a simple screening measure for artemisinin resistance. Artemisinin resistance is highly unlikely if the proportion of patients with parasite densities of <100,000 parasites/μL given the currently recommended 3-day ACT who have a positive smear result on day 3 is <3%; that is, for n patients the observed number with a positive smear result on day 3 does not exceed $(n + 60)/24$.

Artemisinin-based combination treatments (ACTs) are recommended for falciparum malaria throughout the tropical world [1]. Stable resistance to artemisinin and its derivatives is very difficult to induce in the laboratory, and until recently there were no substantiated reports of resistance in the treatment of malaria [2]. Resistance to chloroquine, sulfadoxine-pyrimethamine, and mefloquine were first reported close to the Thai-

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Cambodian border [3–8]. From the same geographic area, there is now evidence of reduced susceptibility to artemisinin derivatives and ACTs with prolonged parasite clearance times and day 42 failure rates up to 20% for artesunate-mefloquine and day 28 failure rates between 13% to 29% for artemether-lumefantrine [2, 9–14]. If artemisinin resistance were to spread as resistance to chloroquine and sulfadoxine-pyrimethamine has spread before, it would threaten malaria control and seriously challenge current initiatives to eliminate malaria. At present, there is no validated molecular marker or in vitro test of artemisinin resistance. We have examined the largest available database of antimalarial clinical trials [15] and detailed studies of patients with hyperparasitemia to characterize parasite clearance after treatment with artemisinin derivatives. The objective was to identify a simple indicator that could be used to screen for artemisinin resistance during standard in vivo efficacy studies and surveillance programs.

METHODS

Background

In 2002, a group of investigators conducting antimalarial drug studies decided to pool their data on therapeutic responses in uncomplicated falciparum malaria obtained in diverse geographic locations with differing malaria transmission intensities [15]. The objective was to characterize the efficacy of different antimalarial drug treatments and to determine the effects of covariates (such as transmission intensity, age, and parasite density) on therapeutic responses. This will now be incorporated into the Worldwide Antimalarial Resistance Network, or WWARN [16]. In total, data on 31,708 individual patients from 85 prospective studies conducted in 25 countries were pooled and analyzed. Of these patients, 17,546 (55%) received an artemisinin derivative, either alone or in combination with a more slowly eliminated antimalarial drug. Many clinical trials excluded patients with high parasite densities. Because the decline in parasite density after antimalarial treatment is exponential (a first-order process) [17–19], the parasite clearance time is directly proportional to the logarithm of the initial parasite count; thus, excluding hyperparasitemia creates a potential confounder. To investigate this further, a large data set for patients studied on the northwestern border of Thailand with parasitemia levels of >4% (termed *hyperparasitemia* in this article) was also studied. The overall objective of this analysis was to provide a statistical basis for prospectively characterizing in vivo resistance to artemisinin and its derivatives.

Clinical and Parasitological Data

Two sources of data were used for this analysis.

World Health Organization Special Programme on Research and Training in Tropical Diseases and Wellcome Trust pooled malaria database. The World Health Organization (WHO) Special Programme on Research and Training in Tropical Diseases and Wellcome Trust (TDR-WT) pooled malaria database comprises data on patients from 85 studies conducted between 1991 and 2005 in 25 countries. Areas of transmission intensity were categorized as high (estimated entomological inoculation rate, >25 infectious bites/person/year), moderate (2–25 infectious bites/person/year), and low (<2 infectious bites/person/year) [15]. Parasite densities were available at enrollment (day 0) and then on days 1 (ie, ~24 h after the start of treatment), 2, 3, and 7, although the exact times of measurement were usually not recorded.

Shoklo Malaria Research Unit hyperparasitemia database. Data from patients in the Shoklo Malaria Research Unit (SMRU) hyperparasitemia database who were admitted with levels of parasitemia of >4% between January 2001 and June 2008 ($n = 1687$) were analyzed. Parasite counts were measured every 6 h until clearance. Exact blood sampling times were recorded.

Blood smears were stained with Giemsa stain, and parasites were counted on the thin or thick smear against 1000 red blood cells or 1000, 500, or 200 white blood cells. The number of parasites per microliter was calculated using measured individual hematocrit values for thin smears or by assuming a white blood cell count of 8000 cells/μL for thick smears. Other data collected included demographic characteristics (age, weight, sex, etc), treatment and doses given, and information on symptoms, signs, temperature, gametocytemia, and hematological and biochemical data. Recurrence of parasitemia during the follow-up period, together with polymerase chain reaction (PCR) genotyping results, were recorded for patients in the TDR-WT database but were not routinely recorded for patients in the SMRU hyperparasitemia database.

Statistical Analysis

Since areas of different transmission intensity differ with respect to patient populations, antimalarial treatment, and length of follow-up, all analyses (unless specified) were performed separately for each level of transmission intensity. This stratification was defined a priori.

Parasite positivity rates (PPRs), or the proportions of patients with parasitemia, were recorded on days 1, 2, and 3. Overall PPRs were calculated as a weighted average of study treatment arm–specific proportions. Confidence intervals (CIs) were estimated using a method for clustered data described by Fleiss et al [20]. The PPRs were compared between groups of studies (determined by region or treatment) by the F test, using grouped logistic regression. CIs for ordinary proportions were calculated using the Wilson method [21].

Associations between positive parasite counts on day 2 or 3 and subsequent recrudescence of infection were assessed by comparison of the cumulative recrudescence proportions, es-

Table 1. Patient Characteristics

Characteristic	TDR-WT database[a]				SMRU HP
	Low	Moderate	High	SMRU	
No. treated with					
Artemisinin derivatives	3867	2077	2083	8984	1590
Artesunate	2123	1825	1881	6185	1590
Dihydroartemisinin	731	252	0	684	0
Artemether	434	0	202	2115	0
Artemisinin	579	0	0	0	0
Years of study	1994–2004	1998–2004	1999–2005	1991–2005	2001–2007
Age					
Median (range), years	16 (1–80)	3 (0.3–65)	2 (0.1–47)	16 (0.2–84)	12 (0.1–63)
<5 years	614 (16)	1595 (77)	1895 (91)	858 (10)	425 (27)
5–15 years	1255 (32)	442 (22)	179 (9)	3333 (37)	529 (33)
>15 years	1998 (52)	40 (2)	9 (0.5)	4793 (53)	623 (39)
Admission parasite density, median (range), parasites/μL	10,468 (8–1,365,523)	25,600 (31–1,787,040)	23,920 (71–540,784)	7448 (7–1,528,753)	271,422 (18,840–2285,920)
Patients with hyperparasitemia (>175,000 parasites/μL)	55 (1)	131 (6)	45 (2)	442 (5)	1395 (88)

NOTE. Data are no. (%) of patients, unless otherwise indicated. Categories are as follows: low, low-transmission areas (<2 infectious bites/person/year), excluding Shoklo Malaria Research Unit (SMRU) studies; moderate, moderate-transmission areas (2–25 infectious bites/person/year); high, high-transmission areas (>25 infectious bites/person/year); SMRU, the uncomplicated malaria studies conducted by the SMRU; SMRU HP, the hyperparasitemic (parasitemia level, >4%) malaria study conducted by the SMRU.

[a] World Health Organization Special Programme on Research and Training in Tropical Diseases and Wellcome Trust (TDR-WT) pooled malaria database.

timated using the Kaplan-Meier method. Patients with missing PCR results were excluded, and patients who were lost to follow-up or who had new infections during follow-up were censored at the last follow-up visit. Studies without PCR genotyping were excluded from this analysis.

The terminal relationship between log-transformed parasite density and time was assumed to be linear [17]. Therefore, if the lower limit of microscopic detection is 50 parasites/μL, P is the admission parasite density, k is the first-order clearance rate constant, and T is the time to parasite clearance then $T = (\log P - \log 50)/k$. As a consequence, the admission parasite density (P) affects the time of clearance and the probability of having a positive count at any specified time. Parasite clearance rate constants (k) for artesunate alone were estimated from patients in the SMRU hyperparasitemia database, who received artesunate only during the first 48 h, analyzing the slope of the log parasite count–time relationship for each patient from linear regression fits, using all measurements taken up to 48 h.

The effects of parasite count and other covariates on the probability of parasite clearance by day 3 were investigated for all patients receiving antimalarial treatments currently recommended by the WHO by logistic regression and were expressed as odds ratios (ORs) and 95% CIs. Random-effects models were used to account for the heterogeneous nature of the data and were fitted separately within each transmission intensity stratum. A pooled model was also fitted, using data from patients who received treatments that were common in at least 2 areas of different endemicity. Because recent evidence has indicated that parasite clearance times have been increasing on the western border of Thailand since 2001 [22], the data from SMRU patients were analyzed separately from the other data sets.

In all analyses, patients who received a blood transfusion were excluded. No patients in the TDR-WT database received blood, whereas 97 patients with hyperparasitemia in the SMRU database (6% of 1687 patients in the SMRU hyperparasitemia database and 5% of all 2068 patients with hyperparasitemia) received transfusions. In the TDR-WT database, patients who received rescue treatment after experiencing early treatment failure were censored at the time of retreatment. In the SMRU hyperparasitemia database, parasite counts were also available after the rescue treatment. Because these patients had the longest initial parasites clearance times, they were included in the analysis, but the characteristics of parasite clearance (PPR and clearance rate) are contrasted with those of other patients.

RESULTS

In total, 18,699 patients treated with an artemisinin derivative were included in this analysis: (1) 8984 with uncomplicated malaria studied by the SMRU and included in the TDR-WT database; (2) 8027 from studies in other countries included in the TDR-WT database (3867 in low-transmission areas, 2077 in moderate-transmission areas, and 2083 in high-transmission areas); and (3) 1590 with hyperparasitemia studied by the SMRU (Table 1). The artemisinin derivative was given either

alone (817 [9%], 226 [3%], and 87 [6%] patients in the 3 groups delineated above, respectively) or, more typically, as part of an ACT (Table 2). Rescue treatments were given to 66 (4%) of the 1590 patients with hyperparasitemia studied by the SMRU and to 48 (0.3%) of the 17,011 patients in the TDR-WT database who experienced early treatment failure or developed severe malaria. These patients were censored at the time of treatment failure. Similarly, an additional 21 patients for whom parasitemia persisted until day 7 were censored on day 7.

Parasite Positivity Rates

On admission, the median parasite density was 1.2×10^4 parasites/μL (range, limit of detection to 1.8×10^6 parasites/μL) among patients with uncomplicated malaria and was 2.7×10^5

Table 2. Antimalarial Treatments Administered

Treatment			No. of patients treated					
Artemisinin derivative	Partner drug (target total dose)	Artemisinin derivative dosage (units per day; target unit dose)	Total	Low	Moderate	High	SMRU	SMRU HP
Artemether	None	7 days (4, 2, 2, 1, 1, 1, 1; 1 mg/kg)	206				206	
	Mefloquine (25 mg/kg)	1 day (10 mg/kg)	19				19	
		3 days (1, 1, 1; 4 mg/kg)	404	224			180	
	Lumefantrine (43.2 mg/kg)	3 days (2, 1, 1; 1.8 mg/kg)	175				175	
	Lumefantrine (64.8 mg/kg)	3 days (2, 2, 2; 1.8 mg/kg)	1826	210		202	1414	
	Lumefantrine (75.6 mg/kg)	5 days (2, 2, 1, 1, 1; 1.8 mg/kg)	121				121	
Artemisinin	None	5 days (2, 1, 1, 1, 1; 10 mg/kg)	114	114				
		7 days (2, 1, 1, 1, 1, 1, 1; 10 mg/kg)	112	112				
	Mefloquine (375 or 500 mg)	1 day (500 mg)	117	117				
	Quinine (30 or 50 mg/kg)	1 day (20 mg/kg)	184	184				
	Doxycycline (12 mg/kg)	1 day (20 mg/kg)	52	52				
Artesunate	None	3 days (1, 1, 1; 4 mg/kg)	4				4	
		5 days (2, 1, 1, 1, 1; 2 mg/kg)	80				80	
		5 days (1, 1, 1, 1, 1; 2 mg/kg)	42				42	
		7 days (2, 2, 2, 2, 2, 1, 1; 1 mg/kg)	403				403	
		7 days (4, 2, 2, 1, 1, 1, 1; 1 mg/kg)	120				33	87
		7 days (2, 1, 1, 2, 2, 2, 2; 1 mg/kg)	2				2	
	Mefloquine (15 mg/kg)	1 day (4 mg/kg)	641	641				
	Mefloquine (25 mg/kg)	1 day (10 mg/kg)	322				322	
		1 day (4 mg/kg)	231	206			25	
		3 days (1, 1, 1; 4 mg/kg)	5296	939			4357	
		5 days (2, 1, 1, 1, 1; 2 mg/kg)	49				49	
		7 days (4, 2, 2, 1, 1, 1, 1; 1 mg/kg)	1437				134	1303[a]
	Sulfadoxine-pyrimethamine (25/1.25 mg/kg)	1 day (4 mg/kg)	669		318	351		
		3 days (1, 1, 1; 4 mg/kg)	1076	143	585	349		
	Amodiaquine (30 mg/kg)	3 days (1, 1, 1; 4 mg/kg)	1874	120	922	832		
	Chloroquine (25 mg/kg)	3 days (1, 1, 1; 4 mg/kg)	423	74		349		
	Atovaquone-proguanil (135/72 mg/kg)	3 days (1, 1, 1; 4 mg/kg)	526				526	
	Antibiotics[b]	7 days	197				21	176
DHA	Piperaquine (50.4 mg/kg)	3 days (1, 1, 1; 2.1 mg/kg)	851	437	252		162	
	Piperaquine (51.2 mg/kg)	3 days (2, 1, 1; 1.6 mg/kg)	560	212			348	
	Piperaquine, trimethoprim, primaquine (12.3/3.5/0.2 mg/kg for 3 days)	3 days (2, 1, 1; 1.2 mg/kg)	82	82				
DHA plus artesunate	Piperaquine (51.2 mg/kg)	3 days (2, 1, 1; 4 mg/kg)	174				174	

NOTE. Categories are as follows: low, low-transmission areas (<2 infectious bites/person/year), excluding Shoklo Malaria Research Unit (SMRU) studies; moderate, moderate-transmission areas (2–25 infectious bites/person/year); high, high-transmission areas (>25 infectious bites/person/year); SMRU, the uncomplicated malaria studies conducted by the SMRU; SMRU HP, the hyperparasitemic (parasitemia level, >4%) malaria study conducted by the SMRU.

[a] Mefloquine was given at 48 and 72 h.

[b] Tetracycline, doxycycline, or clindamycin.

Table 3. Measures of Parasite Clearance

Day, result	TDR-WT database[a]				
	Low (n = 3867)	Moderate (n = 2077)	High (n = 2083)	SMRU (n = 8984)	SMRU HP (n = 1590)
Day 1					
Positive	1751	815	739	5235	1527
Negative	877	350	394	3287	37
Missing	1239	912	950	462	26
PPR (95% CI), %[b]	67 (59–74)	70 (59–81)	65 (60–70)	61 (54–69)	98 (97–98)
Day 2					
Positive	351	301	191	906	1014
Negative	2256	1486	1851	7569	508
Missing	1260	290	41	509	68
PPR (95% CI), %[b]	13.5 (9.4–17.5)	16.8 (9.2–24.4)	9.4 (5.6–13.1)	10.7 (7.8–13.6)	67 (64–69)
Day 3					
Positive	78	69	41	107	355
Negative	2581	1958	2021	8267	1124
Missing	1208	50	21	610	111
PPR (95% CI), %[b]	2.9 (2.0–3.9)	3.4 (0–7.4)	2.0 (0.6–3.3)	1.3 (0.8–1.8)	24 (22–26)

NOTE. Data are no. of patients, unless otherwise indicated. Categories are as follows: low, low-transmission areas (<2 infectious bites/person/year), excluding Shoklo Malaria Research Unit (SMRU) studies; moderate, moderate-transmission areas (2–25 infectious bites/person/year); high, high-transmission areas (>25 infectious bites/person/year); SMRU, the uncomplicated malaria studies conducted by the SMRU; SMRU HP, the hyperparasitemic (parasitemia level, >4%) malaria study conducted by the SMRU.

[a] World Health Organization Special Programme on Research and Training in Tropical Diseases and Wellcome Trust (TDR-WT) pooled malaria database.

[b] The parasite positivity rate (PPR) was adjusted for study intracorrelation.

parasites/μL (range, 1.9×10^5 to 2.3×10^6 parasites/μL) among patients with hyperparasitemia in the SMRU database (Table 1). Parasite clearance times were significantly faster in patients with lower initial densities ($P < .001$). Parasitological responses to artemisinins, either alone or in combination, were slightly faster in high-transmission areas than elsewhere (Table 3), but the differences between areas were not statistically significant. Excluding the SMRU studies, estimated PPRs on day 3 among all patients with uncomplicated malaria treated with artemisinin derivatives were 2.0% (95% CI, 0.6%–3.3%) in the high-transmission areas, 3.4% (95% CI, 0%–7.2%) in the moderate-transmission areas, and 2.9% (95% CI, 2.0%–3.9%) in the low-transmission areas ($P = .364$). For patients with hyperparasitemia ($n = 1583$), the PPR at 72 h was much higher—24% (95% CI, 22%–26%), decreasing to 5.5% (95% CI, 4.5%–6.8%) at 96 h. Of these patients, 66 received rescue treatment; their PPRs at 72 h (44% [95% CI, 33%–57%]) and at 96 h (13% [95% CI, 7%–24%]) were significantly higher than those for the other patients ($P < .001$ and $P = .006$, respectively).

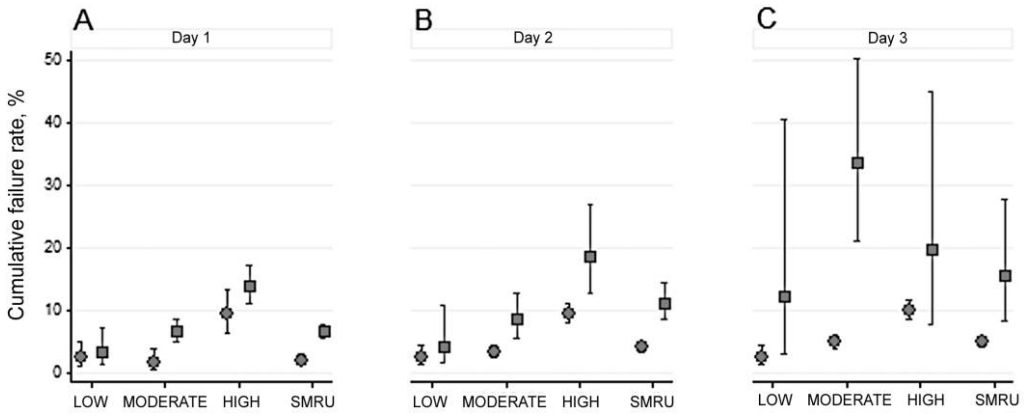

Figure 1. Cumulative risk of recrudescence for patients with a negative (*circles*) or positive (*squares*) parasite count on day 1 (*A*), day 2, (*B*), and day 3 (*C*). SMRU, Shoklo Malaria Research Unit.

Predictors of Subsequent Recrudescence

The presence of parasites on day 3 (ie, ~72 h after the start of treatment) emerged as the best determinant of subsequent recrudescence (Figure 1). In low- and moderate-transmission areas, the risk of recrudescent parasitemia was <5% in patients who cleared parasitemia by day 3. In contrast, among patients with parasitemia on day 3 this risk was 12%, 34%, and 16% in low-transmission areas, in moderate-transmission areas, and among the SMRU patients with uncomplicated malaria, respectively ($P < .001$ for all) (Figure 1*C*). In the high-transmission areas, rates of recrudescence for both groups of patients were higher, but patients with detectable parasitemia on day 3 still had double the risk of recrudescent parasitemia compared with patients who were parasite negative on day 3 ($P = .118$). The positive predictive value of parasite positivity on day 3 in predicting treatment failure is equal to the estimated risks of failure described above, and the negative predictive value is equal to risk of success in patients with a negative count on day 3 (97.5%, 95%, 90%, and 95%, respectively) (Figure 1*C*).

Parasite Clearance Rate Constants

The parasite clearance rate constant (k) could be estimated in 1570 individuals in the SMRU hyperparasitemia database; in 95% ($n = 1495$), the linear regression fit was satisfactory (median $R^2 = 0.92$). Among these patients, the median slope was estimated to be 0.097 per 1 h (range, 0.015–0.333 per 1 h) and had a symmetrical distribution (mean, 0.101; standard deviation, 0.032). Parasite clearance rate constants did not correlate with initial parasitemia ($r_s = .010$; $P = .71$). These slope values correspond to a median half-life of 3.1 h (range, 0.9–19.4 h) or parasite reduction rates ranging from 1.25 to 2.15/h. Parasite clearance among the 63 patients who received rescue treatment was significantly slower than that among the remainder, with a median half-life of 3.6 h (range, 2.4–11.8 h) and a median parasite reduction rate of 1.21/h (range, 1.06–1.33/h) ($P < .001$).

Analysis of Covariates Affecting Parasite Clearance

The OR for failure to clear parasitemia by day 3 per 10-fold increase in admission parasite density was 3.862 (95% CI, 1.712–8.714) in high-transmission areas ($P = .001$), 1.689 (95% CI, 1.041–2.739) in moderate-transmission areas ($P = .034$), and 4.446 (95% CI, 2.813–7.027) in low-transmission areas ($P < .001$), respectively. Similar values were obtained for SMRU patients—an OR of 2.480 (95% CI, 1.849–3.326) for patients with uncomplicated malaria and of 4.191 (95% CI, 2.467–7.121) for the patients with hyperparasitemia ($P < .001$ for both) (Figure 2).

Data on spleen examination were available for 11,340 patients (8699 SMRU patients and 448, 1145, and 1048 patients in the low-, moderate-, and high-transmission areas); there was no difference in parasite clearance rates among patients with or without splenomegaly in each area. No other patient or disease characteristics (age, sex, presence of gametocytes on enrollment) were associated independently with the probability of parasitemia persisting to day 3. The heterogeneity between studies within each area was statistically significant ($P < .001$ for all). The duration of artemisinin treatment (1 or ⩾3 days) and the partner drug administered were important determinants of parasite clearance rates. Overall, 5.8% (108/1849; 95% CI, 1.6%–10.0%) of patients who received artemisinin treatment for 1 day had parasitemia on day 3, compared with 1.4% (187/13,097; 95% CI, 1.0%–1.8%) of patients treated with artemisinin for ⩾3 days ($P < .001$), although the rates varied between partner drugs (Table 4).

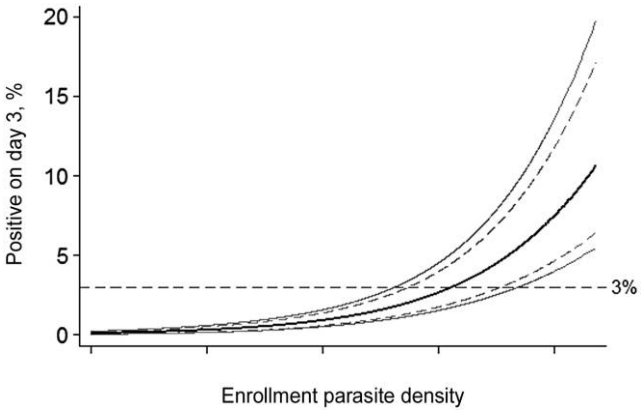

Figure 2. Relationship between enrollment parasite density and the proportion of patients with parasitemia on day 3 after the start of treatment with artemisinin derivatives, estimated using logistic regression with random effects for study site. The outer dashed and continuous lines represent 95% and 99% confidence intervals.

Effects of Partner Drugs

Low-transmission areas. PPRs obtained for artemether-lumefantrine, mefloquine-artemether/artesunate or dihydroartemisinin-piperaquine, after adjustment for initial parasite density, were each significantly lower than those for treatments with artesunate combined with the less efficacious partners amodiaquine or chloroquine (overall OR, 0.148 [95% CI, 0.057–0.387]; $P < .001$). Within these 2 groups of treatments, the PPRs were not significantly different. PPRs for artesunate and sulfadoxine-pyrimethamine were higher than for mefloquine-artemether/artesunate (OR, 0.117 [95% CI, 0.019–0.716]) but were not significantly different from other treatments. After adjustment for treatments and parasite densities, there was no heterogeneity between study sites ($P = .447$).

Among SMRU patients, there were no significant differences between PPRs with different 3-day treatments (test for heterogeneity between studies, $P < .001$).

Moderate-transmission areas. After adjustment for the

Table 4. Parasite Positivity Rates (PPRs) on Day 3 (~72 h), by Artemisinin Derivative and Partner Drug

Length of regimen, artemisinin derivative, partner drug	Low			Moderate			High			SMRU		
	PPR (95% CI), %[a]	No. of study arms	No. of patients	PPR (95% CI), %[a]	No. of study arms	No. of patients	PPR (95% CI), %[a]	No. of study arms	No. of patients	PPR (95% CI), %[a]	No. of study arms	No. of patients
⩾3-day regimen												
Artemether												
None										0.5 (0.4–0.7)	2	194
Mefloquine[b]	0.4 (0–1.0)	2	233							0.6 (0.1–3.3)	1	166
Lumefantrine[b]	2.4 (0–5.6)	2	210				0.0 (0–1.9)	1	201	1.0 (0.3–1.6)	13	1646
Artemisinin												
None	7.1 (4.5–9.6)	2	226									
Artesunate												
None										0.8 (0–1.6)	8	522
Mefloquine[b]	0.3 (0–0.7)	4	373							1.5 (0.5–2.4)	25	4044
Sulfadoxine-pyrimethamine[b]	2.0 (0–5.0)	3	143	0.9 (0–2.1)	9	565	2.0 (1.1–3.0)	2	343			
Amodiaquine[b]	2.6 (0–5.2)	2	115	2.2 (0.8–3.6)	8	910	0.4 (0–0.8)	5	828			
Chloroquine[b]	5.4 (2.1–13.1)	1	74				3.2 (0.9–5.6)	3	343			
Atovaquone-proguanil										0.4 (0.3–0.5)	2	512
DHA												
Piperaquine[b]	0.8 (0.2–1.4)	5	473	0.4 (0–1.0)	3	251				2.6 (1.1–4.2)	6	419
Piperaquine-artesunate										1.3 (0–2.9)	3	159
1-day regimen												
Artesunate												
Mefloquine	5.8 (4.6–6.9)	4	400							1.6 (0.4–2.7)	2	440
Sulfadoxine-pyrimethamine				14 (0–32)	6	301	5.7 (1.4–10)	2	349			

NOTE. The most common treatments with artemisinin derivatives in uncomplicated malaria studies are presented. For details of dosing, see Table 2. Categories are as follows: low, low-transmission areas (<2 infectious bites/person/year), excluding Shoklo Malaria Research Unit (SMRU) studies; moderate, moderate-transmission areas (2–25 infectious bites/person/year); high, high-transmission areas (>25 infectious bites/person/year); SMRU, the uncomplicated malaria studies conducted by the SMRU.

[a] Adjusted for study intracorrelation.

[b] World Health Organization–recommended treatment.

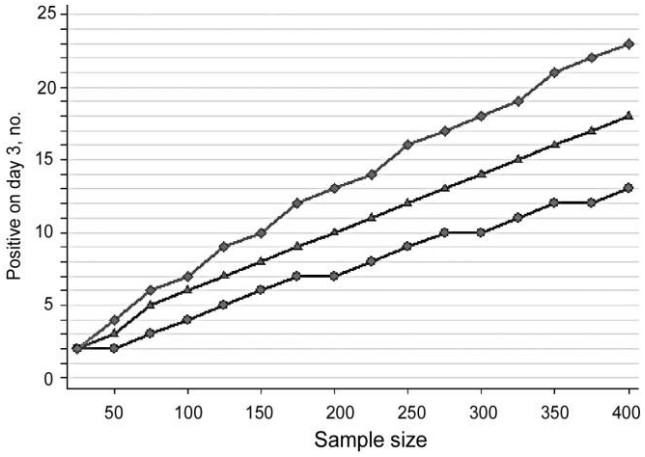

Figure 3. Relationship between sample size and the upper limit of the number of patients with a positive parasite count on day 3 for which the null hypothesis of a true positivity rate of 2% (*circles*), 3% (*triangles*), or 4% (*squares*) cannot be rejected, based on Wilson 95% confidence intervals.

study year and initial parasite density, PPRs were significantly different only between patients treated with artesunate and sulfadoxine-pyrimethamine and those treated with artesunate-amodiaquine (OR, 0.242 [95% CI, 0.084–0.696]; $P = .009$; test for heterogeneity, $P = .497$).

High-transmission areas. In terms of day 3 PPRs, treatment with artesunate-amodiaquine was more effective than treatment with artesunate and sulfadoxine-pyrimethamine (OR, 5.729 [95% CI, 1.473–22.287]; $P = .012$) or with artesunate–chloroquine (OR, 9.167 [95% CI, 2.541–33.061]; $P = .001$; test for heterogeneity, $P = .391$). Artemether-lumefantrine also had significantly lower PRRs than did these 2 latter treatments ($P = .050$ and $P = .006$; OR not calculable because no patients had detectable day 3 parasitemia after treatement with artemether-lumefantrine), which were not statistically different from artesunate-amodiaquine PPRs.

Among 3-day treatments assessed in at least 2 different transmission intensity areas (excluding SMRU patients since the time trend was different from the other areas), after adjustment for year of study and initial parasite density and stratifying for treatment, the combined risk for a positive parasite smear on day 3 was highest in low-transmission settings (OR for high-transmission area, 0.223 [95% CI, 0.103–0.480]; OR for moderate-transmission area, 0.360 [95% CI, 0.159–0.818]; $P = .001$). Artesunate-chloroquine gave the highest day 3 positivity rates (OR for dihydroartemisinin-piperaquine, 0.174 [95% CI, 0.053–0.572]; OR for artesunate-amodiaquine, 0.693 [95% CI, 0.294–1.636]; OR for artesunate and sulfadoxine-pyrimethamine, 0.308 [95% CI, 0.136–0.698]; OR for artemether-lumefantrine, 0.307 [95% CI, 0.094–1.002]; $P = .003$, likelihood ratio test). No relationship was found between total dose of artemisinin or artemisinin derivative received on the first day of treatment or overall and the proportion of positive patients on day 3. Heterogeneity between studies was not significant ($P = .143$).

Derivation of a Simple In Vivo Parasitological Definition of Susceptibility to Artemisinins

Additional analysis was restricted to patients who received artemisinin-derivative regimens currently recommended by the WHO for 3 days or longer (ie, studies with artesunate, artemether, or dihydroartemisinin). Because parasite clearance times increased significantly in the SMRU studies after 2001, only patients from SMRU studies treated before 2001 were included in this analysis.

Among patients with admission parasite densities between 10,000 and 100,000 parasites/μL treated for at least 3 days with artemisinin derivatives, only 1.2% (53/4535; 95% CI, 0.7%−1.6%) had detectable parasitemia on day 3—1.7% (14/830; 95% CI, 0.7%−2.7%) in low-transmission areas, 1.5% (13/863; 95% CI, 0.3%–2.7%) in moderate-transmission areas, 1.2% (14/1152; 95% CI, 0.2%–2.3%) in high-transmission areas, and 0.7% (12/1690; 95% CI, 0.3%–1.1%) in studies conducted by the SMRU before 2001.

Using the highest value of the upper 95% CI bound (~3%) in patients with parasite densities of <100,000 parasites/μL, the probability of observing 7 or more patients with positive day 3 parasitemia in a clinical trial of 100 patients with parasite densities of <100,000 parasites/μL would be 0.03. For sample sizes of <12, no patients should have positive day 3 parasitemia ($P < .05$) if the true positivity rate on day 3 is $\leq$3%. (Figure 3).

For a true positivity rate of <3%, a simple method to calculate the maximum number of patients with positive day 3 parasitemias for studies with $\geq$50 patients is the following: the number of patients with positive day 3 parasitemias (n) should not exceed $(n + 60)/24$.

Among 48 study arms with WHO-recommended treatments (excluding SMRU studies) and including only patients with enrollment parasite counts between 10,000 and 100,000 parasites/μL, 2 had 95% CI lower limits for day 3 PPRs of >3%. Both were relatively ineffective regimens—artesunate-chloroquine in a high-transmission area, and artesunate and sulfadoxine-pyrimethamine in a low-transmission area.

DISCUSSION

Parasite clearance can be analyzed in a number of different ways, depending in part on the frequency with which parasite densities are measured. If blood smears are done frequently enough, as in some research studies, clearance can be assessed as a continuous variable (ie, the continuous relationship between log parasite density and time). Alternatively, the proportion of patients who remain parasite positive (taken as a

parasite density of ⩾50 parasites/μL) at different time points can be assessed. The latter is simpler and therefore more suitable for routine monitoring. In the majority of clinical trials and in clinical practice, parasite densities are measured once daily or less.

Acceleration of parasite clearance is the pharmacodynamic hallmark of the artemisinin derivatives [18]. It results from the killing of circulating ring-stage parasites before they cytoadhere and their subsequent removal by the spleen [23–25]. The other antimalarial drugs have relatively little or no effects on these young circulating parasites [26, 27] and do not prevent cytoadherence in *Plasmodium falciparum* infections [28]. The rate of parasite clearance can therefore be used as a measure of the artemisinin pharmacodynamic effect in vivo [19]. Artemisinin resistance is characterized by prolongation in parasite clearance times [14]. The parasite clearance time is the interval from the start of treatment to the time at which parasite densities fall below the lower limit of detection by light microscopy, which corresponds to a residuum of ~100,000,000 parasites in the body of an adult [29]. Because parasite clearance after artemisinin treatment is rapid, most patients have cleared their peripheral parasitemia by day 3 (~72 h) after the start of treatment. Those few with persistent parasitemia represent a composite of those with the highest starting densities and the tail of a distribution of clearance rates. Artemisinin resistance is therefore associated with an increase in this proportion [14]. In the present very large series, <5% of patients with pretreatment densities of <100,000 parasites/μL had detectable parasitemias on day 3, compared with 25% of patients with parasitemia levels of >4% (corresponding to >100,000 parasites/μL). Thus, clearance time needs to be adjusted for the starting parasite density. With this caveat, the proportion of patients with positive blood smear results on day 3 can be used as a simple measure of artemisinin susceptibility in vivo.

It had been assumed previously that the partner drug would have a negligible effect on parasite clearance after administration of ACT, but the slower responses in patients receiving artesunate-chloroquine than other 3-day ACT regimens with a similar dose of artesunate, as well as the generally more rapid clearance with more effective ACTs (artesunate-mefloquine, dihydroartemisinin-piperaquine, and artemether-lumefantrine), suggests that a significant contribution is made by the partner drug. The significantly higher day 3 positivity rates with 2 mg/kg/day artesunate, artemether, or artemisinin monotherapy regimens further supports the notion that the ACT partner drug contributes to parasite clearance, although 2 mg/kg may not provide maximal ring-stage parasite killing in all patients. The importance of providing more than a single dose of an artemisinin derivative is emphasized by the slower parasite clearance with 1-day regimens.

Immunity contributes to the antimalarial therapeutic response. Although differences were small, parasite clearance was slowest in the low-transmission settings and most rapid in high-transmission settings even with relatively ineffective partner drugs, suggesting that the splenic clearance process for the killed ring-stage parasites may be enhanced. Splenomegaly per se was not associated with accelerated parasite clearance, but splenic structural reorganization and activation in high-transmission areas together with antibody production may enhance clearance function in acute infections.

The day 3 parasite count proved a useful predictor of therapeutic response. Previous studies with monotherapies have shown the value of prolonged parasite clearance in predicting subsequent recrudescence. With mefloquine treatment, persistence of parasitemia to day 4 proved to be the best determinant of treatment failure [30]. Slow parasite clearance reflects a reduced contribution of the artemisinin component of an ACT to overall parasite killing, leaving a larger biomass for the partner drug to remove [29].

Recent data from the Thai-Cambodian border suggest that artemisinin resistance in *P. falciparum* malaria has developed there [2, 13]. This may be a multistage process. It is imperative to determine whether resistance has spread beyond this region or emerged independently elsewhere. The day 3 parasite count is a simple measure that can be followed routinely in in vivo monitoring studies. In a clinical study of n patients presenting with parasite densities of <100,000 parasites/μL, if the number who still have parasitemia on day 3 (72 h) after the start of treatment with ACT exceeds $(n + 60)/24$, then additional, more detailed investigations may be warranted.

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