Host immunity to *Plasmodium falciparum* and the assessment of emerging artemisinin resistance in a multinational cohort

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Artemisinin-resistant falciparum malaria, defined by a slow-clearance phenotype and the presence of *kelch13* mutants, has emerged in the Greater Mekong Subregion. Naturally acquired immunity to malaria clears parasites independent of antimalarial drugs. We hypothesized that between- and within-population variations in host immunity influence parasite clearance after artemisinin treatment and the interpretation of emerging artemisinin resistance. Antibodies specific to 12 *Plasmodium falciparum* sporozoite and blood-stage antigens were determined in 959 patients (from 11 sites in Southeast Asia) participating in a multinational cohort study assessing parasite clearance half-life ($PCt_{1/2}$) after artesunate treatment and *kelch13* mutations. Linear mixed-effects modeling of pooled individual patient data assessed the association between antibody responses and $PCt_{1/2}$. *P. falciparum* antibodies were lowest in areas where the prevalence of *kelch13* mutations and slow $PCt_{1/2}$ were highest [Spearman $\rho = -0.90$ (95% confidence interval, −0.97, −0.65), and Spearman $\rho = -0.94$ (95% confidence interval, −0.98, −0.77), respectively]. *P. falciparum* antibodies were associated with faster $PCt_{1/2}$ (mean difference in $PCt_{1/2}$ according to seropositivity, −0.16 to −0.65 h, depending on antigen); antibodies have a greater effect on the clearance of *kelch13* mutant compared with wild-type parasites (mean difference in $PCt_{1/2}$ according to seropositivity, −0.22 to −0.61 h faster in *kelch13* mutants compared with wild-type parasites). Naturally acquired immunity accelerates the clearance of artemisinin-resistant parasites in patients with falciparum malaria and may confound the current working definition of artemisinin resistance. Immunity may also play an important role in the emergence and transmission potential of artemisinin-resistant parasites.

malaria | artemisinin | drug resistance | immunity | serology

Artemisinin combination therapy (ACT), recommended by the World Health Organization (WHO) as first-line treatment of *Plasmodium falciparum* malaria, was used to treat 337 million patients with malaria in 2014 (1). Global malaria mortality and morbidity have declined in recent years, largely as a result of increased deployment of insecticide-treated bed-nets and ACTs (1). Artemisinin resistance, defined by slow parasite clearance after treatment with an artemisinin derivative, was initially reported in 2007 in Western Cambodia (2, 3). Further independent reports of the slow-clearing phenotype spreading, or emerging independently, came from other areas of Western Cambodia, Thailand, Myanmar, and Vietnam (4–7). In 2014, mutations in the "propeller" region of a *P. falciparum* kelch protein (encoded by the *kelch13* gene) were identified as molecular markers of artemisinin resistance based on their associations with the slow-clearance phenotype (8). Recently, both the slow-clearance phenotype, defined by a long parasite clearance half-life ($PCt_{1/2}$) after artemisinin treatment (9), and *kelch13* mutations were reported by the Tracking Resistance to Artemisinin Collaboration (TRAC), a multinational trial of artesunate efficacy (10). TRAC confirmed that artemisinin-resistant falciparum malaria is firmly established in Western Cambodia, Thailand, Eastern

Significance

Slow-clearing artemisinin-resistant malaria parasites are now well established in the Greater Mekong Subregion. This large multinational therapy efficacy study incorporating clinical data, molecular drug-resistance markers, and immune profiling aimed to understand how variations in population levels of naturally acquired malarial immunity affect the slow-clearing phenotype, emergence of artemisinin resistance-associated mutations, and assessment of the geographical spread of artemisinin resistance. We found that slow-clearing mutant parasites occur at higher frequencies in areas where immunity is lowest, patients with higher immunity have faster clearance times, and immunity has the greatest effect on clearance in patients with slow-clearing mutant parasites. Immunity plays an important role in the emergence of resistant parasites and can confound the World Health Organization's phenotype and genotype definitions of artemisinin resistance.

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Myanmar, and Southern Vietnam, and is emerging in Northern Cambodia and Southern Laos, but it found no evidence for artemisinin resistance in Africa (10).

To facilitate monitoring and surveillance of artemisinin resistance, the WHO now defines confirmed partial artemisinin resistance as ≥5% of *P. falciparum* patients carrying *kelch13* mutations associated with either persistent parasitemia on day 3 or a $PCt_{1/2}$ ≥5 h after artemisinin treatment (11). Suspected artemisinin resistance is defined as ≥5% of patients with *P. falciparum* carrying *kelch13* resistance-associated mutations, ≥10% of patients with parasitemia at day 3, or ≥10% of patients with a $PCt_{1/2}$ ≥5 h after treatment (11). Although detection of molecular markers is unequivocal, there is substantial interindividual variability in parasite clearance. In vivo responsiveness to antimalarials is influenced by additional factors such as patient pharmacokinetic profiles, life cycle stage distribution of the parasites, and levels of host immunity (12). Thus, the predictive values of both the $PCt_{1/2}$ ≥5 h cutoff and the *kelch13* mutations in assessing artemisinin resistance may differ, depending on the contributions of these confounding factors.

Naturally acquired immunity to malaria develops after repeated exposure to parasites, and is acquired faster in high- compared with low-transmission areas (13). *P. falciparum* antibodies are an important component of immunity and can target the sporozoite stage, reducing transmission and infection, and blood-stage parasites (merozoites, infected erythrocytes), reducing parasite multiplication and increasing parasite clearance rates, thereby suppressing parasite densities and clinical symptoms (14, 15). Immunity may therefore confound the interpretation of parasite clearance measures in drug-efficacy studies. The operational implications of an effect of host immunity on parasite clearance measures are that in populations with high levels of immunity and faster parasite clearance, early signs of low-grade drug resistance could go undetected, and conversely, that in populations with lower immunity and slower parasite clearance, a false impression of reduced drug efficacy could arise (16–18). The available immunological evidence for this comes from previous single-study-site investigations, predominantly in high-transmission settings in Africa, which have reported conflicting associations between immunity and treatment failure to historical first-line treatments (e.g., chloroquine, sulfadoxine–pyrimethamine) and ACTs (19–28). Of the four single-site studies looking at artemisinin derivatives, all examined ACT, where associations may be confounded by the partner drug, and all were performed in areas before the emergence of artemisinin resistance or in areas where resistance is yet to arise (24–27). Only one of these studies investigated an outcome measure included in the current WHO definition of artemisinin resistance ($PCt_{1/2}$) and found an unquantified inverse correlation (25). However, single-site studies in areas in which resistance has yet to emerge fail to encapsulate between- and within-population variations in malarial immunity and frequencies of *kelch13* mutations. We hypothesize that between- and within-population variations in host immunity influence parasite clearance after artemisinin treatment, confounding the current WHO working definitions of artemisinin resistance, and consequently the interpretation of the geographical spread of artemisinin resistance. In this multinational study, which includes multiple different transmission settings with varying frequencies of *kelch13* mutations, we determined levels of antibodies specific for a panel of *P. falciparum* antigens and quantified their effect on $PCt_{1/2}$, a key parameter in the WHO definition of artemisinin resistance after exposure to artesunate alone.

Methods

Study Design and Procedures. We studied plasma samples from 959 (of 985) patients with high parasitemia from 11 Southeast Asian sites (*SI Appendix*, Fig. S1, text S1) participating in the TRAC multicenter drug-efficacy randomized control trial. Informed consent was obtained from all patients, and ethical approval was granted by the Oxford Tropical Research Ethics Committee (06/11), Alfred Hospital Committee for Ethics, Australia (485/12) (10). Participants received either 2 or 4 mg/kg artesunate for 3 d, followed by a full course of an ACT. The initial study aimed to enroll 120 patients at each study site, which was achieved in six of the 11 Southeast Asian sites. Patients aged 0.5–65 y with uncomplicated falciparum malaria (parasitemia between 10,000 and 200,000/μL) and fever/history of fever were included. Blood smears were taken for malaria parasite counts at 0, 4, 6, 8, and 12 h, and then every 6 h until two consecutive counts were negative.

Immunoassays. At enrolment, plasma concentrations of total antigen-specific IgG were measured for recombinant *P. falciparum* merozoite, and sporozoite antigens using ELISAs, and IgG to the surface of *P. falciparum*-infected erythrocytes (3D7 strain) containing pigmented trophozoites, as previously described (*SI Appendix*, text S1).

Statistical Analysis. Definitions and methods are detailed in the *SI Appendix*, text S1. Briefly, the primary endpoint, $PCt_{1/2}$ (h) was derived from the parasite clearance estimator (9). $PCt_{1/2}$ is a standardized measure of parasite clearance after antimalarial treatment and is the time needed for parasitemia to be reduced by half during the log-linear phase of parasite clearance. It is independent of initial parasitemia and excludes potential confounding of changes in parasite density after antimalarial drug administration (9). Meta-analysis of the differences in mean $PCt_{1/2}$ according to sero-positivity was performed to provide estimates for each study site and for the overall cohort and heterogeneity was evaluated by the I^2 value. Linear mixed-effects modeling on the pooled individual patient data (adjusting for age and total dose of artesunate, and including a random effect for study site) was performed to assess the association between each antibody response and $PCt_{1/2}$. Interactions between *kelch13* (defined as any mutation above amino acid position 440 with a median $PCt_{1/2}$ ≥5 h and present in at least 5 individuals) and antibody response were assessed using a likelihood ratio test.

Results

The study included 959 patients with falciparum malaria in 11 sites in Southeast Asia (Table 1). *P. falciparum* densities showed no geographical patterns (*SI Appendix*, Fig. S2), whereas median $PCt_{1/2}$ values of ≥5 h and *kelch13* mutation prevalence >50% were found only in Western Cambodia (Pailin and Pursat) and Thailand (Ranong and Srisaket) (10) (Table 1). The prevalence of gametocytemia was also highest in Western Cambodia (Pailin and Pursat) (Table 1 and *SI Appendix*, Fig. S2). We measured levels of antibodies specific for 12 different *P. falciparum* antigens, including a sporozoite antigen [circumsporozoite protein (CSP), a transmission biomarker], relatively conserved merozoite invasion ligands [apical membrane antigen 1 (AMA1); reticulocyte binding ligand homologue 2 (Rh2); erythrocyte binding antigen 175, region 2 ($EBA175_{RII}$); erythrocyte binding antigen 175, region 3 to 5 ($EBA175_{RIII\text{-}V}$)], merozoite surface proteins [merozoite surface protein 1, C-terminal 19 kDa region ($MSP1_{19}$); merozoite surface protein 2, FC27 allele ($MSP2_{FC27}$); merozoite surface protein 2, 3D7 allele ($MSP2_{3D7}$); merozoite surface protein 3 (MSP3); merozoite surface protein 6 (MSP6); merozoite surface protein 7 (MSP7)], and infected-erythrocyte variant surface antigens [infected-erythrocyte variant surface antigens, 3D7 line (VSA_{3D7})], all of which are biomarkers of blood-stage immunity (14, 15) (Fig. 1). Within each site, individual blood-stage antibodies were weakly correlated with age [median (interquartile range [IQR]) ρ across all sites: 0.20 (0.15, 0.21)] and parasite density [−0.10 (−0.20, −0.08)]. Blood-stage antibodies were moderately correlated with each other [median (IQR) of all ρ = 0.54 (0.46–0.63)] and with anti-sporozoite antibodies [median (IQR) ρ = 0.49 (0.45–0.51)]. Antibodies specific for *P. falciparum* varied across study sites (Fig. 1), including those located in the same country. For example, Eastern Thailand (Srisaket) had lower anti-sporozoite and anti-blood-stage antibody levels than the Myanmar–Thailand border region (Mae Sot, Ranong), and Western Cambodia (Pailin, Pursat) had lower antibody levels than Eastern Cambodia (Preah Vihear, Ratanakiri; *SI Appendix*, Figs. S2 and S3).

To assess whether between-population variations in immunity are associated with the emergence of resistance, we plotted $PCt_{1/2}$ values and the prevalence of patients carrying *kelch13* mutations that confer artemisinin resistance (*kelch13* mutants) against a composite measure of blood-stage immunity for each study site (Fig. 2). We observed a strong inverse correlation between

Table 1. Characteristics of 959 study participants according to study site

Country and study site	N	Male (N, %)	Age (median [IQR], R) (y)	Parasite density (median, R)*	Gametocytemia (N, %)	$PCt_{1/2}$ (median, R)*	*kelch13* mutants (%)†
Bangladesh							
Ramu	49	42 (86)	26 [20-35], 10–55	32,154, 10,048–224,196	0 (0)	2.6, 0.7–5.4	0/45 (0)
Cambodia							
Pailin	96	83 (86)	25 [19-38], 10–56	45,216, 2,560–327,062	19 (20)	6.1, 2.4–9	77/96 (80)
Preah Vihear	120	82 (68)	20 [14-29], 4–58	56,582, 13,942–311,237	6 (5)	3.0, 1.2–12.6	22/113 (19)
Pursat	119	109 (91)	25 [19-33.5], 3–60	56,582, 9,797–284,861	22 (18)	5.6, 1.7–11.8	75/114 (66)
Ratanakiri	120	78 (65)	14 [9-19.5], 2–55	62,109, 5,024–310,860	7 (6)	3.0, 0.7–8.8	4/115 (3)
Laos							
Attapeu	85	57 (67)	23 [13-29], 6–60	51,496, 12,811–198,574	6 (7)	2.0, 1.1–9.2	3/85 (4)
Myanmar							
Shwe Kyin	77	64 (83)	24 [19-31], 13–54	64,307, 10,640–420,006	9 (12)	3.1, 1.3–8.6	13/55 (24)
Thailand							
Mae Sot	117	92 (79)	29 [23-37], 18–58	37,806, 2,560–327,062	11 (9)	4.9, 0.6–10.1	42/91 (46)
Ranong	22	16 (73)	32 [26-39], 19–53	45,656, 5,903–94,451	0 (0)	5.3, 2.4–13.8	13/20 (65)
Srisaket	36	36 (100)	28 [22-39], 16–54	28,134, 4,346–192,997	1 (3)	7.0, 1.6–13.9	29/35 (83)
Vietnam							
Binh Phuoc	118	91 (77)	26 [19-39, 4-61	49,738, 9,797–205,230	7 (6)	3.1, 0.7–8.9	24/116 (21)

Data are provided as number N (%) or median with interquartile range [IQR] and/or range (R).
*Interquartile ranges for both parasite density and $PCt_{1/2}$ are shown in *SI Appendix*, Fig. S2 and Fig. 2, respectively.
†A *kelch13* mutation was defined as any mutation above amino acid position 440 with a median $PCt_{1/2} \geq 5$ h and present in at least 5 individuals (10). Discrepancies in numbers of total patients are a result of the presence of mixed infection (both wild-type and mutant alleles present) and/or missing genotypes.

P. falciparum blood-stage antibody responses and $PCt_{1/2}$ values [Spearman $\rho = -0.94$; 95% confidence interval (CI), −0.77, −0.98; $P < 0.0001$], and between anti-blood-stage and anti-sporozoite antibodies with the prevalence of *kelch13* mutants [Spearman $\rho = -0.90$ (95% CI, −0.65, −0.97; $P < 0.0001$) and Spearman $\rho = 0.77$ (95% CI, −0.32, −0.96; $P = 0.0054$), respectively]. In addition, for each doubling of antibody levels toward the individual antigens, there was an approximate 40% decrease [median (interquartile range) odds ratio, 0.59 (0.53–0.72); $P = 0.001$] in the odds of a patient having a *kelch13* resistance-associated mutation.

We then investigated the association between immunity and $PCt_{1/2}$ by initially performing a meta-analysis for each antigen, plotting mean difference (and 95% CI) in $PCt_{1/2}$ in antibody-positive vs. antibody-negative patients at each study site in order of increasing resistance (and decreasing immunity). The largest magnitudes of effect between antibodies and $PCt_{1/2}$ were observed for sites with the highest prevalence of *kelch13* mutations (Ranong and Srisaket in Thailand, Pursat and Pailin in Cambodia), as well as Ramu in Bangladesh (*SI Appendix*, Fig. S5), which had some of the highest levels of blood-stage immunity and where *kelch13* mutations have not been found (Fig. 2). In unadjusted pooled results, seropositivity was associated with reduced $PCt_{1/2}$, ranging from –0.10 ($MSP1_{19}$) to –0.29 h ($MSP2_{3D7}$) with low to moderate heterogeneity in the association between *P. falciparum* antibody response and $PCt_{1/2}$ between study sites [median I^2 (IQR) of all individual meta-analyses = 17.7% (1.7–32.5)].

Because of the limited heterogeneity, we estimated the magnitude of effect for each antibody response on $PCt_{1/2}$ by performing linear mixed-effects modeling of the pooled individual data, adjusting for the confounders of age and artesunate dose. For all *P. falciparum* antigens, seropositivity was associated with faster $PCt_{1/2}$, with the largest magnitude of effect [mean difference (95% CI) in $PCt_{1/2}$] observed for AMA1 −0.38 (−0.69, −0.08, $P = 0.014$); $MSP\text{-}2_{3D7}$, −0.65 (−1.04, −0.26; $P = 0.001$); $MSP2_{FC27}$, −0.43 (−0.88, 0.01; $P = 0.057$); MSP6, −0.44 (−0.68, −0.20; $P = 0.001$); and $EBA175_{RII}$, −0.33 (−0.58, −0.09; $P = 0.009$; Table 2). We then evaluated whether the observed association between immunity and $PCt_{1/2}$ was modified by the presence of a *kelch13* mutation. Analyses of interactions showed that antibody positivity was associated with a reduced $PCt_{1/2}$, and that for some antigens, the strongest magnitude of effect was observed in patients with slow-clearing *kelch13* mutants compared with those with fast-clearing wild-type parasites (Table 2). For example, AMA1 seronegative patients with wild-type parasites had a mean $PCt_{1/2}$ of 2.85 h, and the effect of AMA1 antibodies on $PCt_{1/2}$ were negligible: mean difference (95% CI) in $PCt_{1/2}$ was −0.07 h (−0.43, 0.28 h; $P = 0.693$). In contrast, AMA1-seronegative patients with *kelch13* mutant parasites had a longer mean $PCt_{1/2}$ of 6.95 h, and the effect of AMA1 antibodies was clinically and statistically significant: mean difference, −0.73 h (95% CI, −1.11, −0.35 h; $P = 0.0001$). Other examples whereby the effect of seropositivity on $PCt_{1/2}$ was >0.2 h in *kelch13* mutants compared with wild-types were Rh2, MSP3, MSP6, and MSP7 (all $P < 0.0136$; Table 2). Analyses were also conducted using antibody levels, rather than seropositivity, with similar results (*SI Appendix*, p. 19).

Discussion

In this multinational study of malarial immunity and emergence of artemisinin resistance to date, we show that naturally acquired immunity to *P. falciparum* varies across populations and is lowest in areas where the prevalence of *kelch13* mutations and slow parasite clearance phenotype are highest. *P. falciparum* antibody titers are associated positively with faster parasite clearance rates in these areas of relatively low immunity, and *P. falciparum* antibodies have the greatest effect on parasite clearance rates in the presence of *kelch13* mutations. These findings suggest that host immunity contributes to the emergence and clearance of drug-resistant parasites and have implications for our understanding of the evolution of drug resistance, the spread of drug resistance, and the WHO operational definitions of artemisinin resistance.

The Greater Mekong Subregion, and in particular Western Cambodia, has been the epicenter for the emergence of drug-resistant malaria, with resistance to previous first-line treatments for malaria (e.g., chloroquine, sulfadoxine–pyrimethamine) emerging in the region. Our data show that the highest prevalence of *kelch13* resistance-associated mutations were found in sites in Western Cambodia and Thailand, regions where transmission (measured by antibodies to CSP) and *P. falciparum* blood-stage antibodies was lowest. In areas with low levels of protective blood-stage immunity, *P. falciparum* infections are more likely to progress to symptomatic disease states, which are subsequently treated,

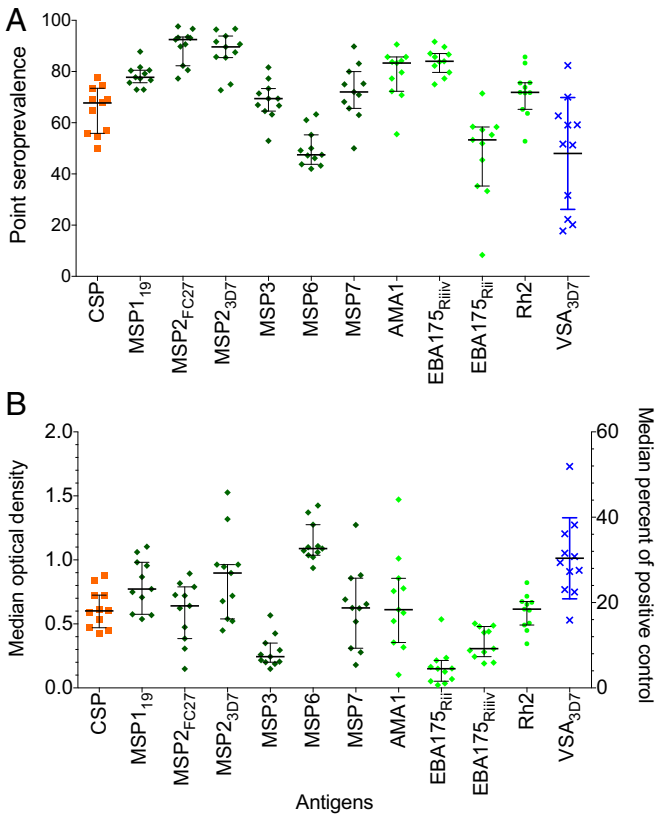

Fig. 1. Seroprevalence and median antibody levels for each *P. falciparum* antigen across all study sites. (*A*) Seroprevalence of and (*B*) median antibody levels to *P. falciparum* antigens representing transmission surrogates (orange), merozoite surface proteins (dark green), merozoite invasion pathway proteins (light green), and infected erythrocyte surface antigens (blue) across all study sites. Seroprevalence and median antibody levels for each site can be found in *SI Appendix*, Figs. S3 and S4. Study sites were ordered from left to right by increasing $PCt_{1/2}$.

exposing parasite populations in these areas to increased drug pressure. Furthermore, low levels of immunity may contribute to the emergence of *kelch13* mutations through mechanisms independent of drug pressure. Initially unfit drug-resistant mutant parasites may be better able to persist in areas of low transmission and low immunity compared with areas of high transmission and high immunity, where there is competition from fitter wild-type parasites and increased recombination breakdown of multigenic resistance mechanisms (29). Studies on the genetic architecture of parasites in the TRAC study suggest lower rates of recombination in Western Cambodia and Eastern Thailand, areas where we observed the lowest levels of anti-sporozoite and anti-blood-stage immunity (30). Low levels of immunity may also facilitate the transmission of resistant parasites from humans to mosquitoes, as low levels of blood-stage immunity increase the probability of gametocyte production (31). Indeed, in the Greater Mekong Subregion, we observed some of the highest prevalences of gametocytemia and *kelch13* mutants in areas with the lowest levels of immunity (e.g., Pursat, Pailin, Mae Sot). These immunological and genetic data from the TRAC study implicate low *P. falciparum* transmission intensity and low immunity in the emergence of mutations that confer artemisinin resistance in the Greater Mekong Subregion and inform our understanding of the evolution and emergence of antimalarial drug resistance in the region.

A delay in parasite clearance after artemisinin treatment is the first sign of emerging resistance before actual treatment failure is observed. In these early stages of emerging resistance, we found that *P. falciparum* blood-stage antibodies were associated with faster $PCt_{1/2}$ after artemisinin treatment, even in low-transmission areas where immunity was lowest. Immunity is therefore an important contributor to variations in $PCt_{1/2}$ between patients. The mean effect of immunity on $PCt_{1/2}$ for associated antigens was around 30 min, with a maximum 95% CI of the true population mean of 1 h, which is striking given the rapid action of artemisinins [median (IQR) $PCt_{1/2}$ of 4,008 profiles, 3.11 (2.33–4.24 h)] (9). The accurate measurement of $PCt_{1/2}$ requires frequent sampling and accurate counting of parasitemia, which is operationally challenging and not available in all settings (9). However, $PCt_{1/2}$ allows for a detailed understanding of artemisinin resistance and prompted the WHO to endorse its use (32). According to the WHO definitions, shifts in the distribution of $PCt_{1/2}$ of 30 min to 1 h may have operational implications in the assessment of artemisinin resistance (32).

First, $PCt_{1/2} \geq 5$ h is a key parameter in validating which *kelch13* mutations (108 nonsynonymous mutations have been identified to date) are associated with resistance, and the effect of immunity on $PCt_{1/2}$ may result in the misclassification of *kelch13* resistance mutations, particularly as different *kelch13* mutations have varying effects on the clearance phenotype (e.g., reported $PCt_{1/2}$ range, 0.6–13.8 h (10). Clear examples of this in the TRAC study are the mutations F446I, N525D, and G538V, with mean $PCt_{1/2}$ of 4.60, 4.70, and 4.65 h, respectively, and classified as mutations that are not associated with resistance but are less than 30 min away from meeting the definition of a resistance-associated mutation (10).

Second, immunity may result in misclassification of artemisinin resistance in patients whose $PCt_{1/2}$ is within 30 min of the $PCt_{1/2}$ cut off of 5 h, which corresponded to 11.5% of patients (4.3% and 18.5% of patients with wild-type and *kelch13* mutant parasites, respectively). The extent of this patient misclassification is concerning, given that these proportions are close to the current WHO

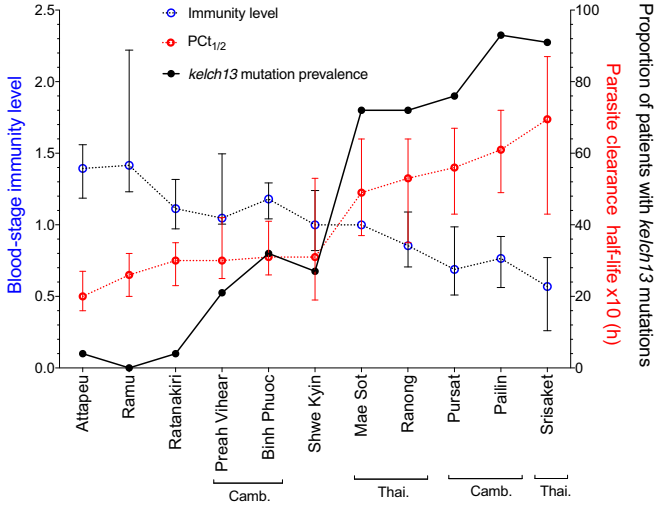

Fig. 2. Relationship between blood-stage immunity level with parasite clearance half-life ($PCt_{1/2}$) and prevalence of *kelch 13* mutations across study sites. For each study site, a composite of immunity to blood-stage antigens (all antigens except CSP) was calculated for each site by obtaining the median optical density (O.D.) values for each antigen within each site, which was then divided by the median of medians for each antigen to standardize the O.D. ranges for different antigens. The blood-stage immunity level obtained and the IQR (left *y* axis, blue circles and whiskers) and the median $PCt_{1/2}$ (right *y* axis, red circles and whiskers) were plotted. The proportion of patients carrying *kelch13* mutations that confer artemisinin resistance is also plotted (black circles and line). Study sites were ordered from left to right by increasing $PCt_{1/2}$. Lines connecting data points are intended only to highlight patterns and not to suggest a continuum between data points. The same pattern was observed for anti-CSP antibodies because anti-blood-stage and anti-CSP antibodies are highly correlated.

Table 2. The association between seroprevalence and $PCt_{1/2}$ (h), according to the presence of *kelch 13* mutants

	All patients (n = 959)		Wild-type (n = 486)		Mutant (n = 302)	
	Mean $PCt_{1/2}$ (h) in sero$^-$	Mean difference if sero$^+$ (h) [95% CI]; P	Mean $PCt_{1/2}$ (h) in sero$^-$	Mean difference if sero$^+$ (h) [95% CI]; P	Mean $PCt_{1/2}$ (h) in sero$^-$	Mean difference if sero$^+$ (h) [95% CI]; P
AMA1	4.67	−0.38 [−0.69,−0.08]; 0.014	2.85	−0.07 [−0.43, 0.28]; 0.693	6.95	−0.73 [−1.11, −0.35]; 0.001
RH2	4.53	−0.22 [−0.50, −0.05]; 0.109	2.84	−0.07 [−0.38, 0.24]; 0.676	6.83	−0.61 [−0.97, −0.24]; 0.001
$EBA175_{RII}$	4.53	−0.33 [−0.58, −0.09]; 0.009	2.86	−0.13 [−0.39, 0.14]; 0.358	6.56	−0.37 [−0.72, −0.02]; 0.039
$EBA175_{RIII\text{-}V}$	4.58	−0.25 [−0.58, 0.09]; 0.147	2.94	−0.16 [−0.54, 0.22]; 0.409	6.75	−0.42 [−0.85, 0.02]; 0.061
$MSP1_{19}$	4.49	−0.16 [−0.45, 0.14]; 0.295	2.87	−0.08 [−0.41, 0.26]; 0.656	6.59	−0.24 [−0.63, 0.15]; 0.233
$MSP2_{3D7}$	4.94	−0.65 [−1.04, −0.26]; 0.001	3.27	−0.52 [−1.03, −0.00]; 0.048	6.92	−0.61 [−1.06, −0.16]; 0.008
$MSP2_{FC27}$	4.76	−0.43 [−0.88, 0.01]; 0.057	3.23	−0.50 [−1.02, 0.09]; 0.104	6.65	−0.27 [−0.79, 0.24]; 0.292
MSP3	4.55	−0.26 [−0.52, −0.01]; 0.045	2.90	−0.14 [−0.42, 0.15]; 0.354	6.70	−0.44 [−0.79, −0.09]; 0.014
MSP6	4.59	−0.44 [−0.68, 0.−20]; 0.001	2.96	−0.30 [−0.56, 0.04]; 0.026	6.64	−0.53 [−0.87, −0.20]; 0.002
MSP7	4.59	−0.30 [−0.58, −0.03]; 0.028	2.93	−0.18 [−0.49, 0.13]; 0.254	6.82	−0.62 [−0.96, −0.27]; 0.001
VSA_{3D7}	4.51	−0.29 [−0.55, −0.03]; 0.032	3.00	−0.34 [−0.61, 0.06]; 0.018	6.41	0.02 [−0.35, 0.39]; 0.899

Linear mixed-effects model, with a random effect for study site and adjustments made for age and artesunate dose, estimate $PCt_{1/2}$ in patients who are seronegative and the mean difference in $PCt_{1/2}$ in patients who are seropositive. To examine whether this association was modified by *kelch13* mutant or wild-type status, an interaction term between *kelch13* and antibody variable was assessed by comparing models with and without the interaction term, using a likelihood ratio test. P values for interaction: AMA1, $P = 0.012$; Rh2, $P = 0.025$; $EB175_{RII}$, $P = 0.272$; $EB175_{RIII\text{-}V}$, $P = 0.385$; $MSP1_{19}$, $P = 0.535$; $MSP2_{3D7}$, $P = 0.789$; $MSP2_{FC27}$, $P = 0.628$; MSP3, $P = 0.186$; MSP6, $P = 0.282$; MSP7, $P = 0.068$; VSA_{3D7}, $P = 0.121$. Models run with continuous $\log_2$ antibody responses can be found in *SI Appendix*, Table S1.

definitions of artemisinin resistance in malaria-endemic populations (confirmed partial resistance, ≥5% of patients with *kelch13* mutants plus $PCt_{1/2} \geq 5$ h; suspected partial resistance, ≥5% with *kelch13* mutants or ≥10% with $PCt_{1/2} \geq 5$ h), and misclassification of the presence of artemisinin resistance at the population level will be in areas of emerging artemisinin resistance, where the distribution of $PCt_{1/2}$ values are on the cusp of the 5-h $PCt_{1/2}$ cutoff included in the population prevalence definition (*SI Appendix*, Fig. S6). In populations with low immunity, a shift toward longer $PCt_{1/2}$ may lead to misclassification of emerging resistance, whereas in populations with high immunity, immune-clearance mechanisms may counteract the propensity for increasing $PCt_{1/2}$ after the emergence of resistant parasites and a conclusion that the population is free from resistance. The potential for missing the emergence of resistance will be greatest where the prevalent *kelch13* mutations confer milder effects on $PCt_{1/2}$. For example, on the Thailand–Myanmar border, the "milder" E252Q mutation predominated as artemisinin resistance emerged, but has now been overtaken by the more "extreme" C580Y mutation. In northern Myanmar, the "milder" F446I now predominates as resistance emerges (33). Potential conclusions that there is no resistance in areas where resistance is beginning to emerge are of significant concern. The confounding effect of immunity in the WHO definitions of artemisinin resistance therefore warrants consideration. For example, in higher-transmission areas, a lowering of the population prevalence of a $PCt_{1/2} \geq 5$ h cutoff from 10% to 5% may be justified. Further studies on the sensitivity and specificity of these definitions in areas of varying immunity, and their operational implications, are required.

Interestingly, we found some evidence that antibodies have little effect on fast-clearing wild-type infections, but a significant effect on $PCt_{1/2}$ in patients with slow-clearing *kelch13* mutant parasites. This may reflect differences in parasite clearance mechanisms between the two strains after treatment with artemisinins. Exposure of wild-type ring-stage parasites to artemisinin derivatives damages and kills the parasite, with the spleen rapidly removing damaged intraerythrocytic ring-stage parasites (a process termed "pitting"), returning the once-infected erythrocytes to circulation (34, 35). Pitting is the main mechanism of parasite clearance after artesunate treatment in nonimmune children in high-transmission areas, whereas in older semi-immune children, immune-mediated parasite clearance mechanisms predominate and parasite clearance by pitting is reduced (36). Therefore, both immune-independent and immune-dependent mechanisms of parasite clearance will result in fast-clearance of wild-type infections regardless of immune status. Conversely, resistant *kelch13* mutant parasites remain phenotypically unchanged after exposure to artemisinin derivatives (37) and will be less susceptible to pitting, with immune-dependent clearance mechanisms playing a greater role in parasite clearance. *Kelch13* mutant parasites that are able to survive after exposure to artemisinins and progress to trophozoites and schizonts (37) can then be cleared through removal of opsonized whole *P. falciparum*-infected erythrocytes or by opsonic phagocytosis of free merozoites (38–40). In addition, antibodies can act to inhibit invasion or act as targets for complement deposition and merozoite lysis, and in this way reduce parasite multiplication rates (41, 42). Immunity therefore has not only more time but a greater range of mechanisms to effectively increase parasite clearance rates of slow-clearing *kelch13* parasites through immune-mediated mechanisms.

A major strength of this study is that it was conducted across multiple populations and transmission settings and included areas in which artemisinin resistance is prevalent and spreading. In addition, we assessed antibody responses against a panel of biomarkers of *P. falciparum* transmission and blood-stage immunity, including both merozoite antigens that are relatively conserved across parasite populations, as well as highly genetically-diverse variant surface antigens. Most antigens were highly immunogenic, with differential recognition according to geographical site and associated with decreases in $PCt_{1/2}$. Although heterogeneity was observed in the association between antibody responses to individual antigens and $PCt_{1/2}$ across study sites, statistical heterogeneity was generally low, validating the generalizability of our findings to other studies and populations. As antibodies determined by ELISA do not produce a common metric measurement, seropositivity data were used in our primary analysis to quantify the effect of immunity on $PCt_{1/2}$ to ensure maximum comparability and translatability to future studies. We have, however, shown that the same conclusions would have been reached if we analyzed antibody levels, rather than seropositivity (SI p26). We have identified a number of relatively conserved merozoite antigens that could be used in future therapeutic efficacy studies of artemisinin resistance. The largest magnitudes of effect were seen with AMA1 and MSP3, vaccine candidates that are established biomarkers of protective immunity across different populations (15), and MSP2, MSP6, and MSP7; interestingly, relatively small effects were observed for $MSP1_{19}$, which is often used, together with AMA1, in serosurveillance studies. Given the variations with respect to individual antigens in populations, future therapeutic efficacy studies should consider using three or more antigens to remove the potential for

spurious associations, and to adjust parasite clearance measures accurately for the confounding effects of immunity and provide the most correct estimates of the prevalence of artemisinin-resistant falciparum malaria in populations.

Our study establishes a role for naturally acquired immunity in the emergence and clearance of artemisinin-resistant parasites. These results inform our understanding of the evolution of drug resistance in the region and have practical implications by providing important parameters for consideration not only in molecular and population definitions of artemisinin resistance but also serological tools to inform artemisinin resistance monitoring and surveillance. The observation that resistance is emerging in areas of low immunity is particularly relevant given that *P. falciparum* transmission is declining in many areas including the Greater Mekong Subregion because of the scale-up of artemisinin resistance containment programs and malaria control programs to achieve national malaria elimination targets (11). As reductions in *P. falciparum* transmission will be accompanied by reductions in immunity, it will be important to understand temporal trends in changing immunity and their effect on the emergence of *kelch13* mutations and the interpretation of $PCt_{1/2}$ values, and consequent assessment of emerging artemisinin resistance. Accurate assessment of the frequency of artemisinin resistance in populations is essential for timely instigation of artemisinin resistance containment and elimination strategies to impede the expansion of artemisinin-resistant parasite populations and to preserve the artemisinin derivatives for the treatment of falciparum malaria.

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