Statistical Analysis Plan

Correlation between K13 mutations and clinical phenotype Study Group

Version 1
10 December 2016

WorldWide Antimalarial Resistance Network (WWARN)

Suggested citation: Statistical Analysis Plan, K13 and Clinical Phenotype Study group

Version History

Version number	Revision(s) & reason for amendment	Release date

WorldWide Antimalarial Resistance Network (WWARN)
www.wwarn.org

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1. Introduction

Artemisinin-based combination therapies (ACTs) are now the mainstay for uncomplicated malaria treatment in most endemic areas and have substantially contributed to the dramatic decrease of malaria incidence and mortality over the last decade (WHO 2015). However, the emergence of Plasmodium falciparum resistance to artemisinin in the Mekong sub-region threatens to jeopardise those gains (Dondorp *et al.* 2009). Resistance to artemisinin has been associated with delayed parasite clearance after ACT or artemisinin monotherapy treatment and a variety of mutations in the propeller region of the K13 gene (Ariey *et al.* 2014).

K13 mutant alleles are now very widely distributed in the Mekong region (Ashley *et al*. 2014; Tun *et al.* 2015; Miotto *et al.* 2015; Wang *et al*. 2015; Huang *et al*. 2015; Spring *et al*. 2015; Thremier *et al*. 2014; Takala-Harrison *et al*. 2015). Parasites with some of these mutations have spread locally, but also have emerged independently in different locations (Takala-Harrison et al. 2014; Miotto et al. 2015). Gene exchange experiments have confirmed that some of the K13 mutations found in parasites from the Mekong region can confer protection against artemisinin exposure in the laboratory (Witkowski *et al*. 2013; Straimer *et al.* 2015).

In addition, molecular surveillance alone has identified K13 mutants in many sites, but without correlation of these alleles with a parasite phenotype, their significance is not yet known. Moreover, the prevalence and role of K13-propeller mutations are poorly known in sub-Saharan Africa. The confirmation that a particular K13 allele does encode the expected parasite phenotype requires either clinical investigation of the parasite clearance (Ariey et al. 2014; Ashley et al. 2014; Huang et al. 2015) or in vitro assessment of the decreased susceptibility of the parasite in the specialized ring-stage survival assay (RSA) (Witkowski et al. 2013). A number of studies assessing the relationship between the K13 molecular markers and delayed parasite clearance have been published recently; this pooled analysis aims to collate published and unpublished studies to explore the relationships between identified K13 mutant alleles and delayed parasite clearance.

1.1 Aim of the study

To assess the relationship between K13 mutations and delayed parasite clearance

1.2 Eligibility criteria for inclusion in pooled analysis

Studies will be deemed eligible for the purpose of this analysis if they meet the following criteria:

- Results of K13 genotyping

AND

- Patients treated with either an ACT or artemisinin monotherapy
- Repeated measure of parasitaemia in the first days of treatment at least every 12 hours (until negative count or at least until day 3) allowing the calculation of parasite clearance half life
- Mg/Kg dosing protocol
- Weight of the patient

Desirable Data (not required for inclusion)

- Patient follow up for a minimum of 28 days post treatment
- PCR genotyping to distinguish reinfection and recrudescence
- Hb at enrolement
- Fever at enrolment
- Gametocyte at enrolment and possible follow up measurement(s)

Study Exclusion criteria - none

Patient Exclusion - none

2. Methodologies

2.1 Data Pooling

The data sets uploaded to the WWARN repository will be standardized using the WWARN Data Management and Statistical Analysis Plans (DMSAP v1.2) for clinical data and pooled into a single database of quality-assured individual patient data. Data will remain the property of the individual donor(s) and publication will be in accordance with an agreed publication plan (WWARN Publication Policy).

2.2 Transmission Intensity

The study sites will be classified into 3 categories: low, medium and high malaria transmission based on the parasite prevalence estimates obtained from the Malaria Atlas Project (Gething, 2011) for specific location and year of study.

2.4 Dosing Calculation

The doses of artemisinin derivatives and the partner compounds received will be calculated from the number of daily tablets administered to each patient. If the daily tablet counts are not available, doses will then be back-calculated using the dosing scheme available from study protocols.

3. Efficacy Endpoints

Primary: Parasite clearance half life (HL)
Secondary: Parasite positivity on day 2
Parasite positivity on day 3
Efficacy response of the treatment at day 28 or 42

4. Covariates Examined

The following covariates will be examined:

- Age
- Treatment and dose
- Transmission intensity
- Parasitaemia
- Fever on enrolment
- Presence of gametocytes on enrolment
- Anemia on enrolment

5. Outline of Statistical Analysis

1. A summary (study profile) of the studies identified and uploaded to the WWARN repository will be presented
2. Table of methodology used in studies (parasitaemia sampling, molecular analysis) will be presented
3. Descriptives and baseline characteristics
    a. A summary of key study characteristics will be outlined
    b. The baseline characteristics of patients in the eligible studies will be summarized by study.
4. Table summarizing distribution of HL (median, quartiles, 5%- 95%-tiles, min max) by mutation, overall and stratified by region, transmission intensity
5. Plot of distribution of HL by mutation, overall and stratified by region, transmission intensity
6. Finite mixture model will be fitted with 2 components (sensitive and resistant) to characterise parasite cleranace in these two populations.
7. Plot of a proportion of patients with HL > x hours by mutation, overall and stratified by region, transmission intensity. X will be defined as a HL for which probability of belonging to sensitive and resistant populations is equal (p=0.5).
8. Plot of a proportion of patients with positive parasite count on Day 2/ Day 3 by mutation will be presented , overall and stratified by region, transmission intensity
9. Comparison of HL in patients with a specific K13 mutation and in patients with WT at the same study sites will be performed using univariate and multivariate regression models with random effect for study site, stratified by region. The multivariate model will be adjusted for the following covariates (data permitted) : age, treatment, AS dose, fever, anemia, initial parasitaemia, presence of gametocytes on enrolment. Separate models for each mutation and WT will be fitted, for comparisions between mutations models including patients with WT and these mutations will be fitted.
10. Plot of distribution of HL in infections with K13 mutations associated with an increase in HL, and distrubition of HL in the remaining infections. ROC evaluation of the “optimal” cutoff for HL for the best separation of the two populations will be performed.

11. Data permitting, survival analysis of the time to recrudescence will be perfomed in a subset of studies with at least 28-days follow-up and PCR genotyping. Patients with WT and most common K13 mutations associated with prolonged HL will be included in the analysis. The multivariate model will be adjusted for the following covariates: mutation, age, treatment, AS dose, fever, anemia, initial parasitaemia, HL, presence of gametocytes on enrolment.

6. Statistical Methodology

6.1 Estimation of parasite clearance

The parasite clearance half live (HL) will be determined using the WWARN parasite clearance estimator tool (PCE) http://www.wwarn.org/tools-resources/toolkit/analyse/parasite-clearance-estimator-pce.

6.2 Mutations included in the analysis

Non-synonymous mutations in in the K13 gene will be included in the analysis. Patients without non-synonymous mutations detected will be treated as having Wild Type. Patients with mixed genotype (wild type/mutation) will be excluded from the analysis.

6.3 Analysis population

Patients without mixed genotype for whom HL could be estimated and the PCE model fitted well to the parasite data will be included in the analysis. Good fit is defined as:

- standard deviations of residuals <2;
- number of data points used to fit the linear part of the curve >2
- duration of lag phase <12h
- pseudo R2-statistics ≥0.8.

Additionally, patients who withdrew or had a record of inadequate dosing were excluded.

6.4 Descriptive statistics

Descriptive statistics will use mean and standard deviation if data are normally distributed, geometric mean and range if data are log-normally distributed (as assessed by Shapiro-Wilk test) , or median and range otherwise. In addition, graphical tools such as histograms and QQ-plots will be used to assess distributional assumptions. For categorical variables, counts, percentages, and frequency distributions will be provided.

6.5 Comparison of HL between patients with different mutations

Linear regression models of the log transformed HL will be fitted, with K13 mutations, age, treatment, AS dose, fever, initial parasitaemia, presence of gametocytes on enrolment as covariates. Random effects for study-site will be used to account for heterogeneity between studies. Residuals will be examined for normality and for a systematic deviations form the model.
Difference between a specific K13 mutation and wild type will be assessed by the Wald test, and the % difference in HL (95% CI) will be calculated as an exponent of the difference of the corresponding regression coefficients.

6.6 Distribution plots

The density function for log-transformed HL will be estimated using kernel estimator. The Epanechnikov kernel will be used with the "optimal" width of the density window around each point. The "optimal" width is calculated as the width that would minimize the mean integrated squared error if the data were Gaussian and a Gaussian kernel were used, so it is not optimal in any global sense.

6.7 Finite mixture modelling

Finite mixture model for log-transformed HL will be fitted with two components, stratified by region. Stata fmm command will be used. HL values corresponding to the posterior probabilityies of 0.5 for both components will be selected as a cutoff for resistant infection.

6.8 ROC analysis

All patients will be devided into two groups: those with infections with K13 mutations associated with an increase in HL ($p<0.05$ for comparison with WT, adjusted for covaraites), and all the remaining patients. Nonparametric ROC analyses will be performed: ROC curve will be plotted and the area under the ROC curve will be calculated. The "optimal" cutoff for HL will be selected using Youden index. Youden index is equl to the difference between the proportion of true positive i.e. sensitivity and the proportion of false negative i.e. 1-specificity:
Youden Index = Sensitivity+ Specificity -1.
The "optimal" cutoff point will be calculated by maximizing Sensitivity + Specificity across various cutoff points.

6.9 Survival regression models

Time to PCR confirmed recrudescence will be modeled using Cox regression. Patient with new infections will be censored at the time of new infection, patients with no PCR resulst will be excluded from the analysis. Random effects in the form of frailty parameters will be used to adjust for study-site effect (Glidden and Vittinghoff 2004). In the Cox regression model, the proportional hazard assumption will be tested based on Schoenfeld residuals(Schoenfeld 1982) and in case it is violated, models with parametric hazard functions such as: Gompertz, Weibull, lognormal and log-logistic will be examined and the best regression model will be selected based on Cox-Snell residuals (Collett 2003).

6.10 Selection of risk factors for the final model

Inclusion of covariates in the final model will be determined based on how they improve the overall model (likelihood ratio test) and if they change the coefficient estimates for other factors and based on the residuals.

For any regression model, the following strategy will be employed to determine independent risk factors. Initially all possible risk factors will be examined in the univariate model, and will be included in model building in the the multivariate analysis. Model with known confounders will be fitted first (K13 mutation type, asexual parasite density, age in categories, transmission intensity baseline model). Variables and covariates will then be added to the baseline model in a stepwise forward fashion and the Likelihood Ratio Test (LRT) i.e. changes in log likelihood *(*$-2\ Log\hat{L}$) will be compared (for nested models) to identify the variables which results in a significant reduction in $-2\ Log\hat{L}$ at 5% level of significance. Akaike's Information Criterion (AIC) will be used to compare competing non-nested models; models with smaller AIC will be preferred.

7. Tools

All statistical analyses will be carried out using *R 3.1.2* (released on 2014-10-31 by The R Foundation for Statistical Computing) or *Stata 13.1* (StataCorp, 4905 Lakeway Drive, College Station, Texas 77845 USA). Nutritional status will be computed using *Stata 13.1.* Using alternative statistical software does not require amendment of this SAP.

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