Title: Characterizing artemisinin resistance in severe malaria (CHARISMA): SMAART sub-study in Uganda

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PROJECT SUMMARY

Title	Characterizing artemisinin resistance in severe malaria (CHARISMA): SMAART sub-study in Uganda
Project Site	Kalongo Hospital, Agago District, Uganda
Description	This protocol is for a sub-study to utilize clinical data and blood samples collected from participants enrolled in the SMAART study (parent study), a prospective observational study of children aged 3 months to 15 years with and without severe malaria. The purpose of this study is to conduct *ex vivo* drug susceptibility assays and determine the presence (or absence) of artemisinin partial resistance among parasites infecting participants of the SMAART study, which is already approved (IRB #: MRRH-2020-10). No additional contact with SMAART participants will be needed for blood samples to be used as part of this study.
Study objectives	1. To characterize and compare the *ex vivo* drug susceptibilities of *P. falciparum* isolates from patients with and without severe malaria. 2. To characterize and compare genomic features of *P. falciparum* isolates infecting patients with and without severe malaria. 3. To assess whether presence of artemisinin partial resistance is associated with poor clinical outcomes in severe malaria patients. 4. To assess whether prior receipt of antimalarials is associated with artemisinin partial resistance in severe malaria patients.
Duration	The anticipated study duration is three years. Enrollment will begin in 2023 and will continue until the sample size is reached.
Sample size	400 children (300 severe malaria cases; 100 non-severe malaria controls)
Study population	The study will collect data and blood samples from 400 SMAART participants who were admitted to Kalongo Hospital with *P. falciparum* malaria confirmed by microscopy.
Selection criteria	The inclusion criteria for the IRB-approved SMAART study at Kalongo District Hospital are as follows: • Children aged 3 months to 15 years • Admitted to the hospital with *P. falciparum* malaria defined by a positive malaria slide • History of fever by self-report or documented abnormal temperature at screening (fever or hypothermia; axillary temperature >37.5°C or <36°C) • *For severe malaria cases only*: Meets the World Health Organization (WHO) and Teule criteria for severe malaria (described further in full protocol) • *For non-severe malaria controls only*: Diagnosed with non-severe *P. falciparum* malaria as per WHO/Teule definitions. The additional inclusion criteria for the CHARISMA study: • Child has provided blood sample at admission • Caregiver and participant have provided informed consent for future use of blood samples in the SMAART study. The exclusion criteria for the CHARISMA study are as follows: • Insufficient amount of blood remaining from SMAART study collection to conduct CHARISMA-specific laboratory assays

Study procedures	Upon admission to Kalongo Hospital and obtaining parental consent, the SMAART study will obtain clinical information, conduct a physical exam, and draw up to 5 ml of blood by venipuncture. Blood used for the CHARISMA study will already be collected by the SMAART study; <u>no additional blood draw will be required.</u>
	
	For the CHARISMA study we will utilize 1-2 ml of this blood sample to:
	• Spot approximately 100 µL onto filter paper
	• Centrifuge the remaining blood to collect:
	  ○ Erythrocyte pellets for *ex vivo* drug susceptibility assays
	  ○ Plasma or serum to determine antimalarial drug levels

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ABBREVIATIONS AND ACRONYMS

IDRC	Infectious Disease Research Collaboration
IRB	Institutional Review Board
MOLAB	Molecular Research Laboratory, Kampala
SD	Standard deviation
SMAART	Severe MAlariA Research and Trials consortium
UCSF	University of California San Francisco
UNCST	Uganda National Council of Science and Technology
WHO	World Health Organization

1.0 BACKGROUND

In most of sub-Saharan African, malaria is the most common cause of hospital admission and under five mortality.[1,2] Despite the scale-up of malaria control efforts and prompt and effective case management, mortality from severe malaria remains unacceptably high (~10-20%).[3] Intravenous artesunate is the recommended therapy for severe malaria.[3]

Artemisinin partial resistance, characterized by delayed parasite clearance after therapy with artemisinins, was first reported on the Thai-Cambodian border in 2008-09, and has since spread through much of Southeast Asia.[4-6] Subsequently, artemisinin partial resistance facilitated the selection of partner drug resistance, resulting in high treatment failure rates of some artemisinin-based combination therapies.[7] Recently, artemisinin partial resistance emerged independently in sub-Saharan Africa, which harbors over 95% of the world's malaria burden.[8-11] The independent emergence of artemisinin partial resistance in Uganda and Rwanda offers a major threat to the control of malaria, and in particular, to the prompt treatment of severe malaria in Africa.

Antimalarial drug susceptibility is assessed by three complementary methodologies. First, clinical trials directly compare the antimalarial treatment or chemoprevention efficacies of regimens of interest.[11-13] However, clinical trial results cannot distinguish relative contributions of parasite and host factors to treatment outcomes. Second, the prevalence of mutations that mediate altered drug sensitivity can be assessed by genomic methods.[14] However, the impacts of parasite genetic polymorphisms on drug susceptibility and specific markers of resistance are incompletely characterized for most drugs.[2] Third, drug susceptibilities of cultured *P. falciparum* can be measured in vitro, including *ex vivo* analyses of fresh clinical isolates.[15]

2.0 RATIONALE

The efficacy of intravenous artesunate, the recommended treatment for severe malaria, is threatened by the emergence of artemisinin partial resistance in East Africa.[16,17] It is of urgent priority to determine whether artemisinin partial resistance impacts on the risk of severe malaria, poor clinical outcomes, and decreased efficacy of intravenous artesunate for the treatment of severe malaria.

3.0 OBJECTIVES

1. To characterize and compare the *ex vivo* drug susceptibilities of *P. falciparum* isolates from patients with and without severe malaria.
2. To characterize and compare genomic features of *P. falciparum* isolates infecting patients with and without severe malaria.
3. To assess whether presence of artemisinin partial resistance is associated with poor clinical outcomes in severe malaria patients.
4. To assess whether prior receipt of antimalarials is associated with artemisinin partial resistance in severe malaria patients.

4.0 RESEARCH DESIGN

4.1 Hypothesis

We hypothesize that artemisinin partial resistance, as assessed based on parasitological or molecular criteria, will be associated with increased risk of severe malaria, poor clinical outcomes, and with decreased efficacy of intravenous artesunate for the treatment of severe malaria.

4.2 Parent study

The CHARISMA study is a sub-study embedded in one of the study sites (Kalongo Hospital) of the Severe MAlariA Research and Trials consortium (SMAART) study, a multi-site prospective observational study of children admitted to the hospital with malaria (IRB approval #: MRRH-2010-10). The aim of the SMAART study is to understand the spectrum and clinical manifestations of malaria across the African continent. To understand differences between severe and non-severe malaria, the SMAART study will enroll two types of children:

(1) hospitalized children with severe malaria (cases; n=300 per site)
(2) time-matched hospitalized children with non-severe malaria (controls; n=100 per site)

For the SMAART study, clinical data and blood samples are taken at admission and participants are followed for 6 months to assess longitudinal health outcomes.

The purpose of the CHARISMA study is to use a portion of the blood sample from the SMAART study to conduct *ex vivo* drug susceptibility assays and determine the presence (or absence) of artemisinin partial resistance among parasites infecting participants of the SMAART study. The SMAART clinical team at Kalongo District Hospital are responsible for patient enrollment, management and follow up. For the CHARISMA study, no additional contact with participants will be needed to analyze blood samples that were collected as part of the SMAART study.

4.2.1 Definition of severe malaria for SMAART study

Severe malaria is defined based on WHO clinical severity criteria or laboratory severity criteria (if laboratory tests are performed as part of clinical care) and/or one or more of the Teule criteria at admission. A description of these criteria is below:

Criteria	Manifestations
WHO clinical criteria	1. Impaired consciousness: prostration (also Teule criteria) or coma 2. 2 or more convulsions within the last 24 hours 3. Respiratory distress (also Teule criteria) 4. Compensated or decompensated shock • Compensated shock is defined as capillary refill ≥3 s or temperature gradient on leg (mid to proximal limb), but no hypotension. • Decompensated shock (hypotension) is defined as systolic blood pressure <70 mm Hg in children 5. Jaundice (in a child with a positive *P. falciparum* malaria on RDT)

	6. Dark or cola coloured urine (blackwater fever)
WHO laboratory criteria	1. Hemoglobin <5g/dl (also Teule criteria) (if routinely done)
Teule criteria	1. HIV (standard test for all hospitalized children) 2. Impaired consciousness: prostration or coma (also WHO clinical criteria) 3. Respiratory distress (also WHO clinical criteria) 4. Hemoglobin <5g/dl (if routinely done) (also WHO clinical criteria)

Teule criteria formally require a temperature >38°C OR <36°C in addition to ≥1 of the clinical signs marked above; however, as these clinical criteria alone define WHO severe malaria, for simplicity of enrollment, and given that the prevalence of HIV infection is expected to be <5%, this temperature criterion will not be required in severe malaria cases for this observational study. These Teule criteria have previously been shown to identify 85% of children with malaria and bacterial co-infections.[12] The reason for requiring a specific set of clinical/laboratory criteria for enrollment as a severe malaria case, rather than relying on physician judgement alone, is to provide an objective standardisation of the population across sites.

4.3 Study population for the CHARISMA study

Samples will be collected from hospitalized children with and without severe malaria at Kalongo District Hospital, one of the SMAART study sites. Kalongo Hospital is located in Northern Uganda, where the prevalence of *P. falciparum* genomic markers of artemisinin partial resistance was recently shown to be high.[11,13,14]

4.3.1 Justification for including children in the study

In highly malaria-endemic areas, including Uganda, malaria (and particularly severe malaria) is primarily a disease of children. Thus, study of severe malaria requires inclusion of children.

4.3.2 Inclusion and exclusion criteria

The inclusion criteria for the SMAART (parent) study at Kalongo District Hospital are as follows:

- Children aged 3 months to 15 years
- Admitted to the hospital with *P. falciparum* malaria defined by a positive malaria slide
- History of fever by self-report or documented abnormal temperature at screening (fever or hypothermia; axillary temperature >37.5°C or <36°C)
- *For severe malaria cases only*: Meets the World Health Organization (WHO) and Teule criteria for severe malaria (described further in full protocol)
- *For non-severe malaria controls only*: Diagnosed with non-severe *P. falciparum* malaria as per WHO/Teule definitions

The additional inclusion criteria for the CHARISMA study are as follows:

- Child has provided blood sample at admission
- Caregiver and participant have provided informed consent for future use of blood samples in the SMAART study.

The exclusion criteria for the CHARISMA study are as follows:

- Insufficient amount of blood remaining from SMAART study collection to conduct CHARISMA-specific laboratory assays

4.4 Study duration and number of subjects

SMAART study enrollment will begin in 2023 and continue until the target sample size of 300 severe malaria patients and 100 non-severe malaria patients has been reached. The maximum duration of the study will be three years.

5.0 STUDY PROCEDURES

5.1 Blood draws

For participants of the SMAART study, up to 5 ml of blood will be collected by venipuncture after enrollment and utilized for a number of tests (see flowchart of SMAART Study Sample Collection below).

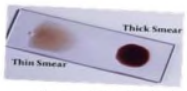
SMAART Sample flow chart for Kalongo Site
(Version 1.0 June 1st 2023)

Point-of-care (bedside) tests at Admission

Screening Bloods

Malaria slide

Full blood count



- ✓ **Positive Malaria slide: Eligible for SMAART study**
- ✓ **Check- Eligibility for Case or Control**

Cases and Control (first two children admitted Monday and Thursday) **sample collection)**
Admission

- ✓ **BLOOD Point of Care diagnostics**
  - Blood glucose **0.05mL**
  - Blood Lactate **0.05mL**
  - **Procalcitonin (PCT)** **0.1mL**
  - **C-Reactive Protein test (CRP)** **0.1mL**
  - **Clinical Chemistry and Ph i-stat machine** **0.04mL**
  - **HIV** **0.05mL**
- ✓ **URINE – For Dipstick Testing/HILMAN CHART**

Study Specific blood samples Admission for storage (with consent)

Lithium heparin 3 mls for plasma storage for batch HRP2

EDTA tube for DNA prep (0.5-1ml)
Human and parasite genotyping

Sample collection during admission

- Malaria parasite clearance (4 hourly first 24 hours then, 8 hourly until 3 negative slides)
- Lactate clearance **if > 2mmol/l (**4hrly first 24 hours, then 8hourly until < 2mmol/l)

For the CHARISMA study, 1-2 ml of the admission blood sample that was collected into tubes containing heparin or another anticoagulant will be used for the following procedures:

- Approximately 100 µL of blood will be spotted onto filter paper for parasite storage of DNA
- The erythrocyte pellet will be used for parasite culture, *ex vivo* drug susceptibility assays, and storage of culture-adapted parasites
- Serum or plasma will be collected and stored for determination of antimalarial drug levels

If the amount of blood remaining after SMAART study procedures is insufficient to carry out CHARISMA laboratory assays, blood samples will not be used for the CHARISMA study.

5.2 Parasite culture

Blood collected from study participants will be transported promptly to the Kalongo Hospital laboratory. Samples will be processed according to our previously published protocols.[15] Key assays will include IC50 (SYBR green detection) and ring survival assays, performed as previously described.[15,16] Drug susceptibilities will be determined for a panel of antimalarial drugs or test compounds relevant for the treatment or prevention of malaria.

5.3 Genotyping of parasite DNA

DNA will be extracted from filter paper blood spots by Chelex extraction or purified using standard methods. DNA will then be characterized by standard genomic methods to identify polymorphisms known or predicted to be associated with drug and diagnostic resistance or to ask other questions related to the biology of malaria parasites.[15,17]

5.4 Plasma drug levels

It is important to determine if levels of circulating antimalarial drugs at the time of diagnosis of malaria are associated with risk of drug resistance. If resources are available, aliquots of plasma or serum collected at the time of malaria diagnosis (see Section 5.1) will be used for the quantification of levels of relevant antimalarial drugs using methods that are standard with our group.[18]

5.5 Clinical Information linked to samples

De-identified clinical information collected from SMAART participants will be shared by the SMAART study statistician under a **data sharing agreement** and linked to samples using a unique identifier. The data will include:

Type of data	Data collected
Basic patient information collected at admission and follow-up data collected for 6 months	• Date of admission • Discharge date • Sex, age • Known sickle cell disease

	• Duration of fever symptoms, temperature at admission • Clinical complications of severe malaria • Hemoglobin if measured routinely at this admission • Any prior healthcare treatments • Length of stay since admission • Development of severe malaria (as defined above) and any transfusions post-admission • In-hospital and post-discharge survival up to 180 days • Weight, height, mid-upper arm circumference • Re-admissions, malaria episodes
Laboratory information from blood samples collected at admission (already collected from SMAART study)	• Parasite and lactate clearance • Genotyping molecular markers of artemisinin resistance • Genotyping molecular markers of diagnostic resistance

5.6 Sample storage

Samples collected as part of this sub-study will be stored under appropriate storage conditions at project laboratories at Kalongo Hospital, Tororo District Hospital, the Central Public Health Laboratories in Kampala, or Mulago Hospital in Kampala.

5.7 Quality control

Quality control will be maintained by using appropriate positive and negative controls for molecular and parasitology experiments, as is standard practice in our laboratory.[10]

5.8 Anticipated study limitations

We do not anticipate major limitations, as all planned procedures are already standard for our research group. Some fresh parasite isolates do not readily grow in culture, and so will not be evaluable for *ex vivo* parasite susceptibility. Similarly, some molecular assays fail. However, in our recent experience success rates for both *ex vivo* culture and molecular assessment of *P. falciparum* polymorphisms have been excellent. A potential limitation is that results from studied isolates, which represent a convenience sample, may not be fully representative of malaria in Uganda or Africa. However, resource limitations obviate a more complex study design, and we anticipate that samples will be reasonably representative of African malaria parasites with and without mediators of drug resistance.

6.0 Data management

For sharing of clinical data collected from the SMAART study, a data sharing agreement will be developed between SMAART consortium, the Medical Research Council at University of College London and UCSF and IDRC. The data sharing agreement will need to be signed by all parties prior to its release to UCSF and IDRC.

Samples for this study will not be linked to patient identifiers, so the risk of loss of study participant confidentiality should be minimal. In addition, consent forms obtained with SMAART participant names are being kept in a locked cabinet.

The Principal Investigator will maintain appropriate medical and research records for this study in compliance with the principles of Good Clinical Practice and regulatory and institutional requirements and in compliance with the requirements for the protection of confidentiality of participants. Only study team members will have access to these records.

Authorized representatives of the sponsor, the ethics committee(s), or regulatory bodies may inspect all documents and records required to be maintained by the investigator. The Principal Investigator or designee will ensure access to facilities and records.

7.0 Analytic plan

7.1 Sample size estimates

The sample size for this study will be fixed based on the enrollment of participants in the SMAART study (i.e., 300 severe and 100 non-severe malaria cases). Based on this sample size, below are the following minimal effect sizes we will be able to detect:

- **Objective 1:** Assuming mean ring-stage survival of 2.5% for non-severe malaria cases (as reported previously in northern Uganda[19]) we will be able to detect a 1% difference in ring survival between severe and non-severe malaria cases with 80% power and an alpha of 0.05.
- **Objective 2:** Assuming a combined prevalence of 34% for the PfK13 C469Y and A675V mutations (associated with artemisinin partial resistance) among non-severe malaria cases as previously reported in northern Uganda,[19] we will be able to detect a 17% difference in the prevalence of PfK13 C469Y and A675V mutations between severe and non-severe malaria cases with 80% power and an alpha of 0.05.
- **Objective 3:** Assuming an average length of hospital stay of 2.5 days (standard deviation (SD) = 1.5) for severe malaria cases,[20] we will be able to detect a mean difference of 0.5 days in hospital stay between individuals with and without artemisinin partial resistance, with 80% power and an alpha of 0.05.
- **Objective 4:** Assuming 34% of children will have partial artemisinin resistance and lumefantrine drug level of 155 ng/mL (SD = 27),[21] we will be able to detect an 8 ng/mL difference in lumefantrine drug levels between children with and without artemisinin partial resistance.

7.2 Statistical analysis plan

All analyses will be conducted using Stata (College Station, Texas, USA) or R (Vienna, Austria). Unadjusted comparisons will be conducted using Pearson's chi-square test or Fisher's exact test (when the frequency of any cell value is <5) for categorical variables and the student t-test or Mann-Whitney test for continuous variables, depending on the degree of normality of underlying distributions. Adjusted associations will be conducted using multivariable logistic regression models for binary outcomes, Poisson regression for count outcomes, and linear regression for continuous variables. For adjusted models, the following variables will be included in multivariable models: age and sex of child, weight, height, mid-upper arm circumference, known sickle cell disease, and month (or season) of admission.

8.0 Protection of human subjects

This study will be conducted in accordance with the principles set forth in the ICH Harmonised Tripartite Guideline for Good Clinical Practice and the US Code of Federal Regulation applicable to clinical studies, 45 CFR 46, whichever affords the greater protection to the participants.

8.1 Institutional review boards

The protocol for the CHARISMA study will be reviewed and approved by all IRBs before the project begins. Any amendments or modifications to this material will also be reviewed and approved by the IRBs prior to implementation. The IRBs will include:

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8.2 Informed consent

Informed consent will be obtained from all SMAART participants, including consent for blood draw and future use of samples. Only blood samples from SMAART participants who consented to future use of samples will be used as part of the CHARISMA study. Thus, no additional consent will be obtained for the purposes of the CHARISMA study.

8.3 Risks and benefits

8.3.1 Risks

No additional risks will be posed by the CHARISMA study, as it will only utilize blood already drawn from the SMAART study.

8.3.3 Benefits

No direct benefits to study participants will be received from this project, but knowledge gained from the CHARISMA study may improve our ability to control malaria and treat severe malaria in Uganda and Africa.

8.4 Costs to the study participants

There will be no cost to the participant or their parents/guardians for participation in this study.

8.5 Reimbursement of study participants

Participants will not receive reimbursement for use of their samples in the CHARISMA study as this is already covered by the SMAART study.

8.6 Confidentiality

Study participant names will not be recorded for this study. Patient identifiers will not be included on any study records.

9.0 Dissemination of results

Results will be shared with collaborators as they are obtained. Results will be available to the Ministry of Health. We will publish results in the medical literature on a regular basis, as has been our group's practice during our research on malaria in Uganda since 1998.

10.0 References

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