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2 Title: Improving methods for analysing anti-malarial drug efficacy trials: molecular correction based
3 on length-polymorphic markers *msp-1*, *msp-2* and *glurp*.

4 Running title: Molecular correction with *msp-1*, *msp-2* and *glurp*.

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Abstract (250 words)

Background.

Drug efficacy trials monitor the continued efficacy of front-line drugs against falciparum malaria. Over-estimates of efficacy result in a country retaining a failing drug as first-line treatment with associated increases in morbidity and mortality, while under-estimating drug effectiveness leads to removal of an effective treatment with substantial practical and economic implications. Trials are challenging: they require long durations of follow-up to detect drug failures, and patients are frequently re-infected during that period. Molecular correction based on parasite genotypes distinguishes reinfections from drug failures to ensure the accuracy of failure rate estimates. Several molecular correction "algorithms" are proposed, but which is most accurate and/or robust remains unknown.

Methods.

We used pharmacological modelling to simulate parasite dynamics and genetic signals that occur in patients enrolled in malaria drug clinical trials. We compared estimates of treatment failure obtained from a selection of proposed molecular correction algorithms against the known "true" failure rate in the model.

Findings.

(i) Molecular correction is essential to avoid substantial over-estimates of drug failure rates. (ii) The current WHO-recommended algorithm consistently under-estimates the true failure rate. (iii) Newly-proposed algorithms produce more accurate failure rate estimates; the most accurate algorithm depends on the choice of drug, trial follow-up length, and transmission intensity. (iv) Long durations of patient follow-up may be counterproductive; large numbers of new infections accumulate and

may be misclassified, over-estimating drug failure rate. (v) Our model was highly consistent with existing *in vivo* data.

Interpretation.

The current WHO-recommended method for molecular correction and analysis of clinical trials should be re-evaluated and updated.

1. Introduction

Malaria is endemic in over 100 countries and causes an estimated 400,000 deaths per annum (1); most deaths are caused by *Plasmodium falciparum* and this study focusses on drug treatment of that species. Prompt treatment of malaria infections is an essential and effective public-health tool, but drug resistance poses a constant threat to effective treatment of falciparum malaria. The World Health Organization (WHO) currently recommends that endemic countries test their first- and second line antimalarial drugs every two years at sentinel sites to confirm their continued efficacy (2) and more frequently if resistance is suspected. The first line treatments in most malaria-endemic countries are artemisinin based combination therapies (ACTs), consisting of an artemisinin component (artesunate (AS), artemether (A or dihydroarteminisin (DHA)), which rapidly clears parasites and a 'partner' drug that ensures eventual parasite clearance and therapeutic cure (3, 4). The clinical consequence is that malaria infections fall rapidly to undetectable levels immediately after ACT treatment initiation. The partner drugs (mefloquine (MQ), lumefantrine (LF), piperaquine (PPQ), amodiaquine, sulphadoxine-pyrimethamine, pyronaridine) all have substantial half-lives. Infections surviving treatment are termed "recrudescences" and may only recover to densities sufficiently high to become detectable once partner drug concentrations have decayed to ineffective concentrations - potentially weeks after treatment. Anti-malarial drug efficacy studies therefore monitor patients for extended periods of time post-treatment to ensure recrudescences are detected. Duration of follow up depends on the half-life of the drug being assessed (2, 5), usually between 4 and 6 weeks (28 to 42 days) (3), sometimes extended to 9 weeks for research purposes. The critical operational problem is that new falciparum clones may be inoculated into patients by mosquitoes during these follow-up periods and these infections (termed "reinfections") must be distinguished from recrudescence to allow accurate estimates of drug efficacy (Figure 1). This is not a trivial problem: Annual entomological inoculation rates (aEIR) of malaria, a measure of malaria exposure in a population, are typically >10 and >100 per patient in areas of moderate to high

transmission, respectively. Moderate to high transmission sites are preferred for clinical trials as morbidity from malaria is high, so trials cover the most at-risk patient populations, and, from a practical viewpoint, patient recruitment is straightforward.

The consensus method for distinguishing recrudescence from reinfections is molecular correction or, equivalently, polymerase chain reaction (PCR)-correction. A genetic profile of the malaria infection of each patient is taken just before treatment, with a second profile taken if the patient develops a detectable malaria infection during follow-up (known as "recurrent" parasitaemia). If the profiles 'match' then the patient is considered to have a recrudescent infection if they do not match the patient is considered to have a reinfection. This 'matching' is simple in principle, but in practice has substantial limitations. The main problem is that individual malaria infections may consist of several genetically-distinct clones. Current genotyping techniques struggle to detect minority clones that are present in relatively low numbers and/or which carry alleles that do not amplify well during the genotyping process. These limitations were recognised early in the development of molecular correction methodology (6-8) and led the WHO and Malaria for Medicines Venture (MMV) to co-sponsor a meeting in 2007 to identify a consensus methodology for molecular correction; their findings were published in 2008 (3). Concerns surrounding the limitations of molecular correction have persisted (9, 10): Previous studies have noted that different algorithms give different results when applied to clinical data (e.g. Table 2 of (10)) and a recent publication quantifying the limitations inherent in PCR detection has led to renewed calls for this methodology to be re-examined (11). There now exist several proposed sets of rules (referred to hereafter as "algorithms"), for interpreting genetic profiles to classify patients (Table 1). The true failure rate is unknown *in vivo*, so it has been impossible to identify which algorithm is most accurate; consequently, the molecular correction field is currently in a state of limbo with several alternative methods giving different results, but with no way of knowing which method is most accurate; furthermore, some of these algorithms are newly proposed and have not been used to return failure rate estimates *in vivo*. There is a clear need for greater precision and improved harmonisation in

molecular correction techniques. Pharmacological simulation methods can be used to recreate data from clinical trials: Since the true failure rate is known *in silico*, it is possible to quantify which algorithm provides the most accurate and/or robust method of analysis. The impact of drug efficacy trials is potentially enormous. Over-estimates of drug efficacy may result in a country retaining a failing drug as first-line treatment with associated increases in morbidity and mortality, while under-estimating drug effectiveness may lead to removal of an effective first-line treatment with substantial practical and economic implications.

2. Methodology

The World Health Organization (WHO) have published a standardised, "consensus" list of terms (12) which we have used throughout this work, with a key exception: Our key term "drug failure" is not equivalent to "treatment failure" because, according to the WHO definitions, late treatment failure (LTF) includes patients who either failed drug treatment (i.e., recrudescence) or acquired a reinfection during follow up (2, 12). The unambiguous term "drug failure" will be used here to indicate that a patient's initial infection was not cleared by drug treatment.

To create a model with which to investigate the accuracy of molecular correction methods, we used a two-stage process implemented in the statistical programming language R (version 3.5.1) (13).

1. Use pharmacological modelling to simulate the parasite dynamics post-treatment in a population of patients enrolled in a clinical trial and track subsequent intra-host *P. falciparum* dynamics in these patients post-treatment.

2. Allocate genetic signals to each simulated parasite clone and calculate the genetic signals detected from a patient's blood sample at a given follow-up day (dependent on a variety of

factors, explored later), then analyse these signals using different algorithms (Table 1) to classify recurrent infections as drug failures or reinfections. This classification was used to generate drug failure rate estimates with comparison to true drug failure rates; thus, determining if improvements in the accuracy of these estimates were obtainable through adoption of novel algorithms.

Malaria parasite dynamics were generated using pharmacological models of malaria drug treatment that have been developed over the last decade (e.g. (14-21)) and previously calibrated and validated for three front-line ACTs: Dihydroarteminisin-Piperaquine (DHA-PPQ), Artemether-Lumefantrine (AR-LF) and Artesunate-Mefloquine (AS-MQ). The key advantage of this approach was that the exact parasitaemia of each malaria clone in each simulated patient at each time point post-treatment was known (Figure 1), as was the true status (i.e. recrudescent or reinfection) of any recurrent infection that occurred in that patient. This allowed testing of how well different PCR correction algorithms classified recurrent infections as “drug failures” or “reinfections”. It also meant the true failure rate of the drug in the simulated trial was known, as this could be calculated directly from models of parasite dynamics (i.e., did all the initial clones clear by the final day of follow-up?). This allowed each algorithm to be tested for accuracy against the true failure rate in the simulation.

2.1 Generation of parasite dynamics post-treatment using pharmacokinetic/pharmacodynamic (PK/PD) models

Parasite dynamics were generated using existing pharmacokinetic/pharmacodynamic (PK/PD) models; these models have been calibrated and validated for a range of ACTs and successfully used to investigate a variety of key research questions (14-18, 22, 23). The PK/PD parameters used to generate these dynamics for each ACT are described in full in Supplemental Material **[SM].** It is important to note that our results were not dependent on any choice of calibration. A full discussion of the variation that use of different PK and PD parameters would induce in our results is included in **[SM]**. Variation was included for all parameters, and we later show that our findings with regards to

158 the relative performance of the molecular correction algorithms were consistent across three
159 different ACTs, multiple PD parameterizations (i.e., changing IC50 to simulate failing / non-failing
160 drugs) and for both a two-compartment and three-compartment model of DHA-PPQ (explored
161 extensively in **[SM]**). Thus, we are confident that PK/PD models of DHA-PPQ, AR-LF and AS-MQ were
162 appropriate means by which to generate parasite dynamics post-treatment for the purposes of this
163 study. Alternative methods were available, i.e. arbitrarily constructing recurrent infections as
164 containing a given proportion recrudescent and/or reinfection, and testing the algorithms' ability to
165 correctly classify them (as is routinely done to construct laboratory mixtures e.g. (11)), or setting
166 distributions of time until recrudescence and/or reinfection and using these distributions to
167 construct recurrent infections. However, the use of an explicit PK/PD model added an additional
168 level of realism to these arbitrary approaches: it was simple, easily scalable, more realistic and
169 allowed for future tuning and testing if novel parameterizations emerge within the field for these
170 and for other antimalarial drugs.

171 While it was obviously not feasible for us to simulate and present *every* possible parameterization to
172 create parasite dynamics likely to occur in trials (though note our included variation covers a large
173 range of possible values), calibrating the models to re-run a specific set of parameters for interested
174 groups is a simple task upon provision of the parameters.

175 **2.1.1 Number of malaria clones per patient**

176 A malaria infection may consist of several genetically-distinct parasite clones and the number of
177 clones in a patient at the time of treatment is termed the multiplicity of infection (MOI). Two MOI
178 distributions were used in our models. A "high MOI" representative of the MOI in an area of intense
179 transmission, in this case Tanzania where MOIs of 1-8 were assigned with probabilities 0.036, 0.402,
180 0.110, 0.110, 0.183, 0.049, 0.061, 0.049 respectively (24). A "low MOI" distribution was based on
181 data from Papua New Guinea with probabilities of 0.460, 0.370, 0.150 and 0.020 for an MOI of 1-4
182 respectively (25); these two distributions were used to check if the accuracy of different algorithms

were consistent across different MOIs. Each clone within the MOI (later called "initial clones") had their starting parasitaemia drawn from a log-uniform distribution spanning from 10^{10} to 10^{11} asexual parasites per person. Previous modelling approaches (25) used 10^{12} parasites as the upper limit of parasitaemia because this level of parasitaemia is likely to be lethal or at least be a parasite density sufficiently high that such patients would not be enrolled in a clinical trial; hence 10^{11} was used as the upper limit for any single clone at the time of treatment.

Reinfections emerging from the liver are illustrated as the grey and orange dotted lines in Figure 1. Reinfections were assumed to emerge from the liver with a parasitaemia of 10^5 and all drugs modelled were assumed to be inactive against the hepatic stages. The rate of emergence reflected the local intensity of malaria transmission and was quantified as the "Force of Infection" (FOI). At the start of the model, each patient was assigned the number of reinfections that would emerge during a year. This number was drawn from a Poisson distribution whose mean value was the FOI. Values for FOI from 0-16 were used to reflect low, medium, and high transmission areas; as a general guide we regarded FOI≤2 as representing a low transmission setting, 2<FOI≤8 as indicative of moderate transmission intensity, and FOI>8 as high transmission; the yearly value was then converted to the number of reinfections occurring during the follow-up period. See **[SM]** for a detailed discussion of FOI values.

2.1.2 Tracking parasite numbers (parasitaemia) over time

Multiple lengths of follow-up are permitted in the WHO guidelines (3) and used in practice (26). The length of the follow-up period affects drug failure rate estimates in two ways: Firstly, a longer follow-up period will allow more time for recrudescent clones to become detectable (i.e. if a patient had parasites that would recrudesce and become detectable on day 60 and the follow-up period was 28 days, this recrudescence would not be observed). Secondly, a longer follow-up period leads to more reinfections emerging in each patient, some of which may be misclassified as recrudescence and inflate failure rate estimates. Accurate, robust analyses need to balance these two risks through

appropriate choice of follow-up duration. WHO guidelines (2) stipulate that patients are checked for recurrent parasitaemia by light microscopy on scheduled days of follow-up. A 28-day follow-up schedule requires patients be examined on days 3, 7, 14, 21 and 28. A 42-day follow-up period uses two additional days i.e. days 35 and 42. A 63-day follow-up period (not recommended in routine surveillance) has scheduled visits as per the 42 days but with 3 extra days i.e. 49, 56 and 63. Novel lengths of follow-up were simulated simply by "ending" the trial on any given day of follow-up, i.e. to investigate a 35-day follow-up length, patients were checked on days 3, 7, 14, 21, 28 and 35.

The parasitaemia of each clone in each patient was tracked and updated each day to reflect two factors. First, the extent of drug killing based on the PK/PD parameters (see calibration of PK/PD parameters in Table S1); second, the growth rate of each clone, which was assumed to be identical for every clone and set to 1.15/day as in previous modelling work (14, 27). The model assumed that if the total parasitaemia (i.e. the sum of parasitaemia of all clones) in a patient at any time, reached 10^{12}, then density-dependent effects, such as fever, set the growth rate of every clone to 0.

The model checked each day of scheduled follow-up to determine whether a patient had enough parasitaemia that a recurrence would be detectable by light microscopy (a recurrence) – parasitaemia was considered detectable if the total number in a patient was $\geq 10^8$ on that day. We note that variance in the limit of detection by light microscopy exists with respect to the skill of the microscopist (28); we have chosen here to assume this limit reflective of an "expert" microscopist (corresponding to roughly 20 parasites / µl of blood).

2.2 Allocation and analysis of genetic data

Each clone, whether an initial clone present at treatment or a reinfection that emerged during the follow-period, was assigned a genetic profile based on three markers: *msp-1, msp-2,* and *glurp,* using previously established distributions for the frequency of alleles. *Msp-1* and *msp-2* allelic frequency distributions and amplicon sizes were derived from 115 or 108 patients from Tanzania (29). *Glurp* distributions were drawn from a collection of field samples described in (11). The length of each

allele and its allelic family (for *msp-1* and *msp-2*) was also noted. The distributions we used gave *msp-1* expected heterozygosity (He) of 0.915, *msp-2* He of 0.963, *glurp* He of 0.956; **[Supplemental File 1]** for full data). It was assumed that the genotypes of initial clones were independent of each other and were also independent of the genotypes of reinfections (i.e. it was assumed there is no local genetic structuring of the malaria population). Note that alleles at *msp-1* and *msp-2*, exist in our distributions as members of three or two distinct families, respectively.

Once the patient parasite dynamics were modelled (as described above), and genetic profiles at the three loci were assigned, our models followed the same process as *in vivo* trials. Blood samples were taken from each patient immediately prior to treatment (the initial or "baseline" sample), and at pre-determined days during the follow-up period. Samples were screened for the presence of *Plasmodium falciparum* by light microscopy and any detected infection is labelled a "recurrence". We simulated the genotyping that would be used *in vivo* to obtain the genetic profiles for all initial and recurrent infections. Recovering the genetic signal that would be observed at time of treatment and at any recurrence reflected the technical limitations of acquiring blood samples and genotyping as follows:

- A "sampling limit" exists; a finite amount of blood is used for genotyping. A parasite clone (and consequently, it's alleles) would not be detected if its density were so low that no parasites are included in the blood sample analysed. Thus, the density and volume of the processed blood sample define the limit of detection. Obviously, this sampling limit differs between methods and laboratories. Typically, the equivalent of 1 µl of whole blood is introduced into PCR. Assuming 5 L of blood in the human body gives a total of 5 x 10^6 µl of blood, For a clone to be detected a minimum of 1 parasite (which carries a single DNA template) would need to be present in 1 µl of blood so there would need to be at least 5 x 10^6 of a given clone present for that clone to be physically sampled in the genotyping process and we also needed to allow for the fact that sub-optimal storage conditions (such

as temperature) frequently occurs in the field and will lead to DNA template breakages, and there is periodical absence from the peripheral blood of sequestered parasites. Consequently, the limit of detection will be much higher than 1 parasite per 1µl of blood. We therefore assumed 10 to 20 parasites per µl would be required to reliably contribute a genetic signal and ensure its detection, corresponding to a total parasitaemia of 5 x 10^7 to 10^8; we selected the upper limit i.e. 10^8, to ensure reliable detection of that clone and because it is consistent with the microscopy detection limit.

- The magnitude of the genetic signal that will be produced by each malaria allele in the blood sample was proportional to the number of parasites carrying that allele.
- An inherent feature of PCR is "template competition" i.e. the relative detectability of alleles at each marker depended on their length, with shorter length alleles being more detectable due to their being better amplified in the PCR process (11) . A linear relationship between allele length and relative detectability was assumed; this was done for simplicity but other relationships, for example log-linear, could also be investigated. The shortest allele in each case was assumed to have a relative detectability of 1 while the longest had a relative detectability of 0.001 i.e. we assumed the shortest allele generated a thousand times the genetic signal of the longest. This number is based on calculations from (11). Families within *msp-1* and *msp-2* were assumed to be amplified by separate reactions (i.e. are not multiplexed), so the effect only occurred between alleles within the same families (*glurp* does not have families so the effect applied to all alleles). The sensitivity of the results to this relative detectability was tested by shortening it to 0.1, see **[SM]**; we later show that it does not affect our results.

The strength of the genetic signal contributed by an allele in a given blood sample was therefore the product of two factors: The number of parasites carrying the allele times the detectability of the allele. Note that genotyping detects alleles, not parasites. Hence, if two (or more) clones within the infection shared the same allele, the signal for that allele was based on the total number of parasites

284 in the two (or more) clones. The final step is to recognise that, in practice, if one allele makes up a
285 large proportion of the genetic signal, then the smaller signals from 'minority' alleles would be
286 rejected as background "noise". We assumed this threshold to be 25% i.e. that signals from alleles
287 that were less than 25% of the highest allelic signal are rejected as "noise", though we test other
288 values of this parameter **[SM]**.

289 We do not explicitly incorporate the effect of malaria sequestration in our simulations. Sequestered
290 stages are not detectable in blood so if a malaria clone is asynchronous in its 48 hours of
291 development, its detectability will differ over consecutive days (30, 31), hence the observation that
292 sampling blood from a patient on two consecutive days greatly improves the genetic detectability of
293 clones in the patient (32, 33). WHO recommend single-day sampling, presumably for logistical
294 reasons and because of ethical considerations to treat infections as soon as possible. We do not wish
295 to enter the debate about the practicality vs desirability of single or consecutive-day sampling but
296 simply note that our results apply to both methodologies. The effect of consecutive-day sampling is
297 to improve genetic detectability of clones and sensitivity analysis of our detection limit (**SM**; Figure
298 S11) shows that improved detectability does not qualitatively affect our conclusions.

299

300 **2.3. Classifying patients according to therapeutic outcome in trials.**

301

302 Analysis of parasitaemia during patient follow-up and, if required, application of molecular
303 correction algorithms to recurrent infections. Four molecular correction algorithms (and a non-PCR
304 corrected "algorithm") were investigated. The current "WHO/MMV" algorithm (3), a "no glurp"
305 algorithm that only considers *msp-1* and *msp-2*, a "≥ 2/3 markers" algorithm that considers *msp-1*,
306 *msp-2* and *glurp* but requires matching alleles at only two markers to classify a recrudescence, and
307 an "allelic family switch" algorithm that considers only *msp-1* and *msp-2* and requires a family shift
308 to classify a recrudescence if the markers are discordant (i.e., one has shared alleles between the

initial and recurrent infections and one does not). Full details of these algorithms are presented in Table 1; they enabled each patient to be classified across four groups as would occur in a real trial i.e.

(i) An early treatment failure (ETF) if a recurrence occurs on or before day 7; note that all such recurrences are regarded as drug failures and molecular correction is not required. In our simulations, on day 3, if total parasitaemia exceeded 10^8 but was <25% of the total parasitaemia of the initial sample, the patient continued in the trial per the WHO protocol (consequently, no genotype was taken of the day 3 sample and no classification was made); if parasites were present at >25% of initial parasitaemia, that patient was classified as an early treatment failure, consistent with the WHO procedure (2) . For the purposes of estimating failure rates in this methodology, we do not distinguish between early treatment failure and recrudescence as both are indicative of drug failure.

(ii) A drug failure if a recurrence was classified as such by a PCR-correction algorithm on Table 1.

(iii) A reinfection if a recurrence was classified as such by a PCR-correction algorithm on Table 1.

(iv) 'Cleared' i.e. no recurrent parasitaemia was detected during follow-up; in these cases, the drug was assumed to have successfully killed all parasites present at time of treatment.

A key objective of this paper was to investigate how well the classification algorithms applied to recurrent infections (Table 1) recovered the true status of recurrent infections. We therefore defined the latter according to parasitaemia data from the PK/PD model (Figure 1).

- True recrudescence was defined as a recurrent infection that contained at least 10^8 parasites from a clone present at time of treatment (this patient is, by definition, a drug

failure). This included patients who have a 'mixed' infection on the day of recurrence i.e. possessed malaria clones that survived treatment plus reinfection clones that were acquired during follow up, providing the former exceed 10^8; note that all clones contributed to the genetic signal of the recurrence as described above.

- True reinfection was defined as a recurrent infection whose blood sample contained only parasites from clone(s) that were reinfection(s) (note that such patients may harbour parasites from original clones if these clones were sub-patent i.e. less than 10^8 parasites).

It was possible that recrudescent clones may not have reached microscopically detectable levels (i.e. parasite numbers are $<10^8$) on the final day of follow-up; such patients would be classified as "cleared" in vivo and thus, a treatment success. However, simulated data have confirmed that it is possible for some patients to still harbour parasites below detection level at the end of follow up (23). Our modelling approach classifies these patients as drug failures.

Note there are only a finite number of alleles at each locus and, thus, two distinct clones of malaria may have had identical allele(s) at one or more marker purely by chance. It followed that reinfections and recrudescences could share alleles, so misclassification of reinfection as recrudescence was possible.

2.4. Estimating drug failure rates in the simulated trials

The model was run for a cohort of 5,000 patients (although any number can be simulated). This is an unrealistically high number for an *in vivo* clinical trial but is ideal for our purposes: A true drug failure rate of 10-12% provided a large number of recurrences (the exact number varied depending on the

ACT, FOI, and length of follow up) that we can test against the various classification algorithms and reduces the uncertainty around results.

The four patient outcomes described above were used to calculate the estimated drug failure rate, $\hat{F}$ in the same manner as outcomes reported *in vivo*). It was assumed, for simplicity, that no patients were lost to follow-up or removed from the trial for any reason other than recurrent parasitaemia. There were three methods for calculating failure rates which differed in how they processed patients with recurrent parasitaemia that had been classified as reinfections, noting that all patients with recurrent parasitaemia would, *in vivo*, be re-treated with another antimalarial (for ethical reasons) and removed from the trial. The three methods were: A non-PCR corrected failure rate, a "per protocol" failure rate and a failure rate obtained using survival analysis. The latter two methods are recommended by the WHO to analyse anti-malarial drug trials (2, 3). Technically, they were calculated as follows using the following nomenclature:

C_o was the number of patients who cleared infection.

nI_o, was the number of patients whose recurrent infections were classified as reinfections.

$\hat{F}$ was the estimated drug failure rate.

N was the total number of patients.

(i) The non-PCR corrected failure rate was obtained by considering all patients with recurrent infections as patients who had failed drug treatment. This method did not require distinguishing between reinfections and recrudescent infections. The failure rate $\widehat{\boldsymbol{F}}$ could then be estimated as:

$$\widehat{\boldsymbol{F}} = \mathbf{1} - \frac{\boldsymbol{C_o}}{\boldsymbol{N}} \qquad \text{Equation 1}$$

(ii) The 'per protocol' method, recommended by WHO (2, 3, 5), simply removed patients who were classified as reinfections from the total number of observations i.e.:

$$\widehat{F} = 1 - \frac{C_o}{N-(nI_o)} \quad \text{Equation 2}$$

(iii) Survival analysis, as recommended by WHO (3), used the survivor function from a Kaplan Meier plot on the final day of follow-up, right-censoring reinfections.

The Kaplan-Meier estimator KM of survivorship at time *t* was obtained as:

$$\widehat{S}(t) = \prod_{ti \le t} \frac{ni-di}{ni} \quad \text{Equation 3}$$

Where *t* was a vector of all timepoints i.e. days of follow-up in which an event occurred in the study population, *ni* was the number of individuals at time *ti* who remained uninfected, and *di* was the number of events (drug failures in this case) that occurred at timepoint *ti*. Plainly, what this method did was calculate the proportion of patients who remained free of recrudescence between consecutive days of follow up, then multiplied all these time periods to obtain the overall probability of 'surviving' recrudescence-free over the whole follow-up period. The advantage was that even those patients who are "censored" (by acquiring a reinfection and leaving the study) will still contribute to the analysis through their inclusion prior to their removal.

The estimator at the final time-point (i.e. the last day of follow-up) was the probability that their treatment was considered a 'success' at the end of the trial. Consequently, it's complement gave the probability that a given individual will fail treatment i.e.

$$\hat{F} = 1 - \hat{S}(t) \quad \text{Equation 4}$$

The final methodological step was to interrogate the modelled data to determine the "true failure rate" – i.e., the drug failure rate calculated directly from the parasitaemia of each patient (thus, not dependent on genotyped data). For each patient in the simulation, an outcome on the final day of follow-up was determined: If, on the final day, the patient had any parasites from any initial clones (i.e. even a single parasite), the patient was denoted as a drug failure. If no parasites had survived from the initial clones present at treatment, that patient was denoted as a treatment success.

The true failure rate, *F*, for the patient population was then calculated:

$$F = \frac{f}{N} \qquad \text{Equation 5}$$

Where *f* was the number of drug failures on the final day of follow-up and *N* was the total number of patients.

This was the "gold standard" metric and cannot be obtained *in* vivo. It was compared to the estimated failure rates obtained from modelling the clinical trial and molecular correction process and allowed us to quantify the accuracy of different methods (i.e., their ability to recover the true failure rate).

2.5 Reanalysis of existing *in vivo* data

Clinical data was obtained from Rwanda (a relatively high transmission area) across 6 sites between 2013 and 2015, where patients were treated with either AR-LF or DHA-PPQ and genotyped at *msp-1, msp-2* and *glurp*. In patients treated with AR-LF, 137 recurrences were observed, of which 110 could be classified as either a reinfection or a recrudescence (it was not possible to classify 27 patients because they had incomplete genetic data). In patients treated with DHA-PPQ, 48 recurrences were observed, of which 43 could be classified as either a reinfection or a recrudescence (it was not possible to classify 5 patients because they had incomplete genetic data). This data was initially presented internally to the National Malaria Control Programme in Rwanda (a manuscript describing clinical efficacy studies for publication is pending).

Clinical data from Cambodia (a relatively low transmission area) was obtained from 6 sites between 2014-2016. Patients were treated with either artesunate plus amodiaquine (AS-AQ), artesunate plus pyronaridine (AS-PYN) or DHA-PPQ, and genotyped at *msp-1*, *msp-2*, and *glurp*. In patients treated with AS-AQ, 12 recurrences were observed, of which 5 could be classified as reinfection or recrudescence (7 patients had incomplete genetic data). In patients treated with AS-PYN, 14 recurrences were observed, of which 12 could be classified as reinfection or recrudescence (2 had incomplete genetic data). In patients treated with DHA-PPQ, 67 recurrences were observed, of which 48 could be classified as reinfection or recrudescence (19 had incomplete genetic data). This data was initially presented internally to the National Malaria Control Programme in Cambodia. A description of the AS-PYN trials has already been published (34).

For all data, the genetic signals (i.e., the *msp-1, msp-2* and *glurp* alleles at the initial sample and any recurrent sample) were re-interpreted using the novel molecular correction algorithms described in Table 1 to investigate how varying the molecular correction algorithm changed the classification (as reinfection or recrudescence) of patients and, consequently, failure rate estimates.

2.6 Data Availability

The R code used to generate the results describe herein is available from the authors. The re-analysed trial data-sets are likewise available from the authors.

3. Results

We identified several types of misclassification of recurrent infections in our experiments:

1. Recrudescent infections could be misclassified as reinfection if the recrudescent allele(s) were not detected during the genotyping of the initial infection i.e. for example they were "minority alleles" (see methods).

2. A recrudescent infection could be misclassified as a reinfection if the recrudescent allele(s) were not detected during the genotype of the recurrent infection i.e. for example they were "minority alleles" (see methods).
3. A reinfection could be misclassified as recrudescent if it shares (by chance) alleles with clones present at time of treatment. The exact number (or type) of alleles that must be shared depended on the molecular correction algorithm chosen (i.e., the "no glurp" algorithm was not affected by sharing an allele at glurp, and the "allelic family switch" algorithm was sensitive to sharing an *msp-1* or *msp-2* family by chance, whereas the other algorithms were not).

While not misclassification of recurrence, another source of bias affected the accuracy of failure rate estimates with respect to the true failure rate:

- A patient who failed to clear their initial infection may have had that infection persisting at a low-lying level, below the limit of detection of detection of light microscopy (assumed, see later, to be 10^8 *total* parasites in all clones), *and* have no reinfection, such that parasites were never detected during follow-up (and thus, no recurrent sample was genotyped); this obviously depends on the duration of follow-up.

3.1 Impact of Algorithm choice on failure rate estimates

Figure 2 shows the failure rates obtained from simulated DHA-PPQ clinical trials using four molecular correction algorithms and the non-corrected algorithm (Table 1), with a follow-up length of 42 days. Both the true failure rate and the estimated failure rate are presented (calculated using survival analysis) as a function of FOI.

The non-corrected algorithm always produced a higher failure rate estimate than any of the four molecular correction algorithms (Figure 2). Failure rate estimates using no correction rose rapidly as FOI increased and at moderate and high levels of transmission estimated failure rates were substantially greater than the true failure rate: At high transmission intensities (FOI of 16) estimated failure rates produced by this algorithm were above 50% - a clear over-estimate of the true failure rate (12%): This pattern occurred because all the additional reinfections that occurred at as FOI increased were misclassified as recrudescence. Conversely, in the absence of any reinfections (when FOI=0), the non-corrected algorithm produced an accurate failure rate estimate by correctly classifying all recurrences as recrudescence (leaving only a slight under-estimate due to patients who had recrudescent parasites at levels of $<10^8$, such that no recurrence occurred during follow-up).

The ability of the four molecular correction algorithms to accurately estimate drug failure rates depended on their ability to correctly classify recrudescences and reinfections. This ability is shown (for an FOI of 8, i.e. a moderate transmission area) in Figure 3. Each algorithm misclassified some proportion of recrudescences and reinfections. The number of recrudescence misclassified as reinfections was consistent as FOI changed, but the number of reinfections misclassified as recrudescence increased as FOI increased – results shown in **[SM]** (note that while results for all parameterizations of AR-LF, AS-MQ and DHA-PPQ are not shown, the proportion of misclassification was extremely robust between drugs). General trends were extremely clear:

- The "WHO/MMV" algorithm consistently under-estimated failure rates at all transmission intensities as shown in Figure 2. The algorithm frequently failed to detect drug failures i.e. it misclassified around 40% of recrudescent infections as reinfections (Figure 3). These misclassifications occurred because of failure to detect recrudescent alleles in either the initial or recurrent blood sample – this algorithm was so stringent (requiring matching alleles at all three markers) that even missing a single allele could result in misclassification. As FOI

499 increased, the estimated failure rate did not change to any meaningful extent because the
500 algorithm correctly classified nearly all reinfections (Figure 3).
501
502 • The "no glurp" algorithm produced slightly higher estimated failure rates than the
503 "WHO/MMV" algorithm across all FOI settings (Figure 2). This occurred because
504 recrudescences were slightly less likely to be misclassified as reinfections while reinfections
505 were slightly more likely to be misclassified as recrudescences than under the "WHO/MMV"
506 algorithm (Figure 3). At low FOI, this difference was small; the high allelic diversity of *msp-1*
507 and *msp-2* meant misclassification of reinfections as recrudescences was rare. The
508 difference between the "no glurp" algorithm and the "WHO/MMV" algorithm increased as
509 FOI increased, but, like the "WHO/MMV" algorithm, the "no glurp" algorithm always under-
510 estimated the true failure rate.
511
512 • The "≥ 2/3 markers" algorithm produced higher estimated failure rates than the "no glurp"
513 algorithm across all FOI levels. This occurred because this algorithm reduced the chance of a
514 recrudescence being misclassified as reinfection (due to failure to detect recrudescent
515 alleles) and increased the chance of a reinfections being misclassified as a recrudescence
516 (Figure 3). Both effects occurred because only needing matching alleles at 2/3 markers gave
517 the algorithm some tolerance to un-detectable alleles.
518
519 • The "allelic family switch" algorithm produced higher estimated failure rates than the "≥ 2/3
520 markers" algorithms at all but the lowest FOI (0-2) settings (Figure 2). A complete family
521 switch in *msp-1* or *msp*-2 in a discordant sample (Table 1) would be sufficient to classify a
522 recrudescence; this led to a similar number of recrudescence being correctly classified as the
523 "≥ 2/3 markers" algorithm, but this algorithm misclassified the largest number of
524 reinfections as recrudescence out of all the molecular correction algorithms – the family

switch could still occur (by chance); the difference in numbers misclassified between the "no glurp" algorithm and the "allelic family switch" algorithm is the result of this misclassification by chance.

3.2 Impact of follow-up length on failure rate estimates

Alternate durations of follow-up length were simulated for DHA-PPQ and their impact on estimated failure rates are shown in Figure 4 for 28, 42 and 63 days of follow-up. Longer durations of follow-up led to larger estimated failure rates for all algorithms. This occurred because longer follow-up (i) allowed more time for recrudescences to become detectable, (ii) allowed more reinfections to emerge, some of which were misclassified as recrudescences (Figure 3).

Under-estimation of the true failure rate occurred with all algorithms when a 28-day follow-up period was chosen. With a 42-day follow-up period, the "allelic family switch" algorithm produced the most accurate failure rate estimate with an FOI of <7, and the "≥ 2/3 markers" algorithm produced the most accurate failure rate estimate with FOI ≥7. As length of follow-up increased to 63 days, the "≥ 2/3 markers" algorithm tended to slightly over-estimate the failure rate. This effect was more apparent as FOI increased. These patterns emerged because only a small number of initial clones recrudesced after 42 days. Figure 5 shows the proportion of recurrent infections on each day of the follow-up period that were truly recrudescent or reinfections. On days 49, 56 and 63, the number of recurrent infections that were truly recrudescent was small. Almost all recurrent infections on these days were reinfections and consequently, inclusion of these three extra days of follow-up inflated the estimated failure rate due to misclassification of these reinfections as recrudescences (as alleles were shared by chance between these reinfections and the initial blood sample). However, the increased failure rate of a 42 day follow-up compared to a 28 day follow-up

(due to both detection of true recrudescence and misclassification of extra reinfections) meant that a 42 day follow-up period analysed with either the "≥ 2/3 markers" or "allelic family switch" algorithm produced more accurate failure rate estimates than the "WHO/MMV" algorithm.

3.3 Results for other drugs / parameterizations / model settings

Additional models for other ACTs are described in **[SM]**. In brief these drugs differed from DHA-PPQ mainly in their persistence of active drug concentrations post-treatment (and hence in their prophylaxis against reinfections). Results for failing AR-LF and AS-MQ were highly consistent with those described above for DHA-PPQ, showing the same qualitative patterns (i.e., that failure rate estimates increase as FOI increases and as the follow-up period increases, that the WHO/MMV algorithm under-estimates, that no correction leads to large over-estimates and that the "≥ 2/3 markers" algorithm was generally accurate across a range of FOI values).

Different prophylactic profiles meant that the most effective duration of follow-up for AR-LF and AS-MQ (as would be expected) differed to DHA-PPQ; using the "≥ 2/3 markers" molecular correction algorithm, a 28 day follow-up for AR-LF appeared to be most accurate at moderate to high FOI. A 49 day follow-up for AS-MQ appeared to be the most accurate with the "≥ 2/3 markers" algorithm but increased accuracy over using the "WHO/MMV" algorithm was also seen with using shorter follow-up periods.

Models of non-failing (i.e. clinically effective) PK/PD calibration of AR-LF and AS-MQ showed that the "≥ 2/3 markers" algorithm slightly under-estimated true failure rate but that this difference was small and there is no evidence that this algorithm would incorrectly identify effective drugs as failing.

Alternative parasite dynamics for DHA-PPQ were generated using a three-compartment model with PK parameters described in (35) to reflect to uncertainty around how PPQ should be modelled (we previously identified and analysed 6 published and distinct PK calibrations for Piperaquine (23));note that PD parameters remained as for the two-compartment assumption, as described in **[SM]**). Parasite dynamics obtained using this three-compartment calibration resulted in a more prophylactic drug (i.e., fewer reinfections became patent) with a lower true failure rate (10%, with unchanged PD parameters). The relative failure rate estimates of the algorithms and the no-correction approach were the same – i.e., that "WHO/MMV" algorithm produces the lowest failure rate estimate, followed by "no glurp", "≥ 2/3 markers", and the allelic family switch algorithm. Failure rate estimates are lower across all algorithms than with the shorter-prophylaxis two-compartment model, and a 63-day follow-up appears to be the most suitable under this calibration; the "≥ 2/3 markers" algorithm produced an accurate failure rate estimate at all but the lowest FOI levels with this follow-up length). Crucially, the key message is the same: The WHO/MMV algorithm under-estimates true failure rate and other algorithms can produce more accurate failure rate estimates. Perhaps the most interesting difference between the two DHA-PPQ PK/PD calibrations is that they suggested, given use of the same molecular correction algorithm, different optimal length of follow-up.

Failure rate estimates were calculated using the per-protocol method (methods) rather than survival analysis. The per-protocol method led to increased failure rate estimates with all algorithms, all ACTs and all follow-up periods. These results are discussed in **[SM]**.

Finally, the simulation was validated by varying the multiplicity of infection (MOI) at time of treatment, the relative detectability of alleles based on length, and the minority allele detection threshold. The results of these analyses are provided in **[SM]** and showed mostly the same qualitative patterns as the results presented above; the one key departure was that assumption of a

minority allele threshold of 5% (reduced from the assumption of 25%, above) lead to slightly increased failure rates and the "no glurp" algorithm being the most accurate at moderate to high FOI.

3.4 Re-analysis of clinical data

Clinical data from Rwanda (a relatively high transmission area) were re-analysed using the proposed molecular correction algorithms (Table 2), and were highly consistent with our models i.e. the "WHO/MMV" algorithm produced the lowest estimated failure rate, followed by "no glurp", then the "≥ 2/3 markers" algorithm, then the "allelic family switch" algorithm. The pattern was quantitively consistent: The "WHO/MMV" algorithm estimated failure rates to be around half that obtained by the "≥ 2/3 markers" algorithm. Results are similarly consistent with re-analysis of a trial from low transmission settings in Cambodia (Table 2). The impact of algorithm choice was not so large in Cambodia because FOI was low: 62 of the recurrences had matching alleles at all 3 loci so were presumably drug failures and would have been classified as such by all four algorithms. There were only 3 potential reinfections (all following DHA-PPQ treatment): 1 had no shared alleles at any locus so was classified as a reinfection under all four algorithms, but the other two patients shared alleles at both *msp-1* and *msp-2* and were only classified as reinfections under the "WHO/MMV" algorithm because no common alleles were noted at *glurp*. In contrast, the other algorithms all classified both patients as being drug failures. In summary, as in the high transmission data, the "WHO/MMV" algorithm had a higher tendency to classify recurrences as reinfections compared to the other algorithms. Note also that, consistent with Figure 4, the choice of algorithm makes little operational difference at low FOI: using the "WHO/MMV" algorithm identified 62 drug failures and three reinfections, while the other algorithms give 64 drug failures and one reinfections, a negligible increase in number of drug failures.

Finally, we reviewed clinical trials that reported failure rates based on no correction and the "WHO/MMV" algorithm (Table 3). The magnitude of differences in failure rate estimates were similar to those noted in the results from our model where the non-corrected algorithm and the "WHO/MMV" algorithm produced the highest and lowest failure rate estimates respectively.

4. Discussion

The key message presented here is that none of the proposed algorithms using *msp-1*, *msp-2* and *glurp* correctly classified all recurrent infections (Figure 3) nor is it likely that such an algorithm exists due to the limitations of the PCR correction process (11). The ability of each algorithm to accurately recover the true failure rate was dependent on the transmission intensity (quantified in these models by FOI) due to the differing propensity of each algorithm to misclassify reinfections as recrudescence (which occurred when alleles are shared by chance or a clone that later recrudesces was not observed in the initial sample); Figure 3. The 2-fold under-estimation of true failure rates that occurred at all FOI levels using the current "WHO/MMV" algorithm is a cause for considerable concern. This under-estimate occurred because this algorithm was extremely stringent – it did not misclassify any reinfections as recrudescence (Figure 3) – but did misclassify some recrudescences as reinfections when a clone that later recrudesced wasn't detected in the initial sample (due to the issues inherent in the PCR methodology with detecting minority alleles and longer alleles). These issues are shared between algorithms; however, "no glurp", "≥ 2/3 markers" and the "allelic family switch" algorithm are all less stringent and misclassified some reinfections as recrudescence (Figure 3), which increased failure rate estimates and accounted – to some extent – for the under-estimation of failure rates.

651 Key to identifying a methodology that gives consistently accurate estimated failure rates is to
652 minimise and balance errors that arise from molecular correction, which are, in turn, influenced by
653 factors including FOI, duration of follow-up, and sensitivity of the PCR protocols. Despite these
654 concerns, these results show that operationally-important increases in accuracy of estimated failure
655 rates for anti-malarial efficacy trials are achievable with alternate genotyping algorithms. It is
656 undesirable to recommend different molecular correction algorithms for different ACTs and
657 transmission intensity levels (as this would be likely to cause confusion), hence the approach of
658 investigating multiple ACTs and varying transmission intensity through FOI to assess if a single
659 algorithm may be identified that gives robust and accurate estimates. Based on the results
660 presented here, it appeared that the "≥ 2/3 markers" algorithm was the most robust in areas of
661 moderate to high transmission, and provided estimated failure rates close (typically within 2
662 percentage units) to the true failure rate (Figure 2, Figure 4, **[SM]**).

663 The other factor that can affect estimates of drug efficacy, given that molecular correction is
664 imperfect, is the duration of follow-up. Recommended duration has gradually increased over the last
665 20 to 30 years, with the objective of capturing all (or at least the majority) of recrudescences.
666 However, the objective of clinical trials is *not* to capture every recrudescence, but to obtain accurate
667 and robust estimates of efficacy. Figure 5 shows that in areas of moderate to high FOI, the penalty
668 for detecting the last few recrudescences by extending the follow-up period was the inclusion of a
669 much larger number of reinfections. These reinfections inflate the estimated failure rate due to the
670 propensity of molecular correction algorithms to misclassify some reinfections as recrudescence
671 (Figure 3). It is obviously preferable to have the shortest follow-up possible while retaining accuracy
672 of failure rate estimates; based on the results shown in Figure 4, and analogous plots for failing AR-
673 LF and AS-MQ (**[SM]**), using the "≥ 2/3 markers" algorithm provided accurate estimates using a
674 follow-up of 28 days for AR-LF, 42 days for DHA-PPQ and 49 days for AS-MQ, all roughly in line with
675 current WHO recommendations (2, 3). Importantly, the accuracy of the estimates with this algorithm
676 appeared to be relatively robust to changes in transmission intensity, quantified in these models by

FOI (the "WHO/MMV" and "no glurp" algorithms were also robust to changes in FOI, but had an under-estimate of failure rate associated with them). Note that a different DHA-PPQ parameterization (i.e., one that is more prophylactic, see **[SM]**) favoured a longer follow-up period more in line with MQ which also has longer prophylaxis post-treatment. The trends across all drugs modelled are clear: it is highly likely that use of the current "WHO/MMV" algorithm will generate substantial (near two-fold) underestimates of failure rates and that switching to an alternative correction algorithm should be considered as matter of urgency.

Technical problems with molecular correction approaches exist (identified and explained in, for example, (9, 11)) which gives rise to the temptation to simply ignore molecular correction and just use uncorrected data. The results presented here strongly suggest that appropriate use of molecular correction is essential. Trials conducted in areas of moderate to high transmission intensity, which are the areas where most malaria morbidity and mortality occur, analysed without molecular correction will lead to severe over-estimates of the true failure rate. This assertion is supported by clinical data (Table 3), which clearly shows that large discrepancies may arise in the absence of molecular correction. Ignoring molecular correction (i.e. non-PCR corrected algorithm in Figure 2 & Figure 4) only produced accurate estimates of failure rates when FOI is very low (a fact generally acknowledged in the literature (2, 3)). However, caution must be taken even when using no-correction in "low" transmission areas. Malaria transmission is highly focal and even if an area is, on average, very low transmission, it is plausible that most patients will be recruited from foci of high transmission where FOI may well be sufficient to invalidate estimates based on no-correction.

The evaluation of different classification algorithms relied on simulated data. This was not ideal but there is no obvious alterative given that key parameters (including the vital one: true failure rate), cannot be directly observed *in vivo*. Confidence in this approach was assured given the past success of pharmacological modelling to correctly reflect and predict clinical data e.g. (4, 14-16, 18, 22), and the consistency of the simulated results with *in vivo* Rwandan and Cambodian data-sets. We

acknowledge that our model may not reflect the *in vivo* parameters of these trials (though see discussion for the parameter space we covered in **[SM]**), however, the purpose of re-analysis of these data were to investigate the change in failure rates from us of proposed algorithms on *in vivo* data – analysis of trial results with these algorithms has not previously taken place. This re-analysis is not dependant on our model parameter space (nor vice versa), and all algorithms require the same data (the *msp-1, msp-2* and *glurp* alleles (and families for the former two)); consequently, this re-analysis showing similar trends to our modelled results is encouraging.

Focus has been on the current WHO-recommended marker loci *msp-1*, *msp-2* and *glurp* and how they may be best used to distinguish recrudescences from reinfections; it would be straightforward to repeat these analyses for different types of molecular data, such as deep sequenced amplicons, microsatellites and SNP barcodes, and this is discussed further in **[SM]**. Notably, reduction of the minority detection threshold to 5% increased the failure rate estimates and altered which algorithm produced the most accurate estimate. We are confident that the length polymorphic markers do not have this level of sensitivity; we analysed this assumption solely to test its effect on our results, however, this threshold emulates more closely the use of amplicon sequencing where minority alleles are easier to detect, and we intend to test the accuracy of failure rate estimates with amplicon sequencing using a similar methodology in the future.

There is concern in the literature that reinfections may share alleles with the initial infection purely by chance and that subsequent misclassification of reinfections as recrudescence would lead to over-estimation of failure rates (9). This could arise in areas of high transmission (7) as increased MOI leads to more alleles in the initial sample; these can later be shared with a reinfection purely by chance. It could also occur in low-transmission areas where genetic diversity is lower and there is more chance of a match by chance. Importantly, we do not observe large-scale over-estimation (e.g. the low impact of FOI on estimated failure rate using the "≥ 2/3 markers" algorithm in Figure 2

& **[SM]**) with increased transmission intensity with either a high MOI (Figure 4) or a low MOI **[SM]**, suggesting these fears are unlikely to have a large impact in practice.

In conclusion, our modelling approach and re-analysis of clinical data both suggest that more accurate and easily implemented algorithms are available to analyse clinical data and the field should consider implementing these methods. Which algorithm will perform best will depend on factors in the patient population/area - our results demonstrate this explicitly for transmission intensity (FOI) and follow-up length. The four algorithms investigated here are not mutually exclusive and are based on the same data. Our firm recommendation is that initial and recurrent samples should be genotyped at all three loci: when using the current "WHO/MMV" algorithm, there is no need to genotype after a mismatch has occurred at one locus, so genotyping is often incomplete. These complete data would allow results obtained from all four algorithms be presented; this maintains consistency with previous analyses based on the "WHO/MMV" algorithm while also providing results that are likely to provide a substantially more robust estimate of malaria drug clinical failure rates.

Contributors statement

SJ wrote the original manuscript, designed the computer models, ran the simulations and analysed the results.

KK designed the computer models and analysed the results.

EMH designed the computer models and analysed the results.

SC performed additional genotyping on Rwandan data samples.

AM provided Rwandan clinical trial genotyped samples.

AU provided Rwandan clinical trial genotyped samples.

DM arranged access to, and additional genotyping of, the field samples from Cambodia and Rwanda.

IF provided technical background on genotyping techniques to calibrate the models.

IH conceived the project, designed the computer models and analysed the results.

All authors were involved in critically and extensively revising the original draft of the manuscript into the finished version presented here.

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Conflict of interests statement

The authors have no conflicts of interests to declare.

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Tables and Figures

Table 1. Molecular correction algorithms proposed to decide whether a patient re-presenting with a recurrent malaria infection during follow up is a recrudescence or a reinfection based on the WHO-recommended genetic markers of *msp-1*, *msp-2 and glurp*. We also summarise the consequences of applying these algorithms for the analysis of clinical trials as quantified by our methodology: the failure rate estimates obtained from each algorithm are shown in Figure 2 & Figure 4.

Table 2. Molecular correction with multiple algorithms from re-analysis of clinical trial data from Rwanda (a high transmission study sites) and Cambodia (a low transmission sites); full details of study sites and methodology are provided in methods

Table 3: The need for molecular correction: a comparison of estimated drug failure rates obtained without correction vs with molecular correction performed according to the current WHO/MMV recommended algorithm. Failure rate was calculated as 1 minus the 28-day adequate clinical and parasitological response reported in the studies (data collated and provided by Drs Jörg Möhrle and Stephan Duparc).

Figure 1: Malaria parasite dynamics following treatment of a hypothetical patient and the need for molecular correction (adopted from Jaki et. al (2013) (25)). Note that parasites only become detectable in the patient's blood by light microscopy once their numbers exceed a detection limit at 10^8 parasites. The blue solid line shows the declining concentration of drug post-treatment as it is eliminated by patient's metabolism. This patient had four malaria clones detectable at time of treatment: The green lines represent initial clones that are cleared by the drug, the red line represents an initial clone that recrudesces. Reinfections periodically emerge from the liver during follow-up in cohorts of ~10^5 parasites per clone (orange line). The grey lines are reinfections that are cleared by the drug. The orange lines are reinfections that are not cleared and survive to reach

patency (i.e. increase in number to>10^8 at which point they are detectable by microscopy). The solid black line is the point during follow-up at which the patient first has a patent recurrent infection i.e. has a parasitaemia sufficiently high that it is detectable by microscopy.

Figure 2 : Analysis of simulated trial data for DHA-PPQ with a follow-up period of 42 days. Estimated failure rates are shown for the different algorithms of molecular correction (Table 1) as a function of Force of Infection (FOI) and are calculated using survival analysis. Mutiplicity of Infection (MOI) is drawn from data from Tanzania – a relativley high transmission area.

Figure 3: Figure showing the ability of the various molecular correction algorithms to correctly classify patients with recurrent malaria. The data are for DHA-PPQ with a 42-day follow-up obtained with a FOI of 8 (i.e. used to obtain the results shown at FOI=8 in Figure 2). Mutiplicity of Infection (MOI) is drawn from data from Tanzania – a relativley high transmission area. The X-axis shows the true status of patients on the day of recurrence (i.e. reinfection or a recrudescence) and the colour-coding shows how these patients were classified by each algorithm. The WHO/MMV recommended algorithm correctly classifies nearly all reinfections, but misclassifies around one third of recrudescences. The "no glurp" algorithm is similar to the WHO/MMV one; it misclassifies only a small number of reinfections, but misclassifies around a third of recrudescences. The "≥2/3" had fewer misclassifications and was also more balanced i.e. misclassified a similar proportion of both reinfections and recrudescences. Finally, the allelic family switch algorithm correctly classifies a large proportion of recrudescences but misclassifies around half of reinfections.

Figure 4 : Analysis of simulated trial data for DHA-PPQ showing the impact of changing follow-up period with follow-up lengths of (A) 28 days, (b) 42 days (as in Figure 2), and (C) 63 days. Estimated failure rates are shown the different molecular correction algorithms (Table 1) as a function of FOI and calculated using survival analysis. Mutiplicity of Infection (MOI) is drawn from data from Tanzania – a relativley high transmission area.

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966 Figure 5 : The true status of recurrent infections on each day of follow-up for a simulated trial of
967 DHA-PPQ with a true failure rate of 12% and an FOI of 8. Mutiplicity of Infection (MOI) is drawn from
968 data from Tanzania – a relativley high transmission area. The total height of the bars indicates the
969 number of recurrent infections detected on that day of follow-up, and the color-coding shows the
970 number of those recurrent infections that were truly recrudescent or reinfections.

971

Algorithm	Reference	Definition	Consequences (identified in the model)
No Correction		All recurrent infections classified as recrudescence	Grossly over-estimates failure rate at higher FOI
WHO/MMV	(2)	Initial and recurrent samples must have shared alleles at all three markers to be classified as recrudescence.	Stringent conditions for recurrences to be classified as recrudescence means that around 50% of true recrudescence are misclassified as reinfections resulting in greatly underestimated failure rates. Most reinfections are correctly classified, so FOI has little impact on estimated failure rate
No glurp	(11)	As for the WHO/MMV algorithm but based on two loci (i.e. *msp-1* and *msp-2; glurp* is omitted as it is prone to genotyping errors).	Largely identical to the WHO/MMV method
≥ 2/3 markers	(11)	As for the WHO/MMV algorithm, but initial and recurrent samples must share alleles at least at two out of three markers to be classified as recrudescence.	Generally intermediate between "no glurp" and "allelic family switch" algorithms

Allelic family switch	(11)	Comparison initially based on *msp-1* and *msp-2*. Identical alleles observed at both markers indicate a recrudescence. Absence of shared alleles at both markers indicate a reinfection. If one marker shares alleles and one does not (i.e. the sample is "discordant"), a complete allelic family shift in the non-sharing marker is required to classify a recurrence as a reinfection	Tendency to misclassify reinfections as recrudescences leads to a dependency on FOI and results in large overestimates of failure rates at higher FOI, though produces accurate failure rate estimates at low FOI.

FOI = Force of Infection, our measure of transmission intensity. FOI is the mean number of malaria infections that emerge in an individual and would become patent in the absence of drug killing over the course of a year.

Table 1. Molecular correction algorithms proposed to decide whether a patient re-presenting with a recurrent malaria infection during follow up is a recrudescence or a reinfection based on the WHO-recommended genetic markers of *msp-1*, *msp-2 and glurp*. We also summarise the consequences of applying these algorithms for the analysis of clinical trials as quantified by our methodology: the failure rate estimates obtained from each algorithm for DHA-PPQ are shown in Figure 2 & Figure 4.

Country	Drug*	Classification of recurrent infection	WHO/MMV	No glurp	≥ 2/3 markers	Allelic family switch
Rwanda	AR-LF	recrudescence	17	27	36	59
		reinfections	93	83	73	51
	DHA-PPQ	recrudescence	3	6	8	18
		reinfections	40	37	35	25
Cambodia	AS-AQ	recrudescence	5	5	5	5
		reinfections	0	0	0	0
	DHA-PPQ	recrudescence	45	47	47	47
		reinfections	3	1	1	1
	AS-PYN	recrudescence	12	12	12	12
		reinfections	0	0	0	0

AS-AQ is artesunate plus amodiaquine; DHA-PPQ is dihydroartemisinin plus piperaquine; AR-LF is artemether plus lumefantrine. AS-PYN is artesunate plus pyronaridine.

Table 2. Molecular correction with multiple algorithms from re-analysis of clinical trial data from Rwanda (a high transmission study sites) and Cambodia (a low transmission sites); full details of study sites and methodology are provided in methods

Drug tested	Uncorrected vs corrected failure rates	Country/yr	Ref
AR-LF AS-AQ	54% vs 10% 42% vs 10%	Burkina Faso, 2014	(34)
AS-AQ	17% vs 6%	Congo, 2013	(35)
AR-LF	22% vs 0%	Tanzania, 2014	(36)
AR-LF	13% vs 0%	Benin, 2016	(37)
AR-LF	9% vs 2%	Mozambique, 2015	(38)
AR-LF	2% vs 1%	India 2015	(39)
AR-LF AS-AQ	16% vs 1% 22% vs 5%	Congo 2012	(40)

AR-LF, artemether plus lumefantrine; AS-AQ, artesunate plus amodiaquine

Table 3: The need for molecular correction: a comparison of estimated drug failure rates obtained without correction vs with molecular correction performed according to the current WHO/MMV recommended algorithm. Failure rate was calculated as 1 minus the 28-day adequate clinical and parasitological response reported in the studies (data collated and provided by Drs Jörg Möhrle and Stephan Duparc).