Host immunity and the assessment of emerging artemisinin resistance in malaria: a multinational cohort study

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Abstract

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 independently of antimalarial drugs. We hypothesise 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 *P. 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 ($PCt_{½}$) after artesunate treatment and *kelch13* mutations. Linear mixed-effects modelling of pooled individual patient data assessed the association between antibody responses and $PCt_{½}$. *P. falciparum* antibodies were lowest in areas where the prevalence of *kelch13* mutations and slow $PCt_{½}$ were highest (Spearman ρ = -0·90 (95% CI: -0·97, -0·65) and -0·94 (-0·98, -0·77) respectively). *P. falciparum* antibodies were associated with faster $PCt_{½}$ (mean difference in $PCt_{½}$ according to seropositivity -0·16 to -0·65 hours depending on antigen); antibodies have a greater impact on the clearance of *kelch13* mutant compared to wild-type parasites (mean difference in $PCt_{½}$ according to seropositivity -0·22 to -0·61 hours faster in *kelch13* mutants compared to 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.

Significance Statement

Slow-clearing artemisinin-resistant malaria parasites are now well established in the Greater Mekong region. 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 impact on 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; Immunity has the greatest impact on clearance in patients with slow-clearing mutant parasites. Immunity plays an important role in the emergence of resistant parasites and can confound WHO's phenotype and genotype definitions of artemisinin resistance.

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Introduction

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 due to increased deployment of insecticide treated bed-nets and ACTs (1). Artemisinin resistance, defined by slow parasite clearance following 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 ($PCt_{½}$) 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 Myanmar and Southern Vietnam, is emerging in Northern Cambodia and Southern Laos, but found no evidence for artemisinin resistance in Africa (10).

To facilitate monitoring and surveillance of artemisinin resistance, WHO now defines 'confirmed partial' artemisinin resistance as ≥5% of *P. falciparum* patients carrying *kelch13* mutations associated with either persistent parasitaemia on day 3, or a $PCt_{½}$ ≥5 hours after artemisinin treatment (11). 'Suspected' artemisinin resistance is defined as ≥5% of *P. falciparum* patients carrying *kelch13* resistance-associated mutations, or

≥10% of patients with parasitaemia at day 3, or ≥10% of patients with a $PCt_{½}$ ≥5 hours after treatment (11). While detection of molecular markers is unequivocal, there is substantial inter-individual 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 $PCt_{½}$ ≥5 hours cut-off 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 to 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, sulphadoxine-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 prior to 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 ($PCt_{½}$) and found an unquantified inverse correlation (25). However, single-site studies in areas where resistance has yet to emerge fail to encapsulate between- and within-population variations in malarial immunity, and frequencies of *kelch13* mutations. We hypothesise 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 first 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 quantify their impact on $PCt_{½}$, 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 (out of 985) patients with high parasitaemia from 11 Southeast Asian sites (See SI Appendix, fig. S1, text S1) participating in the TRAC multicentre drug-efficacy randomized control trial. Informed consent was obtained from all patients and ethical approval granted by the Oxford Tropical Research Ethics Committee (OxTREC 06/11); Alfred Hospital Committee for Ethics, Australia (485/12)) (10). Participants received either 2 or 4 mg/kg artesunate for 3 days followed by a full course of an ACT. The initial study aimed to enrol 120 patients at each study site and this was achieved in 6 of the 11 Southeast Asian sites. Patients aged 0·5-65 years with uncomplicated falciparum malaria (parasitaemia between 10,000-200,000/μL), and fever/ history of fever were included. Blood smears were taken for malaria parasite counts at 0, 4, 6, 8, 12 hours and then every 6 hours until two consecutive counts were negative.

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

Statistical analysis. Definitions and methods are detailed in the SI Appendix text S1. Briefly, the primary end-point, $PCt_{½}$ (hours) was derived from the parasite clearance estimator (9). $PCt_{½}$ is a standardised measure of parasite clearance after antimalarial treatment and is the time needed for parasitaemia to be reduced by half during the log-linear phase of parasite clearance. It is independent of initial parasitaemia and excludes

potential confounding of changes in parasite density after anti-malarial drug administration (9). Meta-analysis of the differences in mean $PCt_{½}$ according to sero-positivity was performed to provide estimates for each study site and overall and heterogeneity was evaluated by the I^2 value. Linear mixed-effects modelling 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 $PCt_{½}$. Interactions between *kelch13* (defined as any mutation above amino acid position 440 with a median $PCt_{½}$ > 5 hours and present in at least 5 individuals) and antibody response were assessed using a likelihood ratio test.

Results

The study included 959 falciparum malaria patients in 11 sites in Southeast Asia (Table 1). *P. falciparum* densities showed no geographical patterns (SI Appendix Fig, S2), while median $PCt_{½}$ values of ≥5 hours 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 gametocytaemia was also highest in Western Cambodia (Pailin and Pursat) (table 1, SI Appendix Fig S2). We measured levels of antibodies specific for 12 different *P. falciparum* antigens, including a sporozoite antigen (CSP, a transmission biomarker), relatively conserved merozoite invasion ligands (AMA1, Rh2, $EBA175_{RII}$, $EBA175_{RIII\text{-}V}$), merozoite surface proteins ($MSP1_{19}$, $MSP2_{fc27}$, $MSP2_{3D7}$, MSP3, MSP6, MSP7), and infected-erythrocyte variant surface antigens (VSA_{3D7}), all are biomarkers of blood-stage immunity (14, 15) (Figure 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 (Figure 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 Fig S2 & S3).

To assess whether between-population variations in immunity are associated with the emergence of resistance, we plotted $PCt_{½}$ 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 (Figure 2). We observed a strong inverse correlation between *P. falciparum* blood-stage antibody responses and $PCt_{½}$ values (Spearman ρ= -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 (-0·90 (-0·65, -0·97); P<0·0001 and 0·77 (-0·32, -0.96); P=0·0054 respectively). Additionally, for each doubling of antibody levels towards the individual antigens there was around a 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 $PCt_{½}$, by initially performing a meta-analysis for each antigen, plotting mean difference (and 95% CI) in $PCt_{½}$ in antibody-positive versus antibody-negative patients at each study site in order

of increasing resistance (and decreasing immunity). The largest magnitudes of effect between antibodies and $PCt_{½}$ 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 (Figure 2). In unadjusted pooled results, seropositivity was associated with reduced $PCt_{½}$, ranging from -0·10 (MSP1-19) to -0·29 hours (MSP2-3D7) with low-to-moderate heterogeneity in the association between *P. falciparum* antibody response and $PCt_{½}$ between study sites (median I^2 [IQR] of all individual meta-analyses = 17·7% [1·7-32·5]).

Due to the limited heterogeneity, we estimated the magnitude of effect for each antibody response on $PCt_{½}$ by performing linear mixed-effects modelling of the pooled individual data, adjusting for confounders age and artesunate dose. For all *P. falciparum* antigens, seropositivity was associated with faster $PCt_{½}$, with the largest magnitude of effect [mean difference (95%CI) in $PCt_{½}$] observed for AMA1 -0·38 (-0·69, -0·08), P = 0·014; MSP-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 $PCt_{½}$ was modified by the presence of a *kelch13* mutation. Analyses of interactions showed that antibody positivity was associated with a reduced $PCt_{½}$ and that for some antigens the strongest magnitude of effect was observed in patients with slow-clearing *kelch13* mutants, compared to those with fast-clearing wild-type parasites (Table 2). For example, AMA1 seronegative patients with wild-type parasites had a mean $PCt_{½}$ of 2·85 hours and the impact of AMA1 antibodies on $PCt_{½}$ were negligible: mean difference (95%CI) in $PCt_{½}$ -0·07 hours (-0·43, 0·28), P = 0·693. In contrast, AMA1 seronegative

patients with *kelch13* mutant parasites had a longer mean $PCt_{½}$ of 6·95 hours and the impact of AMA1 antibodies was clinically and statistically significant: -0.73 hours (-1·11, -0·35), P = 0·0001. Other examples whereby the effect of seropositivity on $PCt_{½}$ was >0·2 hours in *kelch13* mutants compared to 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 p26).

Discussion

In this the first 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 titres are associated positively with faster parasite clearance rates in these areas of relatively low immunity and *P. falciparum* antibodies have the greatest impact 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 on 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, in particular Western Cambodia, has been the epicentre for the emergence of drug resistant malaria, with resistance to previous first-line treatments for malaria (e.g. chloroquine, sulphadoxine-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

were lowest. In areas with low levels of protective blood-stage immunity *P. falciparum* infections are more likely to progress to symptomatic disease, which are subsequently treated, 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 to 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 TRAC 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 novel immunological and genetic data from TRAC 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 $PCt_{½}$ following artemisinin treatment, even in low transmission areas where

291 immunity was lowest. Immunity is therefore an important contributor to variations in
292 $PCt_{½}$ between patients. The mean effect of immunity on $PCt_{½}$ for associated antigens
293 was around 30 minutes, with a maximum 95%CI of the true population mean of one
294 hour, which is striking given the rapid action of artemisinins (median [IQR] $PCt_{½}$ of
295 4,008 profiles, 3.11 [2.33-4.24 hours)(9). The accurate measurement of $PCt_{½}$ requires
296 frequent sampling and accurate counting of parasitaemia, which is operationally
297 challenging and not available in all settings (9). However, $PCt_{½}$ allows for a detailed
298 understanding of artemisinin-resistance and prompted the WHO to endorse its use (32).
299 According to the WHO definitions, shifts in the distribution of $PCt_{½}$ of 30 minutes to one
300 hour may have operational implications in the assessment of artemisinin resistance (32).
301 Firstly, $PCt_{½}$≥5 hours is a key parameter in validating which *kelch13* mutations (108
302 non-synonymous mutations have been identified) are associated with resistance, and
303 the impact of immunity on $PCt_{½}$ may result in the misclassification of *kelch13* resistance-
304 mutations, particularly as different *kelch13* mutations have varying effects on the
305 clearance phenotype (e.g. reported $PCt_{½}$ range 0.6 to 13.8 hours; (10). Clear examples of
306 this in the TRAC study are the mutations F446I, N525D and G538V, with mean $PCt_{½}$ of
307 4.60, 4.70 and 4.65 hours respectively, and classified as mutations not associated with
308 resistance but that are less than 30 minutes away from the definition of a resistance-
309 associated mutation (10). Secondly, immunity may result in misclassification of
310 artemisinin resistance in patients whose $PCt_{½}$ is within 30 minutes of the $PCt_{½}$ cut off of
311 5 hours; which corresponded to 11.5% of patients (4.3% and 18.5% of patients with
312 wild-type and *kelch13* mutant parasites respectively). The extent of this patient
313 misclassification is concerning given that these proportions are close to the current
314 WHO definitions of artemisinin resistance in malaria-endemic populations (confirmed
315 partial resistance, ≥5% of patients with *kelch13* plus $PCt_{½}$≥5 hours; suspected partial

316 resistance, ≥5% with *kelch13* mutant or ≥10% with $PCt_{½}$≥5 hours) and misclassification
317 of the presence of artemisinin resistance at the population level will be in areas of
318 emerging artemisinin resistance where the distribution of $PCt_{½}$ values are on the cusp of
319 the 5-hour $PCt_{½}$ cut-off included in the population prevalence definition (SI Appendix,
320 Fig. S6). In populations with low immunity, a shift towards longer $PCt_{½}$ may lead to
321 misclassification of emerging resistance, whereas in populations with high immunity,
322 immune-clearance mechanisms may counteract the propensity for increasing $PCt_{½}$ after
323 the emergence of resistant parasites and a conclusion of that the population is free from
324 resistance. The potential for missing the emergence of resistance will be greatest where
325 the prevalent *kelch13* genotypes confer milder effects on $PCt_{½}$. For example, on the
326 Thailand-Myanmar border the "milder" E252Q mutation predominated as artemisinin
327 resistance emerged, but has now been overtaken by the more "extreme" C580Y
328 mutation. In northern Myanmar the "milder" F446I now predominates as resistance
329 emerges (33). Potential conclusions that there is no resistance in areas where resistance
330 is beginning to emerge are of significant concern. The confounding effect of immunity in
331 the WHO definitions of artemisinin resistance therefore warrants consideration. For
332 example, in higher transmission areas, a lowering of the population prevalence of a
333 $PCt_{½}$≥5 hours cut-off from 10% to 5% may be justified. Further studies on the sensitivity
334 and specificity of these definitions in areas of varying immunity, and their operational
335 implications, are required.
336
337 Interestingly, we found some evidence that antibodies have little impact on fast-clearing
338 wild-type infections, but a significant impact on $PCt_{½}$ in patients with slow-clearing
339 *kelch13* mutant parasites. This may reflect differences in parasite clearance mechanisms
340 between the two strains following treatment with artemisinins. Exposure of wild-type

ring-stage parasites to artemisinin derivatives damages and kills the parasite, with the spleen rapidly removing damaged intra-erythrocytic ring-stage parasites (a process termed pitting), returning the once-infected erythrocytes to circulation (34, 35). Pitting is the main mechanism of parasite clearance following artesunate treatment in non-immune 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). Additionally, 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 decrease parasite clearance rates of slow-clearing *kelch13* parasites through immune-mediated mechanisms.

A major strength of the study is that it was conducted across multiple populations and transmission settings, and included areas where artemisinin resistance is prevalent and spreading. Additionally, 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

366 genetically diverse variant surface antigens. Most antigens were highly immunogenic,
367 with differential recognition according to geographical site, and associated with
368 decreases in $PCt_{½}$. While heterogeneity was observed in the association between
369 antibody responses to individual antigens and $PCt_{½}$ across study sites, statistical
370 heterogeneity was generally low, validating the generalizability of our findings to other
371 studies and populations. As antibodies determined by ELISA do not produce a common
372 metric measurement, seropositivity data were used in our primary analysis to quantify
373 the impact of immunity on $PCt_{½}$, to ensure maximum comparability and translatability
374 to future studies. We have however shown that the same conclusions would have been
375 reached if we looked at antibody levels rather than seropositivity (SI p26). We have
376 identified a number of relatively conserved merozoite antigens that could be used in
377 future therapeutic efficacy studies of artemisinin resistance. The largest magnitudes of
378 effect were seen with AMA1 and MSP3, vaccine candidates which are established
379 biomarkers of protective immunity across different populations (15), and MSP2, MSP6
380 and MSP7, but interestingly relatively small effects were observed for $MSP1_{19}$ that is
381 often used, together with AMA1, in serosurveillance studies. Given the variations with
382 respect to individual antigens in populations, future therapeutic efficacy studies should
383 consider utilizing three or more antigens to remove the potential for spurious
384 associations in order to adjust parasite clearance measures accurately for the
385 confounding effects of immunity and provide the most correct estimates of the
386 prevalence of artemisinin resistant falciparum malaria in populations.
387
388 Our study establishes a role for naturally acquired immunity in the emergence and
389 clearance of artemisinin-resistant parasites. These results inform our understanding of
390 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 due to 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 impact on the emergence of *kelch13* mutations and the interpretation of $PCt_{½}$ 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 reduce the expansion of artemisinin resistant parasite populations and to preserve the artemisinin derivatives for the treatment of falciparum malaria.

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Figure Legends

Figure 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 & S4. Study sites were ordered from left to right by increasing $PCt_{½}$.

Figure 2 – Relationship between blood-stage immunity level with parasite clearance half-life ($PCt_{½}$) and prevalence of *kelch 13* mutations across study sites. For each study site, a composite of immunity to blood-stage antigens (all antigens excepting CSP) was calculated for each site by obtaining the median O.D. values for each antigen within each site which was then divided by the median of medians for each antigen in order to standardize the O.D. ranges for different antigens. The blood-stage immunity level obtained and IQR (left *y*-axis, blue circles and whiskers) and the median $PCt_{½}$ (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 $PCt_{½}$. Lines connecting data points are intended only to highlight patterns and not to suggest a continuum between data. The same pattern was observed for anti-CSP antibodies because anti-blood-stage and anti-CSP antibodies are highly correlated.