Statistical Analysis Plan

WWARN Primaquine Methaemoglobin Study Group:

Methaemoglobin as a surrogate marker of primaquine antihypnozoite activity in *Plasmodium vivax* malaria

A systematic review and individual patient data meta-analysis

Version 1.1

**WorldWide Antimalarial Resistance Network (WWARN)**

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| Version 1.0 |  | June 2023 |
| Version 1.1 | * Added '... surrogate ...' to the study title for better description of the analysis * Added quinine as one of the schizontocidal treatments considered to include a relevant study * Updated the causal directed acyclic graph for better clarity * Updated the logarithmic transformation to base 2 for improved clinical interpretability of the model estimates * Added a random-effects term for day-7 methaemoglobin concentration to allow estimation of between-site variability of this predictor * Added primaquine duration as an adjustment factor and its interaction term with daily primaquine dose to improve model specification * Updated signalling questions for risk of bias assessment for better clarity | February 2024 |

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# 1. Introduction and Rationale

## 1.1. Background

*Plasmodium vivax* is the most geographically widespread cause of human malaria. Its characteristic ability to form dormant liver-stage hypnozoites leads to cumulative morbidity and mortality over time, as well as confounding malaria control activities, since acute episodes may recur (relapse) weeks to months after an initial infection. Currently, the only widely available drug to prevent relapses is the 8-aminoquinoline primaquine. Primaquine isa prodrug whose active metabolites remain largely unknown and uncharacterised. Preliminary data suggest that methaemoglobin production may be a correlate of the production and exposure to the active metabolites driving drug efficacy (1). This review will synthesise available longitudinal methaemoglobin data in patients with symptomatic vivax malaria treated with primaquine to investigate the utility of using day-7 methemoglobinemia as a pharmacodynamic proxy measurement that predicts treatment failure following radical cure.

## 1.2. Aim of the study

The aim of this study is to investigate the utility of day-7 methemoglobinemia as a pharmacodynamic proxy measure of primaquine antihypnozoite activity in patients with symptomatic *Plasmodium vivax* malaria.

## 1.3. Eligibility criteria for inclusion in analysis

## ****Essential criteria of eligible studies****

* Prospective clinical efficacy studies of uncomplicated vivax malaria; including randomised and non-randomised therapeutic trials, and prospective cohort studies with active follow-up
* A minimum follow-up of 42 days
* Treatment with a daily primaquine regimen given within the first three days of a schizontocidal treatment, i.e., chloroquine or quinine or one of six common artemisinin-based combination therapies (artemether-lumefantrine, artesunate-mefloquine, artesunate-amodiaquine, dihydroartemisinin-piperaquine, artesunate-sulfadoxine-pyrimethamine, artesunate-pyronaridine)

## ****Essential data for inclusion in the analysis****

* Information on schizontocidal treatment and timing, dose, and duration of primaquine
* Baseline data on patient, weight, age, and sex
* Methaemoglobin concentrations measured at baseline and at least once in the first week of follow-up between days 5 and 9

## ****Desirable data for inclusion in the analysis****

* Individual tablet or mg dosing
* Baseline parasitaemia
* Documentation of the supervision of drug administration
* Haemoglobin or haematocrit measured on day 0 and at least once during follow-up
* Qualitative or quantitative assessment of G6PD status
* CYP2D6 status
* Food administration
* History of fever within the last 24 hours at baseline and during follow-up
* Data on vomiting within 1 hour post-administration
* Primaquine/metabolite drug concentrations
* Partner drug levels in terminal elimination phase (e.g., day-7 levels)

## ****Exclusion criteria****

* Pregnancy
* Severe malaria
* Adjunctive antimalarial treatments after the initial schizontocidal treatment

## 1.4. Data Pooling

Embase, Medline, Web of Science, and the Cochrane Library will be systematically searched based on an existing and living systematic review (2); to identify prospective, clinical efficacy studies of acute, uncomplicated vivax malaria published between 1 January 2000 and 29 September 2022 (inclusive) in any language with a minimum active follow-up of 42 days that record methaemoglobin data following daily primaquine administration. Relevant data from unpublished studies will be obtained where possible. Studies that fulfil the study criteria will be targeted through direct email to the corresponding author and/or principal investigator. Once data are uploaded into the WWARN repository, they will be curated and standardised using the WWARN Data Management and Statistical Analysis Plans (3) for clinical data and pooled into a single database of quality-assured individual patient data.

# 2. Outline of Statistical Analysis

## **2.1 Specific objective of the study**

Whether the 7-day methaemoglobin concentration is predictive of the time to first *P. vivax* recurrence, stratified by primaquine dosing regimen.

## **2.2 Primary study endpoint**

Time to first *Plasmodium vivax* recurrence between day 7 and day 120.

## 2.3 Definition of endpoint

*Plasmodium vivax* recurrence is defined as any episodeof *P. vivax* parasitaemia, irrespective of symptoms, between day 7 and day 120 after the initial primaquine administration.

## 2.4 Definition of time zero

Time zero is defined as the time when primaquine treatment is initiated.

## **2.5 Study and patient characteristics**

The following baseline characteristics will be examined:

**Site**: geographical location (region and country), transmission intensity, regional relapse periodicity.

**Patient**: age, sex, weight, height, history of fever (≥37.5°C axillary) in the last 24 hours.

**Drug**: schizontocidal treatment and its mg/kg dose, primaquine use (start day, duration, mg/kg dose), supervision of drug administration, association with food intake, early (within 1 hour) vomiting post-drug administration

**Laboratory**: methaemoglobin concentration, haemoglobin concentration, haematocrit, G6PD qualitative status, CYP2D6 status, primaquine/metabolites drug concentration, schizontocidal drug concentration, parasite density

Children will be considered as aged <15 years with childhood stratified into patients <5 years and those 5 to <15 years if appropriate.

Schizontocidal treatment will be classified as supervised if all doses were directly observed, partially supervised if at least the morning doses of a twice daily regimen was observed, and not-supervised if fewer doses were observed.

Primaquine treatment supervision will be classified as:

* Supervised if all doses were directly observed.
* Partially supervised if >1, but not all, doses were observed.
* Unsupervised if 0 or 1 dose were observed.

In studies with baseline haematocrit measured instead of haemoglobin, haematocrit will be converted to haemoglobin using the following formula (4):

For each study, the locations of study sites will be recorded. Each location will be categorised into:

1. Low, moderate and high transmission settings based on the **observed study site reinfection rate and the** malaria endemicity estimates obtained for subnational regions and year from the Malaria Atlas Project (5).
2. Low (long) and high (short) periodicity of relapses according to Battle’s regions (6), with high periodicity considered to include regions where the median periodicity was ≤42 days. Thus, regions with the two highest periodicities (regions 10 and 12) where the median periodicity is <47 days will be categorised as “high”, and others will be categorised as “low”.

**G6PD deficiency will be classified as deficient (30% activity or a positive qualitative test, e.g., by FST) or normal (30% activity or a negative qualitative test). A second categorisation will be explored to assess patients with intermediate deficiency (30% to <70% activity).**

**CYP2D6 status will be classified by expected phenotype using the activity score system (7,8) to estimate phenotype from genotype. The activity score assigns** values of 0 to 2 based on the *CYP2D6* alleles genotyped in the patient, as follows: zero, no-function alleles (e.g., *\*4, \*4xN, \*5*); 0.25, substantially decreased-function (*\*10*); 0.5, decreased-function (*\*9, \*17, \*29, \*41*); 1, normal-function (*\*1, \*2, \*39*) and 2, increased function *(\*1xN, \*2xN*). The activity score of diplotypes results from the sum of the assigned value to each allele. Patients with an activity score of 0 are designated as poor metabolisers. Patients with an activity score {0.25, 0.5, 0.75, 1} are designated as intermediate metabolisers. Patients with an activity score of >2.25 are designated as ultrarapid metabolisers. Patients with an activity score {1.25, 1.5, 2.0, 2.25} are designated as normal metabolisers (9).

## **2.6 Drug interventions and predictors of interest**

**Drug interventions**

The doses of treatment received, i.e., primaquine, chloroquine, quinine, and artemisinin-based combination therapy, will be calculated from the number of daily tablets administered to each patient. If the daily tablet counts are not available, doses will be back-calculated using the dosing scheme available from study protocols. For each component, a total dose per weight will be calculated for each patient.

**Predictor of interest**

Using individual-level longitudinal data of plasma methaemoglobin (as a percentage of the haemoglobin level), the day-7 methaemoglobin concentration is the main predictor of interest, representing an individual's exposure to the primaquine active metabolites.

Day-7 methaemoglobin concentration (Cday-7) is defined as the methaemoglobin level measured on day 7 after the initial primaquine administration.

Cday-7 will be modelled as the predictor for primaquine antirelapse activity, as guided by a causal directed acyclic graph below.

A screenshot of a computer

Description automatically generated

Under this causal model, methaemoglobin production does not directly affect the risk of *P. vivax* relapse. However, methaemoglobin and relapse share common causes, which may be partially or completely unobserved. Therefore, methaemoglobin concentrations could play a role as a pharmacodynamic proxy of the active metabolites to predict relapse. Potential host factors include, but are not limited to: age, sex, body weight, *CYP2D6* polymorphisms, enzyme maturation (including G6PD), drug-drug interactions, and patient location.

## **2.7 Summary of statistical analyses**

1. ****Description and characteristics of studies****

A profile summary of the relevant studies uploaded to the WWARN repository will be presented to highlight potential heterogeneity.

A summary of the relevant studies will be presented, including (but not restricted to) treatment given, follow-up duration, study populations, description of location by country, transmission intensity, regional relapse periodicity, timing, and dosing of primaquine administration, and day of measurement of methaemoglobin concentrations.

A comparison table of the summary statistics of studies that were eligible but not available will be presented to allow evaluation of inclusion bias related to study selection.

1. ****Baseline characteristics of patients****

A summary of relevant baseline patient characteristics will be presented for all patients, those receiving daily low (5 mg/kg), and high total dose (5 mg/kg) primaquine (10). Variables presented will include age and age group, sex, weight, haemoglobin concentration, methaemoglobin concentration, asexual parasitaemia, presence of fever (axillary temperature 37.5°C or fever recorded), blood schizontocidal treatment and mg/kg dose, mg/kg dose and timing of primaquine (i.e., first day of primaquine treatment), percentage of primaquine administered with food, and host variants (G6PD status, CYP2D6 status).

The distribution of continuous variables will be described using the mean and standard deviation if the data are approximately normally distributed, geometric mean and geometric standard deviation if the data are approximately normally distributed following a log transformation, or the median and interquartile range if the data are non-normally distributed. Tests of statistical significance will not be undertaken for baseline characteristics; rather the clinical importance of any differences in the baseline distributions will be noted.

1. ****Descriptive and survival analyses****

**Primaquine treatment dosing**

A summary of the distribution of total mg/kg primaquine dose will be presented. The distributions will be calculated separately for different age groups, blood schizontocidal drugs, dosing strategies (age-based and weight-based) and regions (6) and presented in tables (mean (SD)) as well as visualised using box-and-whisker plots, histograms, or scatter plots (e.g. mg/kg dosing vs. age or weight).

**Primaquine-induced methaemoglobin production**

A summary of the distribution of methaemoglobin levels will be presented. The distributions after primaquine administration will be visualised using box-and-whisker plots as a function of time since first dose. These plots will be stratified by daily primaquine dose (target doses of 0.25, 0.5 and 1mg/kg) and by duration (7 or 14 days). Additional stratified summaries by schizontocidal drug and age group will be considered.

**Handling of missing methaemoglobin data**

Missing day 7 methaemoglobin concentrations will be linearly imputed using methaemoglobin concentrations measured within 2 days. If only one measurement is available, then the imputation will assume a constant (i.e., that value will be used). If no measurements are available within this timeframe (day 5 to day 9), the participant will be removed.

**Day-7 methaemoglobin concentration to predict time to first recurrence**

* The methaemoglobin concentration on day 7 will be log-transformed to the logarithmic scale (to base 2). An original concentration of zero will remain untransformed.
* Patients will be censored at time of first recurrent vivax parasitaemia (outcome), any malaria parasitaemia, lost-to-follow-up, >60 days blood smear gap or the last day of study, whichever occurs first.
* The primary analysis will only be in patients who are G6PD normal **(30% activity or a negative qualitative test)**. There is evidence that G6PD deficient patients have a considerably lower methaemoglobin response to primaquine treatment while the drug remains efficacious.
* Cox proportional hazards model will be fitted to the time to first recurrence during follow-up (between days 7 and 120) with the day-7 methaemoglobin concentration (percentage, on the log scale), age (years), sex (male, female), daily primaquine dose (mg/kg), primaquine regimen (14-day, 7-day; as a proxy for total primaquine dose), type of schizontocidal drug, and baseline parasite density (parasites per μL blood, on the log scale) as covariates; with shared frailty for study site and day 7 methaemoglobin concentration. An interaction between daily primaquine dose and primaquine regimen will be included. The analysis will be stratified by the duration of primaquine treatment (7 days versus 14 days); adjusted hazard ratios in each stratum will be presented (mean and 95% confidence intervals) along with a meta-analytic estimate.

1. ****Exploratory analyses****

**Secondary endpoints:**

We will explore the secondary endpoints below using the main-analysis framework.

* Any *Plasmodium vivax* recurrence by 4 months (binary endpoint), restricting to studies with at least 4 months follow-up using multivariable logistic regression.
* Absolute change (continuous endpoint) in haemoglobin from baseline to the minimum haemoglobin concentration on day 2 or 3, representing the safety outcome using multivariable linear regression. The model will be adjusted for baseline Hb.

**Alternative summary statistics predictive of the efficacy and safety endpoints:**

**In addition to the day-7 concentration, we will explore two other statistics that summarise primaquine-induced methaemoglobin production: (1) A**rea under the methaemoglobin concentration versus time curve from zero to infinity (AUC[0, ∞)), i.e., the definite integral of the methaemoglobin levels after the initial primaquine administration as a function of time. (2) Maximum methaemoglobin concentration (Cmax), i.e., the peak methaemoglobin level after the initial primaquine administration. These summary statistics will be estimated using population nonlinear mixed-effects modelling. **As in the main analysis, we will** model each of the dose-response methaemoglobin summary statistics to predict the time to first *P. vivax* recurrence. The secondary endpoints will also be modelled using these summary statistics.

Determinants and other correlates of methaemoglobin production:

We will also explore the determinants and predictors of methaemoglobin production under the same causal model; by specifying linear mixed-effects models with day-7 methaemoglobin concentrations (on the log scale) as the endpoint. We will investigate how G6PD status, primaquine and carboxyprimaquine trough levels, haemoglobin concentrations, and CYP2D6 activity scores relate to methaemoglobin production. Potential nonlinearity of the continuous variables will be explored. Our findings may also inform how to best divide CYP2D6 activity scores into a few clinically meaningful categories of primaquine metabolism.

1. ****Risk of bias relating to individual studies****

**The Quality in Prognosis Studies (QUIPS) tool (11) will be adapted to assess potential bias related to individual studies.** A summary of included studies compared to eligible, but unavailable, studies will be presented to highlight potential bias, including items and issues to consider as follows.

Domain 1: The study sample represents the population of interest on key characteristics, sufficient to limit potential bias of the observed relationship between the predictive factor and outcome.

* The source population or population of interest is adequately described.
* The baseline study sample (i.e., individuals entering the study) is adequately described.
* The sampling frame and recruitment are adequately described, including methods to identify the sample sufficient to limit potential bias.
* Period of recruitment is adequately described.
* Place of recruitment (setting, level of endemicity, geographic location) are adequately described.
* Inclusion and exclusion criteria are adequately described).

Domain 2: Loss to follow up (from baseline sample to study population analysed) is not associated with certain characteristics (i.e., the study data adequately represent the sample) sufficient to limit potential bias to the observed relationship between predictive factor and outcome.

* Response rate (i.e., proportion of study sample completing the study and providing outcome data) is adequate.
* Attempts to collect information on participants who dropped out of the study are described.
* Reasons for loss to follow up are provided.
* Participants lost to follow up are adequately described.
* There are no important differences between participants who completed the study and those who did not.

Domain 3: Predictive factor and drug intervention are adequately measured in study participants to sufficiently limit potential bias.

* A clear definition or description of the primaquine regimen and measured methaemoglobin is provided (e.g., including dose, level, duration of exposure, and clear specification of the method of measurement).
* Adequately accurate and reliable measurement of primaquine doses and methaemoglobin concentrations to limit misclassification bias.
* Continuous variables are reported, or clinically relevant cut points (i.e., not data-dependent) are used.
* Method and setting of methaemoglobin measurement are the same for all study participants.
* Adequate proportion of the study sample has complete methaemoglobin data.
* Adequate adherence or supervision of primaquine administration.

Domain 4: Outcome of interest is adequately measured in study participants to sufficiently limit potential bias.

* A clear definition of the outcome is provided, including duration of follow up.
* Method of outcome measurement used is adequately accurate and reliable to limit misclassification bias.
* Method and setting of outcome measurement are the same for all study participants.

Each domain is then rated as high, moderate, or low risk of bias considering the prompting items and considerations.

# 3. PRISMA Statement

The analysis will adhere to the PRISMA-IPD guidelines for reporting systematic reviews and meta-analyses of individual patient data (12).

# 4. Tools

All statistical analyses will be carried out using R. However, when equivalent statistical methods are applied in a different statistical software package, changing the use of statistical software will not require an amendment of this SAP.

# 5. Study Group Governance, Management, Coordination and Publication Policy

**The Primaquine Methaemoglobin Study Group** comprises participating investigators who contribute relevant data sets to the pooled analysis. Data sets will remain the property of the investigator and will not be shared without their consent. Ihsan Fadilah, James Watson, Rob Commons, Kevin Baird, Ric Price, and Nicholas White will oversee the statistical analyses. Participating investigators will be recognised in the publication as contributors under the banner of the Primaquine Methaemoglobin Study Group. A Writing Committee will coordinate activities including data analysis and drafting of publications and reports for complete group review. The Writing Committee will comprise Ihsan Fadilah, James Watson, Rob Commons, Kevin Baird, Ric Price, Nicholas White and other interested investigators. They are responsible for undertaking the data analysis and preparation of the manuscript. Authors will be recognised according to the ICMJE guidelines and the [WWARN publication policy](http://www.wwarn.org/working-together/sharing-data/data-usage) (13).

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