***Title*:** How robust are malaria parasite clearance rates as indicators of drug effectiveness and resistance?

***Running title*:** Malaria parasite clearance rates.

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Abstract

Artemisinin combination therapies (ACTs) are currently the first line drugs for treating uncomplicated falciparum malaria, the most deadly of the human malarias. Malaria parasite clearance rates estimated from patients' blood following ACT treatment have been widely adopted as a measure of drug effectiveness and as surveillance tools for detecting the presence of potential artemisinin drug resistance. This metric has not been investigated in detail, nor have its properties or potential shortcomings been identified. Herein, the pharmacology of drug treatment, parasite biology, and human immunity are combined to investigate the dynamics of parasite clearance following ACT treatment. This approach parsimoniously recovers the principal clinical features and dynamics of clearance. Human immunity is the primary determinant of clearance rates unless, or until, artemisinin killing has fallen to near-ineffective levels. Clearance rates are therefore highly insensitive metrics for surveillance that may lead to over-confidence as even quite substantial reductions in drug sensitivity may not be detected as slower clearance rates. Equally serious is the use of clearance rates to quantify the impact of ACT regime changes as this strategy will plausibly miss even very substantial increases in drug effectiveness. In particular, the malaria community may be missing the opportunity to dramatically increase ACT effectiveness through changes in regimen, particularly through a switch to twice-daily regimens and/or increases in artemisinin dosing levels. The malaria community therefore appears over reliant on a single metric of drug effectiveness, parasite clearance rate that has significant and serious shortcomings.

Introduction

The timely provision of effective antimalarial drugs is a public health priority in most of the developing world (1). The current generation of antimalarial drugs centre on artemisinin-based combination therapies (ACTs) and recent reports that tolerance and/or resistance is evolving to artemisinins (e.g. (2-7)) have caused considerable concerns (e.g. (8-12)). ACTs currently remain largely effective in clearing malaria infections, but reduced parasite clearance rates (i.e. the rate at which parasitaemia declines after treatment (13)) have been widely interpreted as indicating the presence of reduced parasite drug sensitivity to the artemisinin component, and hence indicative of the early stages of resistance (*op cit*). Parasite clearance rates have also been used to evaluate the likely clinical impact of alterations in artemisinin or ACT dosing regimens (e.g. (14)) that may be able to increase ACT effectiveness and hence reduce the threat of resistance. It would therefore seem reasonable to expect that parasite clearance rates are a well validated, demonstrably robust measure of drug effectiveness and resistance. Unfortunately, this appears not to be the case, as reflected in concerns raised in recent commentaries (15-17). Herein, the pharmacology of drug action, parasite biology and human immunity are combined to investigate the dynamics of parasite clearance following ACT treatment. This reveals the basic properties of the metric and allows critical review of the use of parasite clearance rates as an indicator of drug effectiveness and resistance.

The parasite clearance phenotype is as follows. The microscopically observed number of infected red blood cells (iRBCs) following ACT treatment fluctuates for a brief period of around 6 to 20 hours post-treatment (18-20). These initial fluctuations are usually explained by the imbalance between the introduction of new merozoites into the circulation from

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sequestered schizonts and the depletion of circulating iRBCs through sequestration. This is then followed by a sustained, linear decline in log iRBC numbers over the next 40 to 60 hours (18-20) . The slope of this linear decline is the "parasite clearance rate", and there are well-established protocols for its measurement (e.g. (20, 21)). One critical point to note is that artemisinins have a very short half-life of around 40 minutes in humans (22) and are present as short pulses of active concentrations for only 4 to 6 hours post treatment (23). This means the initial artemisinin pulse has effectively been eliminated from the circulating blood by the time the linear clearance dynamics (which define parasite clearance rates) occur. The practical consequence is that iRBC clearance rates measured more than ~6 hours post-treatment cannot be a *direct* measure of artemisinin effectiveness in its first pulse (because the artemisinin is no longer present), but must be an indirect *proxy* measure. Importantly, the subsequent short pulses of artemisinin treatment in an ACT regimen, typically at 24 and 48 hours after first treatment, do not usually show as increased clearance rates at these points, again emphasising the indirect nature of iRBC clearance as measure of current drug killing.

The observed reductions in parasitaemia following drug treatment are invariably referred to as "parasite" clearance rates. In reality, clinical observations consist of counts of the number (actually the density) of iRBC which may contain live, dead or dying malaria parasites (24). This was noted by Kremsner and Krishna (25) who discussed clearance times after drug treatment and concluded that "a circulating parasite might be alive, injured (fatally), or dead in these circumstances". Similarly, Watkins, Woodrow and Marsh (26) state that "the stained blood film, although it can be accurate and reproducible, provides only a total parasite count from which viable and nonviable counts cannot be differentiated". The difference between "parasite clearance" and "iRBC clearance" is not merely semantic: the fact that iRBCs must be cleared (i.e. removed from the blood circulation by the spleen or by other host

mechanisms), rather than the parasite being cleared directly, is important. An iRBC presents a complex target to the human immune system implying that immunity will play a large role in the dynamics of clearance. The impact of host immunity can therefore reduce both the sensitivity and specificity of iRBC clearance as a diagnostic of drug effectiveness. The term "iRBC clearance rate" will be used in this manuscript in place of the more usual "parasite clearance rate" to emphasise what is actually being observed and measured. Readers will be aware that the terms are synonymous, but the former is more technically correct. The principle research question addressed herein is to consider the likely relative contributions of drug effectiveness and host defence mechanisms to the iRBC clearance rates observed in patients after ACT treatment, and to evaluate the use and application of iRBC clearance rates as research and surveillance tools.

Methods

Failure rates to ACTs are currently very low (1) so it is not statistically feasible to compare *in vivo* iRBC clearance rates in patients where drug treatment was successful versus patients where treatment was unsuccessful (for example, Ashley *et al.* (7) reported a cure rate of 98% in their study of 1,241 patients in South East Asia). Even if such a comparison were possible, immunity is likely to affect both therapeutic outcome and iRBC clearance rates, causing a correlation that could be mistaken for causality (as explained later). These circumstances dictate that pharmacological modelling be used to simulate ACT treatment and to investigate the properties of iRBC clearance rates when used as an indicator of drug effectiveness.

Pharmacological model

A pharmacological model of artemisinin drug treatment incorporating drug stage specificity was constructed based upon the standard model first implemented by Hoshen *et al.* (27), and used by several subsequent authors (e.g. (23, 28)). Its construction and calibration is detailed in the Supplemental Material, part 1. Briefly, the parasite population is split into 48 developmental 'age-bins' corresponding to each hour of the *Plasmodium falciparum* 48-hour life cycle. At each hour post-treatment, the drug kills some parasites in each age-bin (if the drug is present and active against that developmental age-bin) and surviving parasites are then moved into the next development stage. Parasites in the 48th age-bin rupture to release new parasites (default of 10 per schizont) and the latter are moved into the 1st age-bin. This enables the number of parasites in each age-bin to be tracked each hour post-treatment.

Drug kill rate units are per hour and are obtained from the more familiar PRR_{48} for the methodological reasons explained is the SI around Equations S1.1. As a reference to interpret these kill rates on an hourly scale, kill rates of 0.19, 0.14 and 0.096, are equivalent to PRR_{48} of 10^{-4} (because $e^{-48x0.19}=10^{-4}$), 10^{-3} and 10^{-2} respectively assuming all parasite stages are equally sensitive. In fact, not all stages are equally sensitive which is why the kill rates for the sensitive stages have to be increased to compensate for the lack of killing in the non-sensitive stages to maintain the same PRR_{48} values (see SI for details).

Previous work (e.g. (27, 28)) typically did not track the fate of parasites once dead within the iRBCs as the studies focussed on drug effectiveness and the clearance dynamics of dead parasites were of no interest. A simple extension was added to this basic methodology: rather than assuming killed parasites are instantaneously removed from the circulation, those killed while inside circulating iRBCs are moved into a "dead-but-circulating" population of iRBCs, which is cleared by the host at a clearance rate determined by host factors. Parasites killed

while in sequestered iRBCs are assumed to die *in situ* and do not re-enter the circulation (see discussion in Supplemental Material, part 1). The same strategy has been used previously by other authors. Hietala and colleagues (29), following Gordi *et al.* (30), fitted a 'spleen clearance' compartment to their pharmacokinetic/pharmacodynamic (PK/PD) analyses of patients treated with the ACT artemether-lumefantrine. They found that inclusion of a spleen clearance rate of 0.26 per hour (equivalent to a half-life of 2.7 hours) as reported in Gordi *et al.* (30) provided a better fit to the data. The term "spleen clearance rate" will be used here to quantify the rate at which iRBC containing dead or dying parasites are removed from the circulation by host defences. It is synonymous with the "spleen and macrophage clearance rate" used previously by Hietala, Gordi and colleagues (29, 30). The use of "spleen clearance rate" is for clarity and to avoid any ambiguity with iRBC clearance but readers will realise that iRBC clearance is a complex drug-dependent process that also depends on immunity, the spleen and possibly other host factors (and, in fact, patients without spleens can still clear their infection) and that "spleen clearance rate" is simply a convenient term covering all these factors; a more detailed discussion of host defences and access to the primary literature can be found elsewhere (e.g.(18)). We also assume that all circulating iRBCs are counted to obtained clearance as guidelines for microscopy in research settings do not distinguish between live and dead parasites (31). There are variants in this procedure. Parasites may be scored as dead/alive based on their morphology (although this is particularly difficult *in vivo* where circulating parasites are predominantly early ring stages) and clearance rates would subsequently be based on the reduction of 'live' parasites (20). Alternatively, direct counts may be replaced by molecular surrogates such as quantitative PCR and clearance quantified as the reduction in qPCR signal (e.g. (32)).

Sensitivity analysis of iRBC clearance rates and drug effectiveness

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169 The parameterisation of the methodology is described in Supplemental Material, part 1.
170 Individual parameter values were varied systematically within calibrations to isolate the
171 effect of changing single parameters (see later discussion of Figure 1). A sensitivity analysis
172 was then performed by simulating 5,000 patients treated with either dihydroartemisinin-
173 piperaquine (DHA-PPQ) or artesunate-mefloquine (AS-MQ). Each patient had an initial
174 parasite number of 10^{12} which may be uniformly distributed across all age bins, or may
175 predominantly be in early ring stages. Note that the initial parasite number has no effect on
176 the subsequent shape of dynamics in the model output except to alter the time until
177 circulating parasites become undetectable. The following four factors were varied during the
178 sensitivity analysis: the artemisinin kill rate, the duration of artemisinin killing after treatment
179 (specified as an integer, i.e. number of hours), the partner drug kill rate and the spleen
180 clearance rate (see Supplemental Material, text surrounding Equation S1.2 for technical
181 details of the drug killing). The correlation was measured between these four factors and drug
182 effectiveness and iRBC clearance rates. Drug effectiveness was quantified using the
183 conventional metric of parasite reduction ratio (PRR_{48}) which is the ratio of the number of
184 parasites present at start of treatment, divided by the number remaining 48 hours later. More
185 effective treatments will kill more parasites and, consequently, will result in a higher PRR_{48}.

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187 Impact of dosing regimen and increasing parasite cell cycle time on iRBC clearance rates

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189 Concerns overs about future effectiveness of ACTs, and the lack of readily available
190 alternatives, have driven attempts to increase clinical effectiveness through changes in their
191 deployment regimens. One such strategy is to increase the dosage given. This is predicted to
192 result in increased duration of drug killing after treatment ((33) and later discussion of Figure

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1). Another strategy is to split the dosage regime. The specific example of splitting the standard three day regimen of DHA-PPQ into twice-daily dosing was then investigated (details in Supplemental Material, part 1). Theory, and intuition, suggest the main impact will be on artemisinin, rather than partner drug, killing (33, 34) so simulations were run with and without PPQ killing, the latter to remove the complicating factors of PPQ drug action.

There has been speculation (e.g. (35)) that mutations in the *P. falciparum* kelch propeller domain (K13) may be associated with increased cell cycle duration. The K13 mutations appear to have reduced drug sensitivity during the hypersensitive early ring stages (36). The likely impact of a simple increase in cell cycle time on iRBC clearance rates was investigated by increasing cell cycle time from 48 hours to 57.6 hours (a 20% increase) or to 72 hours (a 50% increase). The impact of simultaneously increasing cell cycle time and reducing drug sensitivity was investigated by modifying the hypersensitive profile (see Supplemental Material, part 1) so that malaria parasites became insensitive to artemisinin during their hyper-sensitive early ring age-bins.

Results

The model recovered the main features of iRBC clearance dynamics that occur after artemisinin treatment, i.e. the characteristic linear decline in circulating iRBC number following artemisinin treatment was routinely observed. Moreover, this linearity was not affected by additional killing periods that occur in subsequent doses of artemisinins (e.g. Figure 2).

The results from the sensitivity analysis of PRR_{48} and iRBC clearance rates are shown on Table 1. The correlation is high between artemisinin kill rate, partner drug kill rate, duration of artemisinin killing and overall drug effectiveness measured as PRR_{48}. However, negligible correlations were observed between these factors and iRBC clearance rates (recall that PRR_{48} is the change in the total number of living parasites, both circulating and sequestered, whereas iRBC clearance is change in number of circulating iRBC that may contain either living or dead parasites). The main correlation for iRBC clearance rates is with spleen clearance rate, indicating that the latter is the dominant force determining iRBC clearance rates and almost entirely obscures any impact of artemisinin kill rate, partner drug kill rate, duration of artemisinin killing or PRR_{48} on iRBC clearance rates. Drug effectiveness, measured as PRR_{48}, is essentially invisible: the largest correlation between PRR_{48} and iRBC clearance is 0.04 in the simulated datasets whereas the correlation between spleen clearance rate and iRBC clearance is >0.93 in all simulations and generally very close to 1. These are correlation coefficients and squaring their values quantifies the proportion of the variability in iRBC clearance rates that may be explained by the differing factors. Drug effectiveness parameters therefore explains a maximum of $0.16^2 = 2.5\%$ of the variation in the iRBC clearance rates (Table 1), while spleen clearance rates explain between $0.93^2 = 86\%$ and $1^2 = 100\%$ of the variability.

Mutations that affect intrinsic drug susceptibility of malaria parasites were found to act in two main ways (we later discuss the possible impact of changes in cell cycle duration). Firstly, such changes may alter the duration of artemisinin killing after treatment (33), although this will have little impact on iRBC clearance rates unless the duration of killing falls to less that around 2 hours (Figure 1, Panel A), despite the large impact of reduced duration of killing on drug effectiveness (Figure 1, Panel B). This clearly shows that iRBC counts by microscopy are highly insensitive to changes in artemisinin drug effectiveness and can only detect changes once parasite susceptibility to artemisinin

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has fallen to very low levels. Even a reduction in the duration of killing by 83% from 6 hours to 1 hour post-treatment was predicted to only reduce iRBC clearance rates by around 10% (i.e. from around 0.22 to 0.20) despite drug killing (PRR_{48}) falling by factors of up to 10^{10}.

The second way in which the impact of mutation(s) on intrinsic parasite susceptibility to artemisinin may be manifested is in reductions in kill rates. In the model, reduced artemisinin kill rates were found to exhibit little impact on iRBC clearance rate until they reached very low levels. Mutation(s) that reduce artemisinin kill rates below around 20% of wild-type levels may become detectable as reduced iRBC clearance rates although, as might be expected intuitively, the magnitude of this reduced iRBC clearance depends on the stage distribution of parasites at time of treatment (Figure 1 Panel C). Once again, this low sensitivity occurs despite the huge impact that changing artemisinin kill rate has on drug effectiveness (Figure 1, Panel D).

One common method of increasing drug effectiveness in the face of resistance is to increase the amount of drug given to patients (within the limits of toxicity). Pharmacologically, this increases the duration of artemisinin killing after treatment and its predicted impact has already been shown on Figure 1: dose increases which extend the duration of killing for more than around 2 or 3 hours post treatment are unlikely to be detected using iRBC clearance rates (Figure 1, Panel A), despite the huge changes in drug effectiveness that arises from such dose increases (Figure 1, Panel B). This suggests that iRBC clearance rates have low sensitivity for monitoring the impact of drug regimen change based on dose-escalation.

An alternative method to increase drug efficacy, that does not involve increasing the total dose, is to change the dosing regimen. The consequences of splitting the dose of DHA-PPQ into a twice daily dosing regimen are shown in detail on Figure 2. As predicted (34), the drug effectiveness varied substantially (by a factor of 10^8), the PRR_{48} being 1.7×10^4, 9.8×10^7,

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1.8×10^8 and 1.0×10^{12} for Panels A, B, C, and D respectively in Figure 2. Despite these differences in ACT effectiveness, the clearance rates were identical in each panel of Figure 2, suggesting clearance rates are unable to detect even huge changes in drug effectiveness. The impact of the additional doses of artemisinin are quite clear on total parasitaemia (Panel B *versus* Panel A and Panel D *versus* Panel C) but the effects of spleen clearance rates and the constant background killing of PPQ obscure these differences to the extent that observed iRBC clearance rates (blue lines) are not sufficiently sensitive to detect even the substantial impact on total drug killing that occurs as the regimen is split and given twice daily. In this case, the slope of the observed iRBC clearance (blue line) measured on its linear portion between 18 and 48 hours was 0.26 per hour in all cases despite the large differences in artemisinin killing rates (black lines).

The impact of extending the parasites cell cycle time from 48 hours to 57.6 hours (a 20% increase) or to 72 hours (a 50% increases) are shown on Table S1 in Supplemental Material. Changes in iRBC clearance rates are small and occur only when spleen clearance rates are relatively fast, i.e. with half-lives in the region of 2 hours. Moreover the impact is unpredictable, sometimes slowing clearance rates and sometimes increasing them. The largest alterations was of the latter, i.e. clearance rates increasing from 0.34 per hour to 0.43 per hour when cycle time was extended from 48 to 72 hours (Table S1 in Supplemental Material, i.e. the example of DHA-PPQ with an isosensitive profile used to treat an early ring stage infection in a patent whose endogenous clearance rate was 0.35 per hour). It therefore seems unlikely that small to moderate increases in cell cycle time could explain the increasing clearance rates currently being observed in South East Asia. Note that this is only a small pilot exploration designed to reveal whether extending cell cycle time has a consistently large impact. It was assumed that the increase in cell cycle length affected all age-bins equally,

while a more nuanced analysis would investigate more complex patterns where the increase in cell cycle length was due to changes in time spent in specific age bins (such as early rings) (35).

Discussion

The results presented above have such wide ranging implications that the Discussion will be split into four distinct sections to maintain focus and to enable readers to navigate through the separate strands of discussion.

Consistency with previous results

It is widely recognised that immunity affects iRBC clearance rate, high immunity being associated with faster clearance. A review by White (18) specifically noted that "As immunity increases [...] parasite clearance is accelerated so the slopes of parasite clearance curves become steeper". Commentators are also aware of this effect. Uhlemann and Fidock (9), for example, stated that "The shift in parasite clearance rates with time could have various causes, including waning immunity as interventions reduced exposure of patients to parasites". It has long been known that increasing failure rates to other drugs can be due to decreased immunity rather than increased resistance. For example, Greenhouse and colleagues (37) concluded that increasing drug failure rates in their longitudinal study was due to decreasing levels of immunity rather than changes in parasites drug resistance levels. Similarly, Lopera-Mesa *et al.* (38) reported that clearance rates in their study sites most likely reflected differences in patients' immune status. The results presented above show that

318 immunity, which clearly contributes to spleen clearance rates, is most likely the dominant
319 factor determining iRBC clearance rates.
320
321 Clearance rates have been used to quantify drug effectiveness and in surveillance
322 programmes designed to detect drug resistance (e.g. (2-4, 6, 7, 39, 40)) but see (15-17) for
323 critical appraisals of these usages. The theoretical underpinning of their use in this context
324 follows this simple logic. The presence of detectable parasites in a patient three days after
325 treatment is known to be a risk factor for drug failure (e.g. (41)). The iRBC clearance rates
326 partially determine whether or not parasites are detectable at Day 3 (initial parasitaemia also
327 plays a role). Consequently, slower parasite clearance rates must be associated with increased
328 risk of Day 3 positivity and therefore be associated with increased failure rates. This logic
329 appears robust but note the last step, i.e. that slower clearance rates are *associated* with
330 increased failures not necessarily that they *cause* failures.. It is a basic tenet of data analysis
331 that association does not imply causation. It is highly plausible that this association arises
332 from a common factor, human immunity (42), which affects both iRBC clearance rate and
333 eventual probability of treatment failure, and that interpreting this association as causation is
334 logically unsound. Another complicating factor is that malaria infections, especially in high
335 transmission areas, are genetically heterogeneous and clearance rate of the majority of iRBC
336 may not reflect the ultimate fate of the infection (treatment success/failure) as the latter may
337 depend on the presence/absence of low-density resistance genotypes present as minority
338 clones in the infections (e.g.(43)).
339
340 Our simulations allow a detailed consideration of the dynamics of iRBC clearance. This
341 suggests the underlying reason why host immunity is the main determinant of iRBC
342 clearance rates. Artemisinins are present at active concentrations for around 4 to 6 hours post-

343 treatment. The proportion of circulating parasites killed by artemisinin during this period will
344 be called the initial kill burst (IKB). Clearance measures are typically delayed for 6 to 20
345 hours after treatment to allow the log-linear decline in iRBC to become established and
346 measurable (e.g. (21)). This delay is therefore likely to largely exclude the factor we are
347 really interested in measuring, the extent of artemisinin killing in the IKB: artemisinin killing
348 occurs before iRBC clearance rates are estimated so makes no contribution to the subsequent
349 clearance rate of iRBC. The subsequent rate of decline of circulating iRBCs then most likely
350 measures how rapidly host clearance mechanism remove iRBCs containing dead or dying
351 parasites killed during the IKB.
352
353 This interpretation also explains the clinical observation that subsequent doses of artemisinin
354 (indicated as horizontal red lines in Figure 2) have no further impact on clearance rates. The
355 dynamics can be understood as the interactions between the three factors that determine
356 iRBC clearance dynamics, i.e. spleen clearance rates, sequestration rates and new-merozoite-
357 release rates. These rates differ substantially. Spleen clearance rates have half-lives in the
358 region of 2 to 5 hours. Sequestration rates depend on the number and development stages of
359 circulating parasites, but half have been sequestered by age-bin 14 so half-lives may be
360 approximated as 14 hours (although this is more for illustration as it forces an exponential
361 decline onto a much complex sequestration regimen; see Supplemental Material, part 1).
362 Finally, sequestered parasites have a half-life of around (48-14) /2 = 17 hours before their
363 schizonts release new merozoites into the circulation. Sequestration and new-merozoite-
364 release rates are therefore both substantially slower than spleen clearance rates but these rates
365 must be scaled by the number of parasites in each group. The dynamics can therefore be
366 understood as follows: the first few hours of non-linearity occur because the IKB has to
367 establish a sufficient number of iRBC with dead parasites such that the spleen clearance rates

completely dominate the other two factors and hence dominate the overall dynamics of iRBC clearance. Subsequent doses of artemisinin may kill a large proportion of the remaining viable circulating parasites but this will be invisible because, as noted, above, it is impossible to distinguish circulating iRBC with live, dead or dying parasites (26). This interpretation is supported by clinical data from Wootton and colleagues (44) who estimated the proportion of viable parasites among circulating iRBC to be <0.5% following treatment with 2 or 4 mg/kg of artesunate, a clear demonstration that treatment with ACTs results in a huge pool of dead iRBC awaiting spleen clearance.

Implications for assessing drug effectiveness

One of the main opportunities to increase drug effectiveness is by regimen changes, typically increasing the total dosage given to patients and/or changing dosing regimen patterns. This is particularly important given current concerns that artemisinin resistance may be spreading and threating the therapeutic effectiveness of ACTs.

The first option to increase drug effectiveness is to increase the artemisinin dose; this essentially increases the duration of killing after treatment (33). Figure 1, Panels A and B suggest that iRBC clearance rates will be unable to detect even substantial increases in artemisinin killing that occur above a duration of killing threshold of around 2 to 3 hours post-treatment. It is possible to convert this threshold into one based on drug intake doses. We investigated what DHA intake dosages would result in 2 or 3 hours of parasite killing using standard PK/PD modelling with our default dihydroartemisinin (DHA) parameters (Table 1 of (45)). An intake dose of ~0.2 mg/kg DHA resulted in around two hours duration of artemisinin killing after treatment, while an intake dose of ~0.5 mg/kg resulted in a

duration of killing of around three hours (for reference, the currently-recommended DHA dosage is 4 mg/kg giving a duration of killing of around 5 to 6 hours (Figure 3 of (34)). Hence the threshold of 2 to 3 hours on Figure 1, Panel A equates to a DHA intake dose of around 0.2 to 0.5 mg/kg. In practice, this threshold will be higher because there is substantial PK/PD variation in nature and so a considerable proportion of patients treated with 0.5 mg/kg of DHA would have durations of killing much shorter than 3 hours. Using a rule of thumb of 3-fold variation in PK/PD (45, 46) suggests the threshold of detection, above which additional artemisinin killing will not be detected by iRBC clearance rates (Figure 1, Panel A), will probably lie somewhere in the region of $3 \times 0.5 = 1.5$ mg/kg. Angus *et al*. (47) concluded that no further increase in iRBC clearance rates occur above doses around 2 mg/kg. They administered AS which has a higher molecular weight than DHA (384 *versus* 284 g/mol, respectively), meaning their 2 mg/kg of artesunate was equivalent to a $2 \times 284/384 = 1.5$ mg/kg dose of DHA. Their results are therefore highly consistent with the threshold identified in our model (although visual inspection of their raw data (Figures 2 and 3 of (47)) suggests this threshold for detection of increased AS killing by iRBC clearance rates may plausibly be lower than 2 mg/kg). Similarly, Saunders and colleagues (48) reported no difference in iRBC clearance times or rates when dosing with AS at 2, 4 or 6 mg/kg; again these results are highly consistent with our model prediction that all three doses would lie above the detection threshold. Angus *et al*. (47) asserted that no further increase in iRBC clearance rates occurred above 2 mg/kg because higher doses had no further impact on drug killing. A clear alternative interpretation is that their metric, iRBC clearance rates, simply lacked the sensitivity required to detect further increases in parasites killing (Figure 1, Panel A). If the latter interpretation is true, it clearly indicates an opportunity to increase ACT drug effectiveness through the relatively simple expedient of increasing artemisinin dose, at least within the levels restrained by toxicity.

Another strategy to improve drug effectiveness is to split the standard dose and give it more frequently. In ACT this essentially means switching from a single daily dose to twice-daily dosing (as is currently done for artemether-lumefantrine (AM-LF), noting that the need for twice daily dosing is driven by the LF rather than the AM component). The total dose remains unchanged so the twice-daily doses contain half the drug content of the once-daily doses. Pharmacological modelling of clinical data suggested this could increase drug effectiveness (28). Our more recent quantitative PK/PD modelling (34) identified its mechanistic basis (it arises from a law-of diminishing returns in antimalarial drug dosing (33)) and showed that split dosing is a far more effective strategy for improving artemisinin effectiveness than simply increasing the total amount of artemisinin given. Figure 2 illustrates the comparative dynamics of daily and twice-daily dosing in more detail based on current DHA-PPQ regimens and separates out the effect of artemisinin alone (top row) with the effect of including the partner drug PPQ (bottom row). The clear conclusion is that the split-dose strategy will result in increased drug effectiveness but that iRBC clearance rates primarily reflect patient immune status so were similar in all cases and unable to detect the changes in drug effectiveness. Note that this is robust over a range of calibrations and partner drugs; the latter have such long half-lives that our model output suggests that the impact of split dosing is immaterial for partner drugs, it is the artemisinin killing that increases so dramatically with split dose regimens.

Unfortunately, attempts to implement ACT split-dose regimen changes are currently hindered by a trial (14) that evaluated twice-daily regimen changes using iRBC clearance rates as an indicator of drug effectiveness and reported no difference in clearance rates. A key operational question is therefore to decide whether this is a valid measure of drug efficacy, or

whether it reflects an inherent lack of sensitivity in the metric being used to estimate effectiveness. We therefore suggest an alternative interpretation of the results of Das *et al.* (14): No differences in clearance rates occurred between different regimens because the overwhelming impact of immunity on clearance rates would have obscured differences in drug killing between the regimens. The huge costs of developing a new drug and the potential risks to human subjects as drugs enter clinical development, make it operationally and ethically essential to use well-validated clinical indicators of likely efficacy. It seems essential that the malaria community now re-considers drug regimen changes as a means to offset, or even prevent, the early stages of resistance (34).

Implications for monitoring for drug resistance

The most widespread application of clearance rates has occurred in surveys of ACTs in South East Asia where reduced iRBC clearance rates have been routinely interpreted as indicating reduced drug effectiveness due to the onset of artemisinin "resistance" (see (49) for a recent access to the literature and Supplemental Material, part 2 for a discussion on genetic analysis of iRBC clearance rates). The studies have used both artemisinin monotherapy (e.g. (7, 39, 40)) and artemisinins within ACTs (e.g. (3, 50)); the much higher potency of the artemisinin component against circulating stages (compared to its partner drugs within ACTs) means that artemisinins are the main determinants of clearance rates within ACTs so the two types of studies, monotherapies and ACTs, can be viewed as equivalents in terms of their clearance phenotypes (18, 34). The results presented above show that iRBC clearance rates are a highly insensitive surveillance tool for resistance as they can only detect resistance if it is sufficiently strong (or immunogenic, see below) that virtually all parasites within circulating

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iRBC survive treatment. This is presumably the case with the newly-identified K13 mutations (51) which appear to virtually remove parasite hyper-sensitivity in their early ring stages allowing its detection through increased iRBC clearance rates. Note also that it is possible that some partner drugs may kill some circulating parasites which would produce a pool of dead parasites within iRBC that could partially obscure the effects of changing artemisinin sensitivity on iRBC clearance rates (see discussion of the three iRBC clearance factors described above). Consequently, it could be the case that artemisinin resistance may be detected as increased clearance time in ACTs whose partner drugs do not kill circulating parasites, while no such increase in clearance may be noted in ACTs whose partner drugs do kill some circulating iRBC. Hence a strategy of using artemisinin monotherapy to measure iRBC clearance rates (prior to the partner drug being administered) is a preferable strategy.

Clearance rates of iRBCs also have potentially very low specificity as other factors, notably falling patient immunity, may cause slower clearance and be erroneously interpreted as indicating "resistance". At least three reviews (15-17) have pointed out that declining levels of immunity may have contributed to decreased clearance rates observed in South East Asia and have been confused with changes in drug sensitivity levels; see also (52,53) for examples of the subsequent discussion. Given the concerns over the impact of possible artemisinin resistance (*op. cit.*) it seems imperative to properly design a surveillance strategy and recognise the dangers of over-reliance on iRBC clearance rates as the sole surveillance tool. The properties of the K13 mutations, principally their resistance to artemisinins while in circulating early-ring forms (54) combined with possible changes in progression through early (but not later) stages of the parsites' nominal 48-hour cycle (35), seem ready-made to allow their detection through reduced iRBC clearance rate. However there is no guarantee

that other artemisinin resistance mutations will be so obliging, and indeed, it is possible that they may already be present but remain undetected; for example mutations in the ap2-mu gene have been shown to modulate artemisinin sensitivity of both murine and human malaria (55, 56). Plowe (57), for example, noted that K13 need not be the only artemisinin "resistance" gene and we require a surveillance method to detect other mutations. As stressed here, iRBC clearance rates are unlikely to be sufficiently sensitive to detect all manifestations of artemisinin resistance and other surveillance tools, such as screening for genetic sweeps (58, 59) and *in vitro* sensitivity assays, need to be more widely recognised and used in surveillance for resistance.

Conclusions

It is widely recognised that immunity makes a potentially substantial contribution to iRBC clearance rates, and that fitting a "dead-awaiting clearance" class of iRBC improves model fit to clinical data (29, 30). It therefore seems extraordinary that there has been no objective investigation of the impact of host immunity on the use of iRBC clearance rates as surveillance tools for drug resistance and as efficacy tools for evaluating drug regimen changes. This was the impetus for the work presented here. Our model output suggests that host clearance processes, such as immunity, completely dominate the iRBC clearance phenotype unless artemisinin effectiveness is extremely low. This makes iRBC clearance rates highly insensitive to changes in underlying parasite drug sensitivity and to drug effectiveness cause by regimen changes.

The purpose of this study had been to try and open a more objective debate about the use of iRBC clearance rates post-treatment as proxy measures of drug effectiveness and resistance.

It is possible, perhaps even likely, that iRBC clearance rates reflect the level of an individual patient's acquired immunity to malaria (e.g. (38)), with the degree of parasite resistance or drug effectiveness being effectively invisible against this background. The World Health Organization set up an action plan to contain artemisinin resistance in 2011 (60). It was laudable to act on this initial evidence but no serious attempts appear to have been made in the subsequent few years to validate the use of the parasite clearance rate as a good metric of parasite resistance (15-17). The use of iRBC clearance rates as measures of drug effectiveness is particularly worrying, with the likely consequence that regimen changes capable of increasing drug effectiveness may be ignored as they have little impact on iRBC clearance rates ((34); Figure 2 of this manuscript).

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537 Reference List

538

539 1. **World Health Organisation.** 2010. Guidelines for the treatment of malaria, second edition.,
540 Geneva, Switzerland.
541 2. **Dondorp A, Nosten F, Yi P, Das D, Phyo A, Tarning J, Lwin K, Ariey F, Hanpithakpong W, Lee**
542 **S, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An S, Yeung S,**
543 **Singhasivanon P, Day N, Lindegardh N, Socheat D, White N.** 2009. Artemisinin resistance in
544 *Plasmodium falciparum* malaria. N Engl J Med **361:**455 - 467.
545 3. **Phyo AP, Nkhoma S, Stepniewska K, Ashley EA, Nair S, McGready R, Moo CL, Al-Saai S,**
546 **Dondorp AM, Lwin KM, Singhasivanon P, Day NPJ, White NJ, Anderson TJC, Nosten F.** 2012.
547 Emergence of artemisinin-resistant malaria on the western border of Thailand: A
548 longitudinal study. Lancet **379:**1960-1966.
549 4. **Amaratunga C, Sreng S, Suon S, Phelps ES, Stepniewska K, Lim P, Zhou C, Mao S, Anderson**
550 **JM, Lindegardh N, Jiang H, Song J, Su XZ, White NJ, Dondorp AM, Anderson TJC, Fay MP,**
551 **Mu J, Duong S, Fairhurst RM.** 2012. Artemisinin-resistant *Plasmodium falciparum* in Pursat
552 province, western Cambodia: A parasite clearance rate study. Lancet Infect Dis **12:**851-858.
553 5. **Miotto O, Almagro-Garcia J, Manske M, MacInnis B, Campino S, Rockett KA, Amaratunga**
554 **C, Lim P, Suon S, Sreng S, Anderson JM, Duong S, Nguon C, Chuor CM, Saunders D, Se Y,**
555 **Lon C, Fukuda MM, Amenga-Etego L, Hodgson AVO, Asoala V, Imwong M, Takala-Harrison**
556 **S, Nosten F, Su X-z, Ringwald P, Ariey F, Dolecek C, Hien TT, Boni MF, Thai CQ, Amambua-**
557 **Ngwa A, Conway DJ, Djimde AA, Doumbo OK, Zongo I, Ouedraogo J-B, Alcock D, Drury E,**
558 **Auburn S, Koch O, Sanders M, Hubbart C, Maslen G, Ruano-Rubio V, Jyothi D, Miles A,**

O'Brien J, Gamble C, Oyola SO, et al. 2013. Multiple populations of artemisinin-resistant *Plasmodium falciparum* in Cambodia. Nat Genet **45:**648-655.

6. **Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM, Study C.** 2008. Evidence of artemisinin-resistant Malaria in Western Cambodia. N Engl J Med **359:**2619-2620.

7. **Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, Sreng S, Anderson JM, Mao S, Sam B, Sopha C, Chuor CM, Nguon C, Sovannaroth S, Pukrittayakamee S, Jittamala P, Chotivanich K, Chutasmit K, Suchatsoonthorn C, Runcharoen R, Hien TT, Thuy-Nhien NT, Thanh NV, Phu NH, Htut Y, Han K-T, Aye KH, Mokuolu OA, Olaosebikan RR, Folaranmi OO, Mayxay M, Khanthavong M, Hongvanthong B, Newton PN, Onyamboko MA, Fanello CI, Tshefu AK, Mishra N, Valecha N, Phyo AP, Nosten F, Yi P, Tripura R, Borrmann S, Bashraheil M, Peshu J, Faiz MA, Ghose A, Hossain MA, Samad R, et al.** 2014. Spread of Artemisinin Resistance in *Plasmodium falciparum* Malaria. N Engl J Med **371:**411-423.

8. **Breman JG.** 2012. Resistance to artemisinin-based combination therapy. Lancet Infect Dis **12:**820-822.

9. **Uhlemann A-C, Fidock DA.** 2012. Loss of malarial susceptibility to artemisinin in Thailand. Lancet **379:**1928-1930.

10. **Fairhurst RM, Nayyar GML, Breman JG, Hallett R, Vennerstrom JL, Duong S, Ringwald P, Wellems TE, Plowe CV, Dondorp AM.** 2012. Artemisinin-resistant malaria: Research challenges, opportunities, and public health implications. Am J Trop Med Hyg **87:**231-241.

11. **Talisuna AO, Karema C, Ogutu B, Juma E, Logedi J, Nyandigisi A, Mulenga M, Mbacham WF, Roper C, Guerin PJ, D'Alessandro U, Snow RW.** 2012. Mitigating the threat of artemisinin resistance in Africa: Improvement of drug-resistance surveillance and response systems. Lancet Infect Dis **12:**888-895.

12. **Dondorp AM, Yeung S, White L, Nguon C, Day NPJ, Socheat D, von Seidlein L.** 2010. Artemisinin resistance: current status and scenarios for containment. Nat Rev Micro **8:**272-280.

13. **Worldwide Antimalarial Resistance Network.** Parasite Clearance Estimator (PCE). http://wwwwwarnorg/toolkit/data-management/parasite-clearance-estimator

14. **Das D, Tripura R, Phyo AP, Lwin KM, Tarning J, Lee SJ, Hanpithakpong W, Stepniewska K, Menard D, Ringwald P, Silamut K, Imwong M, Chotivanich K, Yi P, Day NPJ, Lindegardh N, Socheat D, Nguon C, White NJ, Nosten F, Dondorp AM.** 2012. Effect of high dose or split dose artesunate on parasite clearance in artemisinin resistant falciparum malaria. Clin Infect Dis **56:**e48-e58.

15. **Krishna S, Kremsner PG.** 2013. Antidogmatic approaches to artemisinin resistance: reappraisal as treatment failure with artemisinin combination therapy. Trends Parasitol **29:**313-317.

16. **Ferreira PE, Culleton R, Gil JP, Meshnick SR.** 2013. Artemisinin resistance in Plasmodium falciparum: what is it really? Trends Parasitol **29:**318-320.

17. **Meshnick S.** 2012. Perspective: Artemisinin-resistant malaria and the wolf. Am J Trop Med Hyg **87:**783-784.

18. **White NJ.** 2011. The parasite clearance curve. Malar J **10:**278.

19. **Day NPJ, Diep PT, Ly PT, Sinh DX, Loc PP, Van Chuong L, Chau TTH, Mai NTH, Bethell DB, Phu NH, Hien TT, White NJ.** 1996. Clearance kinetics of parasites and pigment-containing leukocytes in severe malaria. Blood **88:**4694-4700.

20. **Flegg JA, Guerin PJ, White NJ, Stepniewska K.** 2011. Standardizing the measurement of parasite clearance in falciparum malaria: The parasite clearance estimator. Malar J **10**.

21. **Jamsen K, Duffull S, Tarning J, Price R, Simpson J.** 2013. A robust design for identification of the Parasite Clearance Estimator. Malar J **12:**410.

22. **World Health Organisation.** 2011. Methods and techniques for assessing exposure to antimalarial drugs in clinical field studies. Geneva.

23. **Zaloumis S, Humberstone A, Charman S, Price R, Moehrle J, Gamo-Benito J, McCaw J, Jamsen K, Smith K, Simpson J.** 2012. Assessing the utility of an anti-malarial

pharmacokinetic-pharmacodynamic model for aiding drug clinical development. Malar J **11:**303.

24. **Warhurst DC, Williams JE.** 1996. Laboratory diagnosis of malaria. J Clin Pathol **49:**533-538.

25. **Kremsner PPG, Krishna PS.** 2004. Antimalarial combinations. Lancet **364:**285-294.

26. **Watkins WM, Woodrow C, Marsh K.** 1993. Falciparum malaria: Differential effects of antimalarial drugs on ex vivo parasite viability during the critical early phase of therapy. Am J Trop Med Hyg **49:**106-112.

27. **Hoshen MB, Na-Bangchang K, Stein WD, Ginsburg H.** 2000. Mathematical modelling of the chemotherapy of *Plasmodium falciparum* malaria with artesunate: postulation of 'dormancy', a partial cytostatic effect of the drug, and its implication for treatment regimens. Parasitol **121:**237-246.

28. **Saralamba S, Pan-Ngum W, Maude RJ, Lee SJ, Tarning J, Lindegårdh N, Chotivanich K, Nosten F, Day NPJ, Socheat D, White NJ, Dondorp AM, White LJ.** 2011. Intrahost modeling of artemisinin resistance in *Plasmodium falciparum*. Proc Natl Acad Sci USA **108:**397-402.

29. **Hietala SF, Mårtensson A, Ngasala B, Dahlström S, Lindegårdh N, Annerberg A, Premji Z, Färnert A, Gil P, Björkman A, Ashton M.** 2010. Population pharmacokinetics and pharmacodynamics of artemether and lumefantrine during combination treatment in children with uncomplicated falciparum malaria in Tanzania. Antimicrob Agents Chemother **54:**4780-4788.

30. **Gordi T, Xie R, Jusko WJ.** 2005. Semi-mechanistic pharmacokinetic/pharmacodynamic modelling of the antimalarial effect of artemisinin. Br J Clin Pharmacol **60:**594-604.

31. **World Health Organisation.** 2015. Microscopy for the detection, identification and quantification of malaria parasites on stained thick and thin blood films in research settings. http://wwwwhoint/tdr/publications/microscopy_detec_ident_quantif/en/.

32. **Beshir KB, Sutherland CJ, Sawa P, Drakeley CJ, Okell L, Mweresa CK, Omar SA, Shekalaghe SA, Kaur H, Ndaro A, Chilongola J, Schallig HDFH, Sauerwein RW, Hallett RL, Bousema T.**

2013. Residual *Plasmodium falciparum* Parasitemia in Kenyan Children After Artemisinin-Combination Therapy Is Associated With Increased Transmission to Mosquitoes and Parasite Recurrence. J Infect Dis doi:10.1093/infdis/jit431.

33. **Hastings I, Hodel EM.** 2014. Pharmacological considerations in the design of anti-malarial drug combination therapies - is matching half-lives enough? Malar J **13:**62.

34. **Kay K, Hodel EM, Hastings IM.** Altering antimalarial drug regimens may dramatically enhance and restore drug effectiveness. Submitted to AAC as a companion paper.

35. **Mok S, Ashley EA, Ferreira PE, Zhu L, Lin Z, Yeo T, Chotivanich K, Imwong M, Pukrittayakamee S, Dhorda M, Nguon C, Lim P, Amaratunga C, Suon S, Hien TT, Htut Y, Faiz MA, Onyamboko MA, Mayxay M, Newton PN, Tripura R, Woodrow CJ, Miotto O, Kwiatkowski DP, Nosten F, Day NPJ, Preiser PR, White NJ, Dondorp AM, Fairhurst RM, Bozdech Z.** 2015. Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance. Science **347:**431-435.

36. **Klonis N, Xie SC, McCaw JM, Crespo-Ortiz MP, Zaloumis SG, Simpson JA, Tilley L.** 2013. Altered temporal response of malaria parasites determines differential sensitivity to artemisinin. Proc Natl Acad Sci USA **110:**5157-5162.

37. **Greenhouse B, Slater M, Njama-Meya D, Nzarubara B, Maiteki-Sebuguzi C, Clark TD, Staedke SG, Kamya MR, Hubbard A, Rosenthal PJ, Dorsey G.** 2009. Decreasing efficacy of antimalarial combination therapy in Uganda is explained by decreasing host immunity rather than increasing drug resistance. J Infect Dis **199:**758-765.

38. **Lopera-Mesa TM, Doumbia S, Chiang S, Zeituni AE, Konate DS, Doumbouya M, Keita AS, Stepniewska K, Traore K, Diakite SAS, Ndiaye D, Sa JM, Anderson JM, Fay MP, Long CA, Diakite M, Fairhurst RM.** 2013. *Plasmodium falciparum* clearance rates in response to artesunate in Malian children with malaria: Effect of acquired immunity. J Infect Dis **207:**1655-1663.

662 39. **Bethell D, Se Y, Lon C, Tyner S, Saunders D, Sriwichai S, Darapiseth S, Teja-Isavadharm P,**
663 **Khemawoot P, Schaecher K, Ruttvisutinunt W, Lin J, Kuntawungin W, Gosi P, Timmermans**
664 **A, Smith B, Socheat D, Fukuda MM.** 2011. Artesunate Dose Escalation for the Treatment of
665 Uncomplicated Malaria in a Region of Reported Artemisinin Resistance: A Randomized
666 Clinical Trial. PLoS ONE **6:**e19283.
667 40. **Kyaw MP, Nyunt MH, Chit K, Aye MM, Aye KH, Aye MM, Lindegardh N, Tarning J, Imwong**
668 **M, Jacob CG, Rasmussen C, Perin J, Ringwald P, Nyunt MM.** 2013. Reduced Susceptibility of
669 *Plasmodium falciparum* to Artesunate in Southern Myanmar. PLoS ONE **8:**e57689.
670 41. **Stepniewska K, Ashley E, Lee SJ, Anstey N, Barnes KI, Binh TQ, D'Alessandro U, Day NPJ, De**
671 **Vries PJ, Dorsey G, Guthmann JP, Mayxay M, Newton PN, Olliaro P, Osorio L, Price RN,**
672 **Rowland M, Smithuis F, Taylor WRJ, Nosten F, White NJ.** 2010. In vivo parasitological
673 measures of artemisinin susceptibility. J Infect Dis **201:**570-579.
674 42. **Rogerson SJ, Wijesinghe RS, Meshnick SR.** 2010. Host immunity as a determinant of
675 treatment outcome in *Plasmodium falciparum* malaria. Lancet Infect Dis **10:**51-59.
676 43. **Henriques G, Hallett RL, Beshir KB, Gadalla NB, Johnson RE, Burrow R, van Schalkwyk DA,**
677 **Sawa P, Omar SA, Clark TG, Bousema T, Sutherland CJ.** 2014. Directional Selection at the
678 pfmdr1, pfcrt, pfubp1, and pfap2mu Loci of *Plasmodium falciparum* in Kenyan Children
679 Treated With ACT. J Infect Dis **210:**2001-2008.
680 44. **Wootton DG, Opara H, Biagini GA, Kanjala MK, Duparc S, Kirby PL, Woessner M, Neate C,**
681 **Nyirenda M, Blencowe H, Dube-Mbeye Q, Kanyok T, Ward S, Molyneux M, Dunyo S,**
682 **Winstanley PA.** 2008. Open-label comparative clinical study of chlorproguanil–dapsone fixed
683 dose combination (Lapdap™) alone or with three different doses of artesunate for
684 uncomplicated *Plasmodium falciparum* malaria. PLoS ONE **3:**e1779.
685 45. **Winter K, Hastings IM.** 2011. Development, evaluation and application of an *in silico* model
686 for antimalarial drug treatment and failure. Antimicrob Agents Chemother **55:**3380-3392.

687 46. **Kay K, Hastings IM.** 2013. Improving pharmacokinetic-pharmacodynamic modeling to
688 investigate anti-infective chemotherapy with application to the current generation of
689 antimalarial drugs. PLoS Comput Biol **9:**e1003151.
690 47. **Angus BJ, Thaiaporn I, Chanthapadith K, Suputtamongkol Y, White NJ.** 2002. Oral
691 artesunate dose-response relationship in acute falciparum malaria. Antimicrob Agents
692 Chemother **46:**778-782.
693 48. **Saunders D, Khemawoot P, Vanachayangkul P, Siripokasupkul R, Bethell D, Tyner S, Se Y,**
694 **Rutvisuttinunt W, Sriwichai S, Chanthap L, Lin J, Timmermans A, Socheat D, Ringwald P,**
695 **Noedl H, Smith B, Fukuda M, Teja-Isavadharm P.** 2012. Pharmacokinetics and
696 pharmacodynamics of oral artesunate monotherapy in patients with uncomplicated
697 *Plasmodium falciparum* Malaria in Western Cambodia. Antimicrob Agents Chemother
698 **56:**5484-5493.
699 49. **Greenwood B.** 2014. Treatment of malaria - A continuing challenge. N Engl J Med **371:**474-
700 475.
701 50. **Spring MD, Lin JT, Manning JE, Vanachayangkul P, Somethy S, Bun R, Se Y, Chann S,**
702 **Ittiverakul M, Sia-ngam P, Kuntawunginn W, Arsanok M, Buathong N, Chaorattanakawee**
703 **S, Gosi P, Ta-aksorn W, Chanarat N, Sundrakes S, Kong N, Heng TK, Nou S, Teja-isavadharm**
704 **P, Pichyangkul S, Phann ST, Balasubramanian S, Juliano JJ, Meshnick SR, Chour CM, Prom S,**
705 **Lanteri CA, Lon C, Saunders DL.** 2015. Dihydroartemisinin-piperaquine failure associated
706 with a triple mutant including kelch13 C580Y in Cambodia: an observational cohort study.
707 Lancet Infect Dis **15:**683-691.
708 51. **Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois A-C, Khim N, Kim S, Duru V,**
709 **Bouchier C, Ma L, Lim P, Leang R, Duong S, Sreng S, Suon S, Chuor CM, Bout DM, Menard S,**
710 **Rogers WO, Genton B, Fandeur T, Miotto O, Ringwald P, Le Bras J, Berry A, Barale J-C,**
711 **Fairhurst RM, Benoit-Vical F, Mercereau-Puijalon O, Menard D.** 2014. A molecular marker
712 of artemisinin-resistant *Plasmodium falciparum* malaria. Nature **505:**50-55.

52. **Dondorp AM, Ringwald P.** 2013. Artemisinin resistance is a clear and present danger. Trends Parasitol **29:**359-360.

53. **Krishna S, Kremsner PG.** 2013. Artemisinin resistance needs to be defined rigorously to be understood: Response to Dondorp and Ringwald. Trends Parasitol **29:**361-362.

54. **Straimer J, Gnädig NF, Witkowski B, Amaratunga C, Duru V, Ramadani AP, Dacheux M, Khim N, Zhang L, Lam S, Gregory PD, Urnov FD, Mercereau-Puijalon O, Benoit-Vical F, Fairhurst RM, Ménard D, Fidock DA.** 2015. K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates. Science **347:**428-431.

55. **Hunt P, Afonso A, Creasey A, Culleton R, Sidhu ABS, Logan J, Valderramos SG, McNae I, Cheesman S, Rosario Vd, Carter R, Fidock DA, Cravo P.** 2007. Gene encoding a deubiquitinating enzyme is mutated in artesunate- and chloroquine-resistant rodent malaria parasites. Mol Microbiol **65:**27-40.

56. **Henriques G, van Schalkwyk DA, Burrow R, Warhurst DC, Thompson E, Baker DA, Fidock DA, Hallett R, Flueck C, Sutherland CJ.** 2015. The Mu Subunit of Plasmodium falciparum Clathrin-Associated Adaptor Protein 2 Modulates In Vitro Parasite Response to Artemisinin and Quinine. Antimicrob Agents Chemother **59:**2540-2547.

57. **Plowe CV.** 2014. Malaria: Resistance nailed. Nature **505:**30-31.

58. **Anderson T, Nkhoma S, Ecker A, Fidock D.** 2011. How can we identify parasite genes that underlie antimalarial drug resistance? Pharmacogenomics **12:**59-85.

59. **Mwangi JM, Ranford-Cartwright LC.** 2013. Genetic and genomic approaches for the discovery of parasite genes involved in antimalarial drug resistance. Parasitol **140:**1455-1467.

60. **World Health Organisation.** 2011. Global Plan for Artemisinin resistance containment. Geneva.

Table 1. Sensitivity analysis: correlation coefficients between drug effectiveness (measured as parasite reduction ratio, PRR_{48}), infected red blood cell clearance rates (iRBCcr), and underlying drug and host parameters. Model parameters investigated were the duration of artemisinin killing after dosing (Art. duration), the magnitude of artemisinin killing rate (Art. kill rate), the magnitude of partner drug kill rate (Partner kill rate), and the spleen clearance rate of circulating iRBC containing dead parasites. The drugs investigated are **(A)** dihydroartemisinin-piperaquine (DHA-PPQ) and **(B)** artesunate-mefloquine (AS-MQ). Two artemisinin sensitivity profiles are investigated (the iso- and hyper-sensitivity profiles) and starting stage distribution of parasites may be either uniform or early ring stage. See Supplemental Material, part 1 for more explanation and technical details.

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751 (A) DHA-PPQ

Artemisinin sensitivity profile	Isosensitivity				Hypersensitivity			
Parasite distribution at time of treatment	uniform		early ring		uniform		early ring	
Outcome:	PRR_{48}	iRBCcr	PRR_{48}	iRBCcr	PRR_{48}	iRBCcr	PRR_{48}	iRBCcr
Art. Duration (hr)	0.18	0.05	0.17	0.02	0.18	0.08	0.21	0.08
Art kill rate (hr^{-1})	0.19	0.05	0.18	0.08	0.19	0.10	0.22	0.15
Partner kill rate (hr^{-1})	0.14	0.03	0.14	0.02	0.15	0.04	0.14	0.09
PRR_{48}	-	-0.01	-	0.00	-	0.04	-	0.03
Spleen clearance rate (hr^{-1})	-	0.99	-	0.99	-	0.99	-	0.93

752

753 (B) AS-MQ

Artemisinin sensitivity profile	Isosensitivity				Hypersensitivity			
Parasite distribution at time of treatment	uniform		early ring		uniform		early ring	
Outcome:	PRR_{48}	iRBCcr	PRR_{48}	iRBCcr	PRR_{48}	iRBCcr	PRR_{48}	iRBCcr
Art. Duration (hr)	0.19	0.02	0.20	0.01	0.19	0.01	0.19	0.07
Art kill rate (hr^{-1})	0.21	0.04	0.22	0.05	0.20	0.06	0.21	0.16

Partner kill rate (hr^{-1})	0.14	0.00	0.15	0.02	0.13	0.01	0.14	0.11
PRR_{48}	-	0.01	-	0.00	-	0.01	-	0.04
Spleen clearance rate (hr^{-1})	-	1.00	-	0.99	-	0.99	-	0.93

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Figure 1. The lack of sensitivity of clearance rates to changes in artemisinin killing.

Changes in artemisinin killing may arise in two ways. Firstly, the duration of killing post-treatment will alter if parasites evolve resistance (measured as a reduced concentration at which drug killing is half its maximum value) or if the intake dose given to patients is changed. The left hand column shows the effect of varying the duration of artemisinin killing from 1 to 8 hours after each dose (the default value being 6 hours). Panel (A) shows the impact on observed infected red blood cell (iRBC) clearance rate. Panel (B) shows the impact on drug effectiveness quantified as the parasite reduction ratio (PRR_{48}). Secondly, the artemisinin kill rate may changes as parasites evolve resistance. The right hand column shows the effect of varying the artemisinin kill rate from 10% to 120% of the default value. Panel (C) shows the impact on observed iRBC clearance rate. Panel (D) shows the impact on drug effectiveness quantified as the PRR_{48}.

The two drugs investigated were artesunate-mefloquine (AS-MQ) and dihydroartemisinin-piperaquine (DHA-PPQ). Parasite sensitivity to AS and DHA follows "isosensitivity" or "hypersensitivity" PD profiles, respectively, and the dosing was either once- or twice-daily. All simulations had spleen clearance rates set to 0.231 per hour (equivalent to a clearance half-life of 3 hours). See Supplemental Material, part 1 for technical details. Note that the red and green dotted lines are superimposed on panels B and D, as are the blue and black dotted lines. Note also that PRR_{48} does not fall to zero as partner drug killing alone would achieve a PRR_{48} of around 10^3.

Figure 2. An example of the lack of sensitivity of parasite clearance rates to changes in drug effectiveness caused by regimen changes.

Blue lines are "parasite clearance rates", i.e. the number of circulating infected red blood cells (iRBCs) containing either live or dead parasites. Green lines are the number of circulating iRBCs containing live parasites. Black lines are total parasitaemias, i.e. the total number of live parasites in both circulating and sequestered iRBCs. Red horizontal bars indicate when DHA is present at active concentrations and the grey horizontal band indicates the parasite detection limit below which circulating parasites cannot be realistically detected or counted by routine microscopy. The drug simulated is dihydroartemisinin (DHA) alone (top row) and in combination with piperaquine (PPQ) (bottom row). The left hand column is the drug(s) given once per day over three days, and the right hand column is an alternative regimen when the same total amount of drug(s) is given but split into twice-daily doses given over three days. Spleen clearance rate of iRBCs containing dead parasites is assumed to be 0.26 per hour as estimated in Gordi *et al*. (30), equivalent to a spleen clearance half-life of 2.7 hours. The drug sensitivity profiles follow the hypersensitivity model, i.e. where early ring stages are hypersensitive to DHA. The infection at start of treatment was primarily in early ring stages (mean = 10.5 hours, standard deviation = 5 hours). The modelling details are provided in Supplemental Material, part 1.