"PROGRAM FOR RESISTANCE, IMMUNOLOGY, SURVEILLANCE, AND MODELLING OF MALARIA"

(PRISM)

Study Title: Cohort and entomology studies to compare malaria metrics in the Tororo and Busia Districts of Uganda

Short Title: PRISM Border Cohort Study

Sponsored by: National Institute of Allergy and Infectious Diseases (NIAID)

DMID Funding Mechanism: NIH/NIAID U19AI089674

Principal Investigators: Moses R. Kamya, MBChB, MPH, PhD; Grant Dorsey, MD, PhD

DMID Program Officer: Malla Rao, DrPH, M Eng

Protocol Version Number: 10.0, dated: 08 June 2023

STATEMENT OF COMPLIANCE

The study will be carried out in accordance with Good Clinical Practice (GCP) as required by the following:

- US Code of Federal Regulations applicable to Clinical Studies (45 CFR 46)
- ICH GCP E6
- Ugandan National Council for Science and Technology
- Completion of Human Subjects Protection Training
- NIH/NIAID Clinical Terms of Award

SIGNATURE PAGE

The signature below constitutes the approval of this protocol and the attachments, and provides the necessary assurances that this study will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and applicable US federal regulations and ICH guidelines.

Site Investigator: Professor Moses Kamya, MBChB, MPH, PhD

Signed: _____

Name

Title

ABBREVIATIONS AND ACRONYMS

ACT	Artemisinin-based combination therapy
AL	Artemether-lumefantrine
CRF	Case record form
DHS	Demographic and health surveys
DMFA	Direct membrane feeding assay
EIR	Entomological inoculation rate
GCP	Good clinical practice
GFATM	Global Fund to Fight AIDS, Tuberculosis and Malaria
HMIS	Health Management Information System
IPTp	Intermittent preventive therapy in pregnancy
IRS	Indoor residual spraying (of insecticide)
LLIN	Long-lasting insecticide-treated net
MIS	Malaria indicator survey
PMI	President's Malaria Initiative
PPY	Per person years
PRISM	Program for Resistance, Immunology and Modelling of Malaria
TDH	Tororo District Hospital
TRA	Transmission reducing activity
QA	Quality assurance
UMSP	Uganda Malaria Surveillance Project
WHO	World Health Organization

STUDY SYNOPSIS

Title	**Cohort and entomology studies to compare malaria metrics in the Tororo and Busia Districts of Uganda**
Description	A prospective observational study will be carried out among households and household members to measure clinical and entomologic measures of malaria burden and transmission intensity.
Study site	Households and study participants will be enrolled from an area bordering Tororo and Busia Districts. A designated study clinic will be located at Tororo District Hospital.
Study objectives	**Objectives of epidemiology studies** 1. To compare the incidence of malaria, parasite prevalence, and the molecular force of infection among cohort study participants living in areas with different transmission intensity. 2. To characterize factors determining the malarial force of infection. 3. To determine factors affecting the duration, density, and clinical consequences of blood stage malaria infection. 4. To assess the associations between overnight travel and the risk of malaria infection. 5. To compare measures of transmission intensity including the human biting rate, sporozoite rate, and the entomological inoculation rate at the household level in areas with different transmission intensity. 6. To compare the species composition of mosquito vectors and the host source of mosquito blood meals in areas with different transmission intensity. 7. To identify pathogens responsible for non-malarial febrile illness among cohort study participants. **Objectives of transmission studies** 1. To characterize factors associated with gametocyte production. 2. To evaluate the impacts of human, and parasite, and mosquito factors on parasite infectivity to mosquito vectors. 3. To characterize the human infectious reservoir for malaria in areas with different transmission intensity. **Objectives of immunology studies** 1. To compare antibody responses to malaria antigens and associations between antibodies responses and protection from /exposure to infection with malaria parasites in areas with different transmission intensity. 2. To compare malaria specific cellular immune responses in naturally exposed children and adults in areas with different transmission intensity. **Objectives of resistance studies** 1. To assess the impact of genotypic markers of antimalarial drug resistance on malaria transmission. 2. To assess the impact of genotypic markers of insecticide resistance on malaria transmission.
Sample Size	All eligible household members from80 randomly selected houses located in the area bordering Tororo and Busia Districts
Selection Criteria	<u>Eligibility criteria for households:</u> 1. Have at least 2 household residents < 5 years of age. 2. No more than 7 permanent residents current residing in the household. 3. No plans for the household to move out of the study area in the next 2 years. 4. Willingness to participate in entomological surveillance studies. <u>Eligibility criteria for household members:</u> 1. Household considered their primary residence. 2. Agreement to come to the study clinic for any febrile illness and scheduled routine visits. 3. Agreement to avoid antimalarial medications administered outside the study. 4. Provision of written informed consent (from parent or guardian in case of children).
Participant Follow up	Households and study participants will be followed up to March 31st 2024 depending on availability of resources. Study participants will receive care for all their health care needs as per local standards at a designated study clinic open every day. Study participants will be encouraged to come to the study clinic whenever they are ill. Study participants will be seen at the study clinic every 4 weeks for routine assessments and to collect blood for laboratory studies. Entomological studies will be conducted at the households every 2 weeks for collection of mosquitoes.

TABLE OF CONTENTS

1.0 KEY ROLES

INVESTIGATORS/COLLABORATORS

Moses Kamya, MBChB, MPH, PhD
Professor, Department of Medicine, Makerere University, Kampala, Uganda
Director, Infectious Disease Research Collaboration, Kampala, Uganda
Email: mkamya@infocom.co.ug
Role on project: Principal Investigator

Grant Dorsey, MD, PhD
Professor, Department of Medicine, University of California, San Francisco, USA
Email: grant.dorsey@ucsf.edu
Role on project: Principal investigator

Sarah Staedke, MD, PhD
Professor of Malaria and Global Health, London School of Hygiene and Tropical Medicine, UK
Email: sarah.staedke@lshtm.ac.uk
Role on project: Co-investigator

Chris Drakeley, BSc, PhD
Professor of Immunology and Infection, London School of Hygiene and Tropical Medicine, UK
Email: chris.drakeley@lshtm.ac.uk
Role on project: Co-investigator

TeunBousema, PhD
Professor of Epidemiology of Tropical Infectious Diseases, Radboud Institute for Health Sciences, Netherlands
Email: Teun.Bousema@radboudumc.nl
Role on project: Co-investigator

Bryan Greenhouse, MD, MA
Associate Professor, University of California, San Francisco, USA
Email: bryan.greenhouse@ucsf.edu
Role on project: Co-Investigator

Martin Donnelly, MA, MSc, PhD
Professor, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, UK
Email: Martin.Donnelly@lstmed.ac.uk
Role on project: Co-investigator

Philip Rosenthal, MD
Professor, Department of Medicine, University of California, San Francisco, USA
Email: Philip.Rosenthal@ucsf.edu

Role on project: Co-investigator

Samuel Nsobya, MSc, PhD
Senior Lecturer, Department of Pathology, School of Biomedical Science, Makerere University College of Health Sciences
Laboratory Director, Molecular Research Laboratory, Infectious Disease Research Collaboration
Email: snsobya@idrc-uganda.org
Role on project: Co-investigator

Joaniter Nankabirwa, MBChB, MSc, PhD
Epidemiologist, Infectious Diseases Research Collaboration, Kampala, Uganda
Email: jnankabirwa@yahoo.co.uk
Role on project: Co-investigator

Emmanuel Arinaitwe, MBChB, MPH
Epidemiologist, Infectious Diseases Research Collaboration, Kampala, Uganda
Email: earinaitwe@idrc-uganda.org
Role on project: Co-investigator

Henry Ddumba Mawejje, MSc
Entomologist, Infectious Diseases Research Collaboration, Kampala, Uganda
Email:mawejjehenry@yahoo.com
Role on project: Co-investigator

Isaac Ssewanyana, MSc
Immunologist, Infectious Diseases Research Collaboration, Kampala, Uganda
Email:sewyisaac@yahoo.co.uk
Role on project: Co-investigator

John Rek, MBChB, MSc
Epidemiologist, Infectious Diseases Research Collaboration, Kampala, Uganda
Email:jrek@idrc-uganda.org
Role on project: Co-investigator

Isabel Rodriquez-Barraquer, MD, PhD
Assistant Professor, Department of Medicine, University of California, San Francisco, USA
Email: monedita@gmail.com
Role on project: Co-investigator

Prasanna Jagannathan, MD
Assistant Professor, Department of Medicine, Stanford University, USA
Email: prasj@stanford.edu
Role on project: Co-investigator

David Smith, PhD
Professor of Global Health, University of Washington, USA
Email: smitdave@uw.edu
Role on project: Collaborator

PARTICIPATING SITES

Infectious Diseases Research Collaboration (IDRC)

Address: Plot 2C Nakasero Road, P.O. Box 7475, Kampala, Uganda
Contact Person: Catherine Tugaineyo
Phone Number: +256 (0)-312281479
Email: ctugaineyo@idrc-uganda.org

London School of Hygiene & Tropical Medicine (LSHTM)

Address: Keppel Street, London, WC1E 7HT, UK
Contact Person: Laure Disson
Phone Number: +44 (0) 20 7927 2936
Fax Number: +44 (0)20 7637 4314
Email: laure.disson@lshtm.ac.uk

University of California, San Francisco (UCSF)

Address: San Francisco General Hospital, 1001 Potrero Avenue,
Building 30, Room 402/408, San Francisco, CA 94110, USA
Contact Person: Tamara Clark
Phone Number: +1 (415) 206-8790
Fax Number: +1 (415) 648-8425
Email: tamara.clark@ucsf.edu

Liverpool School of Tropical Medicine (LSTM)

Address: Pembroke Place, Liverpool, L3 5QA, UK
Contact Person: Helen McCormack
Phone Number: +44 (0) 151 705 3293
Email: Helen.McCormack@lstmed.ac.uk

Radboud University Medical Center

Address: Geert Grooteplein Zuid 26-28, 6500 HB Nijmegen, the Netherlands
Contact Person: Annemieke Jansens
Phone number: +31 (0) 24 3610577
Email: Annemieke.Jansens@radboudumc.nl

Stanford University

Address: 300 Pasteur Drive, Stanford, CA 94305
Contact Person: Dalia Vanderzee

Phone Number: +1 (650) 724-5243
Email: dvanderz@stanford.edu

INSTITUTIONAL REVIEW BOARDS

Makerere University, School of Medicine - Research and Ethics Committee (SOMREC)
Address: Makerere University, School of Medicine, Office of the Dean, PO Box 7072, Kampala, Uganda
REC Chair: Prof. Ponsiano Ochama
Phone Number: +256 (0) 414-530020
Fax Number: +256 (0) 414-531091

University of California, San Francisco, Human Research Protection Program & IRB
Address**:** Office of Ethics and Compliance Research, 3333 California Street Suite 315, San Francisco, CA 94143, U.S.A.
HRPP Interim Director: Michael C. Roach, MHSA, JD, CIP, CHRC
Phone Number: +1-415-476-1814
Email: IRB@ucsf.edu

London School of Hygiene and Tropical Medicine Ethics Committee
Address: Keppel Street, London, WC1E 7HT, UK
Contact Person: Rebecca Carter
Phone Number: +44 (0) 20 7636 8636
Email: Ethics@lshtm.ac.uk

Stanford University, Research Compliance Office
Address: 3000 El Camino Real Five Palo Alto Square, 4th Floor, Palo Alto, CA 94306
Sr. HRPP Lead: Celia Molvin
Phone Number: +1 (650) 724-7141 or +1 (650) 723-0082
Email: irbeducation@lists.stanford.edu

2.0 BACKGROUND INFORMATION AND SCIENTIFIC RATIONALE

2.1 Background Information

2.1.1 Burden of malaria in sub-Saharan Africa and Uganda

Despite recent progress, malaria remains a major global health problem, accounting for and estimated 219 million cases and 435,000 deaths in 2017, with over 90% of cases and deaths occurring in sub-Saharan Africa[1]. There are several reasons why malaria disproportionately affects sub-Saharan Africa. First, most infections are due to *Plasmodium falciparum*, the cause of the most difficult to treat and severe form of the disease. Second, this region is also home to the most efficient malaria mosquito vectors, members of the *Anopheles gambiae* complex and *An. Funestus* complex. Third, most African countries are "the poorest of the poor", lacking the basic infrastructure and resources necessary to mount sustainable malaria control efforts. Uganda, where the proposed work will be located, is emblematic of the immense problem that malaria poses for African countries. Uganda currently ranks 4th in terms of number of malaria cases and 11th in terms of number of malaria deaths globally [2]. Malaria is endemic in over 95% of the country, with some of the highest malaria transmission intensities reported in the world [3]. Malaria is the leading cause of morbidity and mortality in Uganda, accounting for 30-50% of outpatient visits and 15-20% of hospital admissions [4].

2.1.2 Malaria control in Sub-Saharan Africa and Uganda

Primary malaria control interventions currently being implemented in Africa include long-lasting insecticide treated nets (LLINs), indoor residual spraying of insecticide (IRS), and effective case management with artemisinin-based combination therapies (ACTs). In the last decade increases in funding have led to a dramatic scale up of malaria control intervention in Uganda and many other African countries. The World Health Organization (WHO) recommends universal coverage of LLINs, and Uganda has conducted two universal LLIN distribution campaigns, from 2013-14 and 2017-18, leading to a marked increase in the proportion of households owning at least one LLIN [5]. Like many other African countries, Uganda has also renewed efforts to provide vector control through the use of IRS. Uganda's IRS program was reinitiated in 2006, after a gap of 40 years, and it is currently being implemented in 14 of 112 districts. However, a major challenge with IRS is its high cost, limiting coverage to less than 5% of the population [5]. ACTs have become the recommended treatment for malaria throughout Africa. Uganda adopted artemether-lumefantrine (AL) as its first-line therapy in 2004, and the supply of AL at health facilities has greatly increased, with the proportion of children treated for malaria who received AL increasing from 39% in 2009 to 87% in 2014 [5]. Although the scale up of control interventions has been well documented in Uganda, quality data estimating the impact of these interventions on clinically relevant outcomes is limited. The most recent WHO World Malaria Report states that it was not possible to monitor trends in malaria morbidity and mortality from Uganda due to inconsistencies in reporting and laboratory confirmed cases[1]. Thus, there is a critical need to generate an evidence base to support the rational use of control interventions to maximize their impact.

2.1.3 Surveillance and monitoring the burden of malaria in Uganda

Most malaria endemic countries rely heavily on health facility-based surveillance to monitor the malaria burden. In this surveillance system, cases diagnosed as malaria at health facilities are reported through a national system and trends are monitored over time. In Uganda, malaria cases are reported through a Health Management Information System (HMIS) and analysed at national level. While these data are collected routinely from presumably the majority of health facilities in the country, they are often limited due to incomplete reporting, failure of the system to capture cases that occur outside the formal health care system, and the absence of laboratory confirmation, particularly where the vast majority of reported cases of malaria are not laboratory confirmed [6,7]. As a result these data are grossly sub-optimal to monitor the changing clinical epidemiology of malaria in the setting of increasing intervention coverage. Indeed, national reports on trends in malaria cases from Uganda derived from the HMIS system often simply reflect the number of cases of fever captured through HMIS[8].Another source of malaria data in Uganda is periodic national cross-sectional surveys. Population-based demographic and health surveys, such as a 2014 Malaria Indictor Survey in Uganda, provide useful statistics on a range of malaria indicators from nationally representative household samples[5]. However they are very expensive to conduct, making them unfavourable to conduct frequently and unattractive to rely on for routine malaria surveillance in a resource limited setting.

Gold standard metrics for estimating the burden of malaria include the entomological inoculation rate (EIR), malaria incidence, and the malarial force of infection. However these metrics are challenging to routinely enumerate as the most accurate method involves well designed longitudinal studies in which a representative well described population sample is followed through time and incident cases systematically identified. These cohort studies minimize undetected cases that often limit other methods of estimating malaria trends, utilize valid case definitions and standardized methodology of case ascertainment and often collect useful data beyond malaria counts. However, they are labour-intensive, require specialized skills and not implementable over a wide enough geographical scale for routine surveillance purposes. Longitudinal studies may therefore provide gold-standard method for assessing malaria incidence against which other existing methodologies are compared.

2.1.4 PRISM longitudinal surveillance studies

To address the need for improved malaria surveillance in Uganda, we initiated the Program for Resistance, Immunology, Surveillance, and Modelling of Malaria (PRISM) project in 2011. The PRISM project initially collected comprehensive surveillance data at 3 sites with differing malaria epidemiology: 1) Walukuba – an urban site in Central Uganda with relatively low transmission intensity; 2) Kihihi – a rural site in Western Uganda with moderate transmission intensity; and 3) Nagongera – a rural site in Eastern Uganda with historically high transmission intensity. Catchment areas were comprised of the surrounding sub-counties, with populations ranging from 30-40,000 residents. All households within the 3 sub-counties were enumerated and mapped to create a sampling frame for recruitment. Comprehensive surveillance studies include longitudinal cohorts and entomological surveys. For the cohort studies, 100 household were randomly selected from each study site and all children aged 0.5-10 years and 1 adult primary caregiver from each household were enrolled in August 2011. The cohorts were dynamic, such that all newly eligible children were

enrolled and study participants who reach 11 years of age were excluded. In August 2013 an additional 31 houses were enrolled to replace households that had been withdrawn. All study participants were given an LLIN at enrolment and followed at dedicated study clinics for all health care needs. Routine evaluations were done on all participants every 1-3 months including blood smears and collection of blood for future laboratory testing. Entomological surveys were also conducted once a month using miniature CDC light traps in each of the same households participating in the cohort studies. Female anopheles mosquitoes were counted, morphology characterized, species identification performed using gross dissection and PCR, and the presence of sporozoites tested using ELISA.

The cohort and entomology studies continued at all 3 sites through June 2016. A total of 1,402 cohort participants were enrolled, covering 4,704 person years of follow-up. We diagnosed 5,934 episodes of malaria, with an incidence ranging from 0.29 to 2.36 episodes per person year (PPY) among children and 0.13 to 0.46 episodes PPY among adults across the 3 sites. Interestingly, only 14 episodes (0.2%) met WHO criteria for severe malaria and there have been no deaths due to malaria, strongly suggesting that with the provision of prompt and effective diagnosis and therapy with ACT, severe malaria and malaria mortality can be virtually eliminated in our setting. Through our entomology surveys we collected a total of 158,088 female anopheline mosquitoes. The predominant species were *An. gambiaes.s., An. arabiensis*, and *An.funestus*, but species composition varied across the 3 sites. Sporozoite rates were higher in the 2 higher transmission intensity sites, with the overall annual entomological inoculation rate ranging from 2.7 infectious bites PPY in Walukuba to 166 PPY in Nagongera. Data from our cohort and entomology studies have resulted in a number of publications, including studies on the role of poor housing and poverty on the risk of malaria [9,10], associations between urbanicity and malaria risk [11], the descriptive epidemiology of malaria across our 3 sites [12], the impact of LLINs and IRS on malaria burden [13] and a detailed analysis of the parasite reservoir among our cohort participants[14]. The cohort and entomological surveillance studies in Walukuba and Kihihi were completed at the end of June 2016. Given our interest in studying the epidemiology of malaria following the implementation of IRS, we have continued surveillance activities in Nagongera under our existing protocols.

2.1.5 Epidemiology of malaria in Nagongera and the impact of control interventions

IRS was first implemented in Tororo District in December 2014. The IRS program began by using 3 rounds of bendiocarb, a carbamate class of insecticide, spaced ~ 6 months apart. In June-July 2016, the formulation of insecticide used for IRS was changed to Actellic, a longer acting organophosphate class of insecticide, with plans to continue spraying approximately once a year. Following the implementation of IRS in Nagongera, we have seen dramatic declines in transmission intensity, the incidence of malaria, and parasite prevalence. The EIR has reduced from ~ 200 infectious bites per person per year prior to the implementation of IRS to < 10 infectious bites per person per year after IRS (Figure 1).

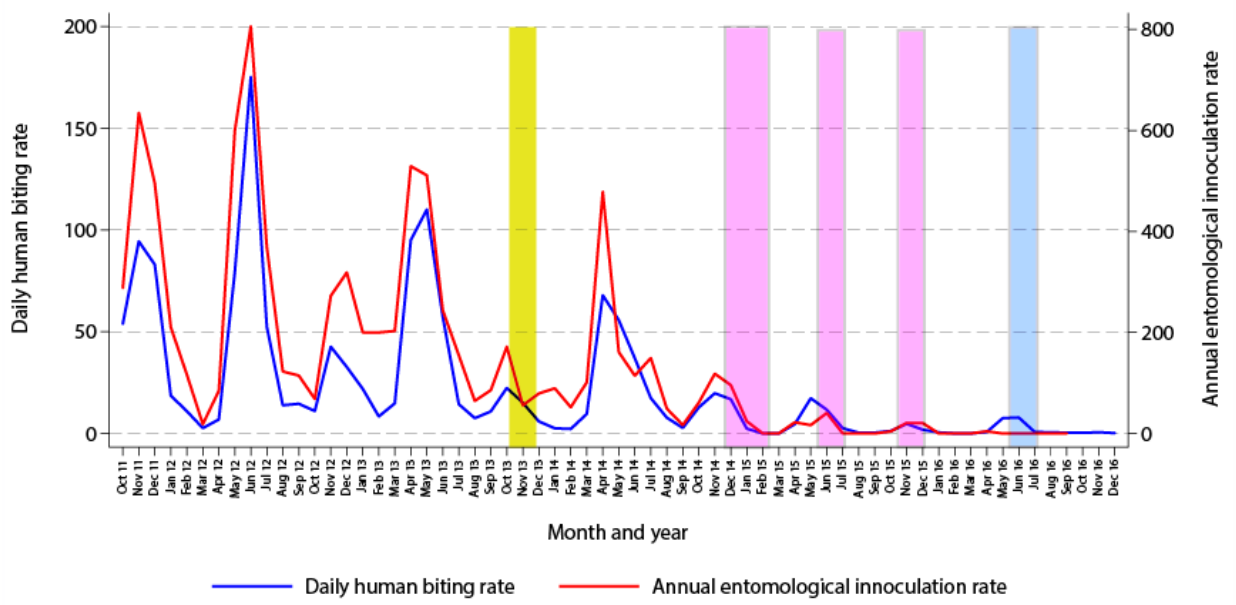


Figure 1: Yellow vertical bar = Universal LLIN distribution; Pink vertical bars = rounds of IRS with bendiocarb; Blue vertical bars = round of IRS with Actellic

Among children 0.5-10 years of age the incidence of malaria reduced from ~ 3 to less than 0.5 episodes per person year, the prevalence of microscopic parasitemia has reduced from ~ 30% to ~ 10%, and the prevalence of microscopic + sub-microscopic parasitemia has reduced from ~ 70% to ~ 25% (Figure 2).

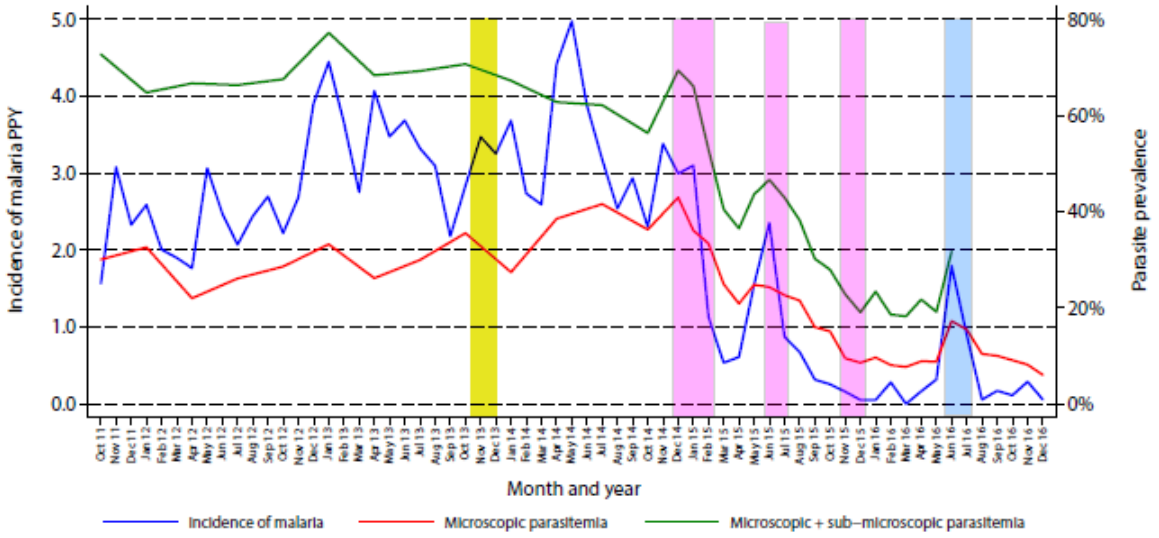


Figure 2: Yellow vertical bar = Universal LLIN distribution; Pink vertical bars = rounds of IRS with bendiocarb; Blue vertical bars = round of IRS with Actellic

2.1.6 PRISM 2 Cohort Study

In October 2017 we began the PRISM 2 cohort study to further investigate the epidemiology of malaria in a historically high transmission area that has seen a dramatic decline in the burden of malaria following the implementation of widely available malaria control interventions including malaria case management with ACTs, universal LLIN distribution, and sustained IRS. The overall of

objective of the PRISM 2 Cohort Study is to improve our understanding of factors that influence malaria infection, disease, and transmission in a setting of changing transmission intensity and population level control interventions. In this study we have enrolled all members of 80 randomly selected households from Nagongera sub-county. Recruitment is dynamic such that people are withdrawn from the study when they leave the household and new participants are enrolled when they moved into the household. Withdrawn participants are also re-enrolled when they permanently return to the households. Similar to our previous cohort studies, all study participants are given an LLIN at enrolment and followed at a dedicated study clinic for all health care needs. Malaria incidence is measured using passive surveillance, with malaria defined as a fever with a positive blood smear. Routine evaluations are done on all participants every 28 days including thick smears and collection of blood for assessment of parasitemia using a highly sensitive quantitative qPCR assay. Entomological surveys are conducted every 14 days in all rooms where participants are sleeping using miniature CDC light traps. Female anopheles mosquitoes are counted, morphology characterized, species identification performed using gross dissection and PCR, and the presence of sporozoites tested using ELISA.

Through March 2019 we have enrolled a total of 509 study participants, 20 of whom have been withdrawn and 489 who continued to be followed. A total of 10,416 female anopheles mosquitoes have been collected after 5,430 CDC light trap collections giving an overall daily human bite rate of 1.92 per person per night. The human biting rate has been close to zero except for a peak prior to the last round of IRS followed by a gradual decline (Figure 3). Over 99% of the mosquitoes that have been speciated have been *Anopheles arabiensis* and sporozoites have been detected in only 8 of 9743 mosquitoes tested (sporozoite rate < 0.1%), giving an overall annual entomological inoculation rate of < 0.002 infectious bites per person per year.

The incidence of malaria has been very low with only 24 cases of malaria after 682 person years of follow-up. The highest incidence of malaria has been in children 5-15 years of age with 0.06 episodes per person years. Overall parasite prevalence measured at the time of monthly routine visits has been 2.1% by microscopy and 11.7% by qPCR (Table 1). There have been only 2 episodes of malaria since January 2019 and both were temporally associated with travel outside of Tororo District.

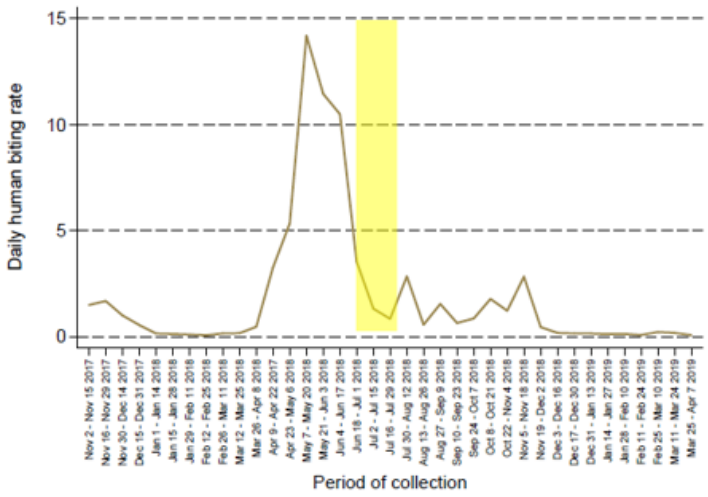


Figure 3: Temporal changes in daily human biting rate
Yellow vertical bar = round of IRS with Actellic

Table 1. Incidence of malaria and parasite prevalence from PRISM 2 cohort study

Measure	Age groups			
	All	< 5 years	5-15 years	> 15 years
Person years of follow-up	682	188	266	228
Incident episodes of malaria	24	6	15	3
Incidence of malaria PPY	0.04	0.03	0.06	0.01
Parasite prevalence by microscopy	193/9032 (2.1%)	34/2506 (1.4%)	135/3519 (3.8%)	24/3007 (0.8%)
Parasite prevalence by qPCR	1054/9026 (11.7%)	119/2505 (4.8%)	560/3516 (15.9%)	375/3005 (12.5%)

Parasite prevalence by qPCR was initially between 20-25% in participants over 5 years of age and ~ 10% in children < 5 years of age. Parasite prevalence gradually decreased before increasing just before the last round of IRS. Following the last round of IRS parasite prevalence has decreased again reaching unprecedentedly low levels in March of 2019 of < 10% in participants over 5 years of age and 0% in participants < 5 years of age (Figure 4). These data suggest that after over 4 years of sustained IRS in Tororo, malaria has almost been eliminated and the parasite reservoir has been reduced to very low levels. Currently laboratory studies are ongoing to genotype longitudinal samples among participants with parasitemia to distinguish new infections from persistent infections and estimate the molecular force of infection.

Figure 4: Temporal changes in parasite prevalence measured at the time of routine visits using qPCR stratified by age groups.
Yellow vertical bar = round of IRS with Actellic

In addition to the basic epidemiological studies described above, we are also conducting membrane feeding assays to estimate the infectiousness of the human reservoir. Using convenient sampling, blood is being collected from cohort participants that have malaria or asymptomatic parasitemia at the time of their previous routine visit to feed on mosquitoes. Mosquitoes are dissected after 9-10 days to assess whether they have been infected with malaria parasites. As of March 2019 we had successfully completed 329 membrane feeding assays of which 31 (9.4%) were infectious. The highest rates of infections have been in children < 5 years of age (15.4%) followed by children 5-15 years of age (11.4%). Interestingly, none of the 71 assays conducted in participants over 15 years of age have been infectious. Stratified by parasite status, infectious rates have been highest in participants with asymptomatic microscopic parasitemia (26.2%) followed by those with symptomatic malaria (10.0%) and those with sub-microscopic parasitemia (7.0%). Interestingly, since January 2019 only 1 of 74 (1.4%) samples tested has been infectious. These data demonstrate our

ability to successfully carry out membrane feeding assays in large numbers and document an infectious reservoir among children that appears to be waning over time.

2.1.7 Malaria surveillance data from Busia District

Busia District is located in south-eastern Uganda and is bordered by Tororo District to the north, Kenya to the east, and Lake Victoria to the south (Figure 5). IRS has never been implemented in Busia District, which is known to be an area of very high malaria transmission intensity. Our research group has been active in Busia since 2016 including clinical trials at Masafu General Hospital, cross-sectional surveys as part of cluster RCT of different LLINs, and health facility-based surveillance at Lumino Level IV HC. Data from these research activities have documented very high parasite prevalence among pregnant women, children, and patients presenting to the health center (Table 2).

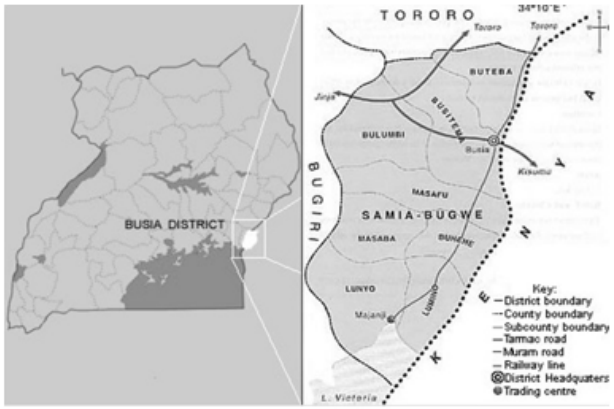


Figure 5: Map of Busia District

Table 2. Malaria metrics from Busia District

Source of data	Dates of collection	Parasite prevalence by microscopy
Pregnant women presenting for ANC at Masafu Hospital	September 2016 – May 2017	401/782 (51.3%)
Children 2-10 years of age from cross-sectional survey	April 2017	115/269 (42.8%)
Test positivity rate among patients with suspected malaria at Lumino HC IV	June 2018 – March 2019	9720/15693 (60.9%)

2.2 Study Rationale

2.2.1 Rationale for epidemiology studies

In this study we will conduct comprehensive surveillance, including a cohort of individuals and entomology surveys within the households of cohort participants, in a contiguous area covering both sides of the border between Tororo and Busia Districts. These highly detailed longitudinal studies will allow us to compare key malaria metrics such as malaria incidence, repeated measures of parasite prevalence, and a variety of entomological measures across a continuum of transmission intensity from a high transmission area where IRS has never been implemented (Busia) to a low transmission area where sustained IRS has been implemented for over 4 years (Tororo). In addition, we will take advantage of unique access to linked entomologic, parasitologic, and clinical data to quantify the impact of environmental and host factors on the establishment and maintenance of infection. Central to these efforts will be our ability to detect, distinguish, and follow genetically

distinct parasites in the blood of individuals over time, allowing us to accurately measure the force of infection (FOI or rate of acquisition of blood stage infections), follow the trajectory of these infections within a host, and relate these metrics to entomologic and epidemiologic data. We will then integrate these individual-level relationships to characterize their epidemiologic consequences and model the impact of interventions.

We will also examine the role of overnight travel on malaria risk. Currently, our understanding of how often people travel (with overnight stay) in Uganda between areas of low and high malaria transmission intensity is limited, and to our knowledge there are limited longitudinal data on travel and risk of malaria infection. Moreover, little is known about modifiable risk factors associated with this kind of travel that could be targeted through malaria control interventions to decrease the risk of infection, and there are no national guidelines on malaria prevention during travel for Ugandans within Uganda. Therefore, this research will provide a key step in understanding the extent of travel (with overnight stay) and its association with malaria infection in Uganda. Risk factors identified will be instrumental in malaria prevention in this population.

Related to this work on characterizing malaria epidemiology is identifying alternative causes of febrile illness. In particular, as transmission declines, as is seen with successful malaria control such as IRS, non-malarial illnesses become a higher proportion of the burden of febrile illness; this may be further augmented by the recent global emergence of the SARS-CoV-2 pandemic. In addition, even when participants had malaria parasites in their blood, alternative pathogens may contribute to or be primarily responsible for the illness presentation. Therefore, we will undertake measurements to identify alternative causes of febrile illness in cohort participants.Additionally, information on subjects' vaccination history will provide important context for understanding the risk to and breadth of pathogens that are responsible for non-malarial febrile illness.

2.2.2 Rationale for transmission studies

An in-depth understanding of transmission of malaria from the human host to the mosquito vector is required to develop and target improved interventions to further reduce the burden of malaria to its ultimate elimination. However, our comprehension of what governs natural malaria transmission is far from complete. We know that malaria transmission depends on the presence of mature gametocytes in the peripheral blood, yet the rate at which gametocytes are produced, and factors associated with gametocyte commitment, maturation and infectivity, remain poorly elucidated. Few studies have accurately quantified the likelihood of transmission from the infected host to the vector, and fewer still the contribution of different age groups within populations to overall transmission. In high transmission settings, sub-microscopic infections are an important contribution to the infectious reservoir, and may threaten efforts to control and eliminate malaria. However, data on the infectious reservoir in different settings, and our understanding of the role that clinical and asymptomatic infections, either as microscopically-detectable and sub-microscopic infections, play in this reservoir, are limited. In this project, we will utilize blood and mosquito samples collected in Tororo and Busia Districts to assess experimental infectivity to mosquitoes, and natural mosquito sampling to address key knowledge gaps. We will utilize the framework of our study cohort together with novel molecular diagnostics to investigate commitment to gametocyte production, gametocyte maturation and infectivity in relation to human host, parasite and environmental characteristics. We

will perform repeated assessments of the infectivity of different age groups to locally relevant *Anopheles* mosquitoes using two sources of mosquitoes. We will also perform a direct comparison of transmission efficiency between membrane feeding and direct skin feeding. Direct skin feeding typically results in higher mosquito infection rates than membrane feeding assays [15,16]. To interpret the predictive value of membrane feeding experiments (that are acceptable across a wider age-range and more scalable) in the context of more natural assessments of transmission potential by direct skin feeding, paired assessments of mosquito infection rates by membrane feeding assays and direct skin feeding experiments will be performed for a selection of older individuals (≥8 years). Finally, we will quantify the contact between humans and mosquitoes for different age groups over time and space. This work will be tightly integrated with the epidemiology studies, allowing us to address research questions structured across the parasite life-cycle, and to provide information to help guide strategies for the control and ultimate elimination of malaria in Uganda.

2.2.3 Rationale for immunology studies

Immunity is critical in modulating morbidity and mortality from falciparum malaria in endemic areas, where some individuals are infected hundreds of times per year. Indeed, malaria has exerted significant selection pressure on the human genome as evidenced by the association of malaria parasite prevalence with the sickle cell trait (HbAS) and other hemoglobinopathies[17,18]. These and other host genetic polymorphisms can exert protective effects through innate or acquired mechanisms, but known polymorphisms only account for a fraction of the observed variation [19,20]. Antibodies are known to be an important mediator of acquired immunity, as demonstrated by experiments in which the passive transfer of immune serum cleared parasitemia and alleviated symptoms in subjects with malaria [21].In addition, naturally acquired malaria-specific cellular immune responses are likely to play a critical role in protection against malaria. As with host polymorphisms, the specific immune responses associated with protection are only partially elucidated and likely to be complex[22].

A major barrier in identifying host genetic and immunologic correlates of protection, and even more basic relationships such as the impacts of repeated exposure and age (which may act independent of repeated exposure e.g. via maturation of the immune system), has been the limited characterization of clinical immune phenotypes. Immunity can prevent establishment of blood stage infection, reduce parasite densities (i.e. anti-parasite immunity), modify the fever response (i.e. anti-disease immunity), reduce the severity of disease and probability of death, or reduce the duration of an infection. However, most previous studies have only evaluated limited manifestations of immunity, e.g. case-control studies of severe malaria[23]. Thus, despite a substantial research effort, there are still fundamental questions about the protective effect of immunity in relation to age and exposure as well as the time scales over which these immune responses wax and wane[22]. Some essential challenges in answering these questions are the heterogeneous nature of malaria exposure between populations and among individuals within a population; the fact that malaria immunity is a complex phenotype that develops differently in different individuals due to varied exposure, host genetics, parasite genetics, chance, or other unknown factors; and finally that changing patterns of malaria exposure (e.g. driven by climate or control measures) can interact with all of these factors in a complex fashion. In the proposed study, we will probe these and related questions in greater depth,

taking advantage of an innovative study design tailored to address limitations of prior surveillance studies.

2.2.4 Rationale for studies of antimalarial drug and insecticide resistance

The control of malaria is seriously threatened by resistance to drugs and insecticides. Considering antimalarial drugs, current first-line artemisinin-based combination therapies (ACTs) continue to offer excellent efficacy in Africa, but widespread resistance in Southeast Asia is of great concern, as the spread of resistance to ACTs to Africa may have devastating consequences[1]. Considering insecticides, resistance to all major classes is present in African anopheline mosquitoes, potentially jeopardizing key control measures[24]. Thus, longitudinal surveillance to assess resistance of *P. falciparum* to leading antimalarial drugs and anopheline mosquitoes to available insecticides is of very high priority.

The interplay between parasite resistance and fitness is of profound importance in determining the ability of drug resistance to become established. Limited studies have generally shown that parasites with resistance-mediating mutations are out-competed by wild-type (WT) in culture and in vivo. For example, parasites containing key *pfmdr1* SNPs were out-competed by WT in co-culture experiments [25]. However, results have been complex. For example, evaluations of seasonal parasite prevalence suggest that parasites with WT *pfmdr1* N86 outcompete those with the mutant sequence when selective drug pressure is low, but mixed isolates from Uganda showed selection in culture of the mutant 86Y genotype [26,27]. Practically most meaningful are studies of clinical isolates. In Malawi, cessation of CQ use was followed by the return of CQ-sensitive malaria[28]. In Uganda, regular use of AL led to significantly different *pfcrt* and *pfmdr1* genotypes than did regular use of DP[29]. Arguably the most important aspect of parasite fitness is transmissibility, but studies in this area are limited. Murine parasites selected for resistance to pyrimethamine had slowed mosquito development, but those selected for enhanced virulence and decreased pyrimethamine sensitivity had no apparent defect in mosquito development[30]. In *P. falciparum*, enhanced transmissibility of CQ- and SP-resistant parasites was seen[31]. In contrast, in Zambia prevalences of *pfdhfr* SP-resistance mutations and the *pfcrt* 76T mutation were both greater in parasites infecting humans than those infecting mosquitoes[32,33]. In our studies in Uganda, compared with parasites infecting humans, those infecting *Anopheles gambiae*s.s. were enriched for the *pfmdr1* 86Y ($P = 0.0001$) and *pfcrt* 76T ($P = 0.0412$) mutant alleles. Mosquito insecticide resistance patterns may also play a key role in transmissibility. In West African lab studies, some insecticide resistance mutations increased susceptibility to *P. falciparum* infection[34]. In Tanzania, we showed that wild-caught *A. gambiaeVgsc-1014S/kdr* homozygotes had a significant increase in sporozoite rate compared to WT (8.0% vs 2.4%; p=0.026)[35]. Against a backdrop of increasing insecticide resistance there is an urgent need for two related lines of inquiry, first to study mosquito infection by resistant parasites, and second to study *P. falciparum* infection in resistant mosquitoes.

2.3 Potential Risks and Benefits

2.3.1 Risks and Discomforts

Privacy - Care will be taken to protect the privacy of subjects and parents/guardians, as described in this protocol. However, there is a risk that others may inadvertently see patients' medical information, and thus their privacy may be compromised.

Finger Pricks and Venipuncture – Routine sampling by phlebotomy will be done at enrolment and every 4 weeks during follow-up. Finger prick / heel stick sampling will be done at the time of each non-routine visit if the participant is febrile. Additional sampling of blood by phlebotomy will be done at the time malaria is diagnosed and at specific timepoints following symptomatic malaria, non-malarial fever, or asymptomatic malaria infections in select individuals as described in more detail below. Precautions will be taken to ensure that the volumes of blood taken do not exceed maximum recommended volumes over any 30 day period as outlined in Appendix C. These volumes are too small to experience any side-effects from the blood drawing although a mild and transient feeling of discomfort/bruising may occur at the site of sampling. Risks of these procedures include pain, transient bleeding and soft-tissue infection.

Direct skin feeding – Although direct skin feeding has been performed on children from the age of 5 onwards[15], we chose to include only older individuals (≥8 years) in this study since it is the first of its kind in Uganda. An increasing number of sites in Africa are implementing direct skin feeding assays as part of clinical trials. The health hazard that mosquito colonies form for exposed study participants is negligible because viral infections that can cause disease in humans have never been detected in *Anopheles* colonies that are maintained in controlled insectaries and vertical transmission of pathogens (essential to result in infected colony mosquitoes) has never been described in viruses infecting anophelines. Although this has prompted several labs to perform direct skin feeding assays without prior screening of mosquito colonies (recently Medical Research Council in Fajara , The Gambia and KEMRI-CDC in Kisumu, Kenya) [36,37], we will take an extra precaution. Following procedures followed by the Centers of Disease Control (CDC) in Atlanta, the National Institute of Health in Bethesda (NIH) and the Malaria Research & Training Centre in Bamako, Mali (MRTC), we screen our mosquito colony for viral infections at a reference laboratory of the World Health Organization (Pasteur Institut, Dakar) and used a screened human blood source for colony maintenance. We will extensively monitor and report on the tolerability of direct skin feeding assays and provide antihistamine cream after the direct skin-feeding assay is completed.

2.3.2 Known Potential Benefits

Benefits of participation include receiving rapid clinical care from the doctors and nurses on the research staff in the dedicated study clinic as per local standards. This will include care for all malaria and non-malaria illnesses. Additionally, the information which we get from this study will help Uganda and other countries to decide on the simplest, cheapest and best way of observing the burden of malaria in our population and increase our understanding on how people living in different settings develop immunity to malaria.

3.0 STUDY OBJECTIVES

The overall objective of this proposal is to improve our understanding of factors that influence malaria infection, disease, and transmission in a setting with varied transmission intensity and

population level control interventions. Specific objectives according to the types of studies that will be undertaking are listed below.

3.1 Objectives of epidemiology studies

1. To compare the incidence of malaria, parasite prevalence, and the molecular force of infection among cohort study participants living in areas with different transmission intensity.
2. To characterize factors determining the malarial force of infection.
3. To determine factors affecting the duration, density, and clinical consequences of blood stage malaria infection.
4. To assess the associations between overnight travel and the risk of malaria infection.
5. To compare measures of transmission intensity including the human biting rate, sporozoite rate, and the entomological inoculation rate at the household level in areas with different transmission intensity.
6. To compare the species composition of mosquito vectors and the host source of mosquito blood meals in areas with different transmission intensity.
7. To identify pathogens responsible for non-malarial febrile illness among cohort study participants.

3.2 Objectives of transmission studies

1. To characterize factors associated with gametocyte production.
2. To evaluate the impacts of human, parasite, and mosquito factors on parasite infectivity to mosquito vectors.
3. To characterize the human infectious reservoir for malariain areas with different transmission intensity.

3.3 Objectives of immunology studies

1. To compare antibody responses to malaria antigens and associations between antibodies responses and protection from /exposure to infection with malaria parasite sin areas with different transmission intensity.
2. To compare malaria specific cellular immune responses in naturally exposed children and adults in areas with different transmission intensity.

3.4 Objectives of resistance studies

1. To assess the impact of genotypic markers of antimalarial drug resistance on malaria transmission.
2. To assess the impact of genotypic markers of insecticide resistance on malaria transmission.

4.0 OVERALL STUDY DESIGN

We propose to conduct a prospective cohort study and associated longitudinal entomological surveillance to collect clinical data and biological samples for complementary laboratory studies. We will enroll 80 randomly selected households and all eligible household members from a catchment area that extends across a low transmission setting where IRS has been sustained for over 4 years (Tororo District) to a high transmission setting where IRS has not been implement (Busia District).

Participants enrolled in this study will be followed up to March 31st 2024 depending on availability of resources.

5.0 STUDY POPULATION

5.1 Study Site

The study will be conducted in a contiguous catchment area within 3 parishes across the border of Tororo District (Osukuru and Kayoro parishes) and Busia District (Buteba parish) in Eastern Uganda (Figure 6). Tororo District is predominantly a rural setting with historically high transmission intensity. Entomology studies conducted in Tororo estimated the entomological inoculation rate (EIR) to be 562 infective bites per person per year in 2001-02 and 310 infective bites per person per year in 2011-12 [3,12,38]. Following the implementation of IRS in Tororo District in December 2014, there has been a dramatic decline in transmission intensity with the EIR now estimated to be < 1 infective bite per person per year. Busia District borders Tororo District to the south and is also a predominately rural area. IRS has never been implemented in Busia District and transmission intensity is estimated to be very high based on various surveillance studies conducted by our group (see section 2.1.7). The change of study sites from Nagongera subcounty to the 3 parishes across the border of Tororo and Busia districts will allow us collect data similar to what is being collected in PRISM 2 but across areas of varying malaria transmission intensity. A dedicated study clinic has been established within Tororo District Hospital. The furthest distance a study participant needs to move from their home to the study clinic for a routine or sick visit is estimated at 20km.

5.2 Selection of Study Population

5.2.1 Enumeration survey

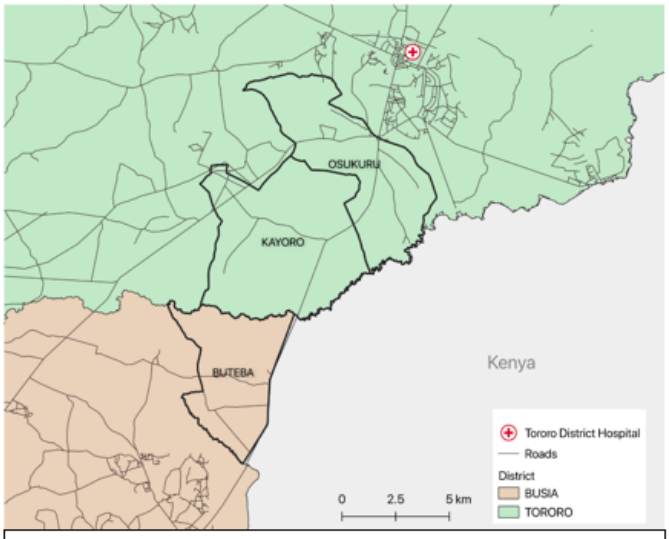


Figure 6: Map of study catchment area

To generate a sampling frame for selection of households to participate in the cohort study we will enumerate and map all households within our catchment area (Figure 6). Prior to the start of the study, investigators will meet with local officials and community representatives to discuss the study and plans for the household enumeration. Using a map of the boundaries of our catchment area, project personnel will systematically cover the entire area within the boundaries to identify and enumerate all households. A household will be defined as any single permanent or semi-permanent dwelling structure acting as the primary residence for a person or group of people that generally cook and eat together. Some households may include members who sleep in other dwelling structures within the same compound, if the members are still dependent on the head of household in the main household. All households identified will be assigned sequential unique IDs. Household locations will be mapped using hand-held GPS receivers. Readings will be taken from the door of the household, if possible, or from a point that is most representative of the household. At each household, a reading

will be taken every five seconds for 2 minutes, and the average values from these readings will be recorded (Easting, Northing, and Altitude) in UTM units. Only GPS coordinates will be picked from the households. No additional data will be collected during the enumeration survey.

5.2.2 Cross-sectional survey

To better inform the selection of households for participation in the cohort study we will conduct a cross-sectional survey of up to 300 households from our enumeration list to generate spatial estimates of parasite prevalence for children 2-10 years of age within our catchment area.

5.2.2.1 Screening of households for cross-sectional survey

Households will be randomly selected for screening from our enumeration list. When a household is identified, study personnel will briefly describe the purpose of the survey to the head of the household (or their designate) in the appropriate language, and screen for eligibility. Households with at least one household resident between 2-10 years of age present will be eligible for the cross-sectional survey.

5.2.2.2 Informed consent for cross-sectional survey

A detailed description of the informed consent procedures is provided in section 9.0. Briefly, study personnel will carry out the informed consent discussion with the parent(s) or guardian(s) of children. Informed consent will be conducted in the appropriate language and a translator will be used if necessary. Consent forms will be available in English and the local languages (. Following the informed consent discussion, parents/guardians will be asked by the study personnel to sign a written consent form for their child(ren) to participate in a research study. Written assent to participate in the study will also be obtained from children aged 8 years and older at the time of screening. If an adult respondent or parent/guardian is unable to read or write, their fingerprint will substitute for a signature, and a signature from a witness to the informed consent procedures will be obtained.

5.2.2.3 Screening and recruitment of participants for cross-sectional survey

All children aged 2-10 years from eligible households who are present will be eligible for participation in the clinical survey.

The inclusion criteria are:

1 Child aged 2-10 years
2 Usual resident who was present in the sampled household on the night before the survey
3 Agreement of parent/guardian to provide informed consent
4 Agreement of child aged 8 years or older to provide assent

The exclusion criterion is:

1 Child not home on day of survey

5.2.2.4 Cross-sectional survey procedures

The cross-sectional survey will include measurement of temperature, subjective fever and a finger-prick blood sample for measurement of thick blood smear and collection of dried blood spots. Participants who have a temperature of $\geq$ 38.0^{o}C, or who report fever in the past 48 hours, will have an RDT performed. Febrile participants will be treated with paracetamol as appropriate. Participants with a positive RDT and no evidence of severe malaria will be treated with AL. Participants with a positive RDT and evidence of danger signs of severe disease will be referred for further evaluation and treatment. Any participant with other concerning clinical symptoms will also be referred to an appropriate health care facility at the discretion of the study personnel. Thick smears will be assessed for the presence of asexual parasites as described in section 6.3.1. These data will be used to generate a spatial distribution of parasite prevalence across our catchment area and assist in the selection of study households for participation in our cohort study as described below.

5.2.3 Selection of study households for participation in cohort study

Our catchment area will be divided into areas of high, medium, and low parasite prevalence based on data obtained from the cross-sectional survey. Random lists of households will be generated for each of the 3 strata of parasite prevalence using our enumeration list. Study personnel will approach households on the randomly selected household lists in the order in which they were selected. Our goal will be to recruit 20 households from the high parasite prevalence strata, 40 households from the medium parasite prevalence strata, and 20 households from the low parasite prevalence strata (80 household total). Households will be eligible for recruitment if the meet the following criteria:

1) Have at least two members of the household aged 5 years or younger
2) No more than 7 permanent residents currently residing in the household
3) No plans for the household to move from our study catchment area in the next 2 years
4) Willingness to participate in entomological surveillance studies (see section 6.2)

Selection criteria have been established to ensure that 1) there is sufficient representation of younger children, and 2) the number of household members does not exceed our capacity for follow-up. If a household is eligible and the head of the household is interested in the study, all permanent household members will be asked to come to the study clinic for screening and enrollment. If the head of the household is not home during the initial contact, the household will be re-visited on at least 3 other occasions over a 2-week period before elimination from the sample selection process.

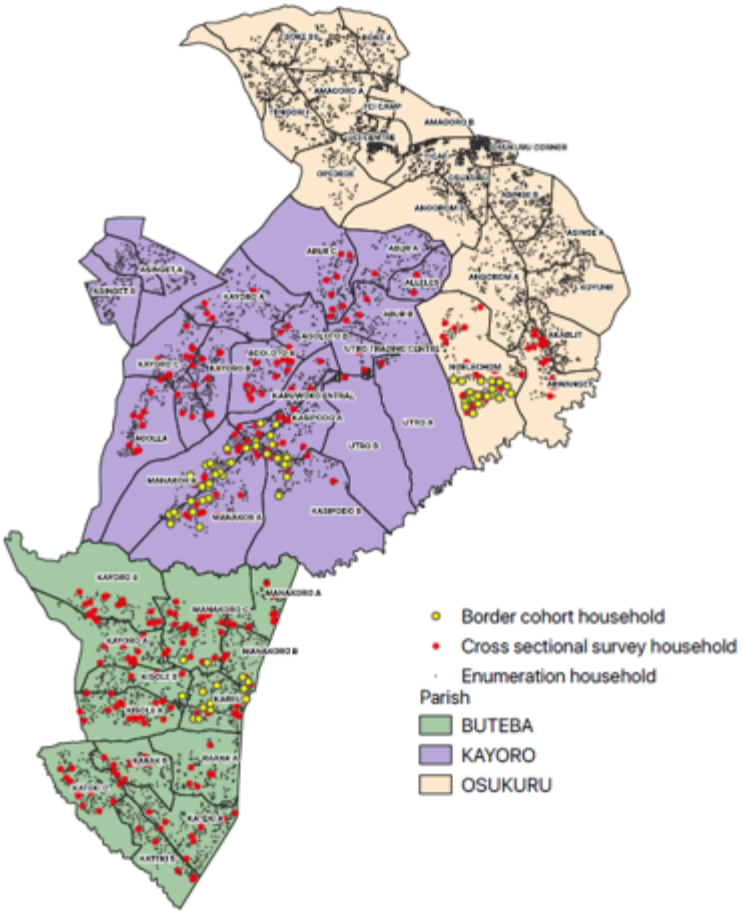
5.2.4 Modification of selection of study households for participation in the cohort study

Between August 10th and September 2nd 2020, 80 households were enrolled in the cohort study following the procedures outlined in section 5.2.3. This included 20 houses from the Buteba Parish of Busia District, 40 houses from the Kayoro Parish of Tororo District, and 20 houses from the Osukuru Parish of Tororo District (Supplemental Figure). Preliminary findings confirmed that baseline parasite prevalence was high among participants living in households enrolled from Busia District, as expected. However, baseline parasite prevalence was also high among participants living in

households from Tororo District (Supplemental Table). The houses enrolled from Tororo were chosen to be close to the Tororo District border, assuming that parasite prevalence would be significantly lower than Busia District given that IRS has only been implemented in Tororo District. However, it appears that the impact of IRS is substantially lower than expected among houses close to the Tororo border. As the study objectives are highly dependent on enrolling houses with a clear gradient in transmission intensity, selection of households for participation in the cohort study will be modified as follows:

- Participants from the 20 houses enrolled from Osukuru Parish, Tororo District will be dropped from the study
- Participants from 10 houses randomly selected from 24 houses enrolled from Kayoro Parish, Tororo District in an area closest to the border with Busia District will be dropped from the study
- The study team will visit the households dropped to explain reasons for exclusion and when their follow-up period will end
- 30 replacement houses will be enrolled from Tororo District following the same procedures outlined in section 5.2.3 and all eligible household members will be enrolled as outlined in sections 6.1.1 and 6.1.2. 15 houses will be enrolled from an area of Osukuru Parish farthest from the Tororo border and 15 houses will be enrolled from an area of Kayoro Parish farthest from the Tororo border.

Supplemental Figure.



Supplemental Table. Data at the level of each cohort study participant at enrollment

Characteristics	Busia	Tororo
Parasite positive by microscopy, n (%)	24 (23.8%)	66 (18.9%)
Parasite positive by microscopy stratified by age categories, n (%)		
< 5 years	10 (22.2%)	21 (15.9%)
5-15 years	7 (35.0%)	35 (35.7%)
16 years or older	7 (19.4%)	10 (8.4%)
Parasite positive by qPCR, n (%)	47 (46.5%)	153 (43.8%)
Parasite positive by qPCR stratified by age categories, n (%)		
< 5 years	15 (33.3%)	45 (34.1%)
5-15 years	13 (65.0%)	60 (61.2%)
16 years or older	19 (52.8%)	48 40.3(%)

5.2.5 Recruitment of new households to replace households that have withdrawn from the study

In 2022, the study will re-open the household enrollment process, following the same selection process as described above in section 5.2.3, to replace households that have been withdrawn from

the study (i.e. all residents of a particular household have been withdrawn from the study). We will only enroll an equal number of households that have been withdrawn such that the total number of households being followed at any time will not exceed the original sample size of 80 households. Cohort study participants enrolled from new household will follow the same procedures as currently enrolled households.

6.0 STUDY PROCEDURES AND EVALUATIONS

6.1 Cohort study

6.1.1 Study participant screening

The screening visit and all subsequent study visits will take place at our designated study clinic at Tororo District Hospital (TDH). Study clinicians will assess eligibility of potential study participants using the following selection criteria:

1) Household considered their primary residence
2) Agreement to come to the study clinic for any febrile illness and scheduled routine visits
3) Agreement to avoid antimalarial medications outside the study
4) Provision of written informed consent (for parent or guardian in case of children)

The cohort will enrol all members of the household irrespective of their age and will be dynamic such that over the course of the study any permanent residents that join the household will be screened for enrolment. Participants and/or guardians will be requested to bring any documents (including a vaccine card) where their vaccinations and dates are written down, to the baseline evaluation.

6.1.2 Study enrolment

After cohort participants are deemed eligible for enrolment and informed consent for study participation is obtained, they shall be enrolled. See Section 9.0 for Informed Consent and Assent Process. Study participants will be given a unique study number which will be linked to their household ID number and will undergo a standard baseline evaluation. The baseline evaluation will include a history and physical examination accompanied with filling out standard case record form, and a vaccination history case record form (Appendix E). Subjects will have blood collected by venipuncture (2.5-15 mL depending on the weight of the study participant as specified in Table 3) for hemoglobin measurement, thick blood smears, and storage of blood and filter paper samples for future studies. Subjects found to have a fever (tympanic temperature $\geq$ 38.0°C) or history of fever in the previous 24 hours and a positive thick blood smear will be diagnosed with malaria and treated with AL while those with other illnesses will be treated according to local guidelines (see section 6.1.3.2). Following enrolment, a household survey appointment will be scheduled to collect household-level information on the use of malaria interventions and treatment seeking practices (Appendix A). All household members will be provided access to a long-lasting insecticide treated bed net (LLIN) immediately after the household survey is completed.

6.1.3 Cohort follow-up and procedures

The overall principle for this observational study is to provide study participants with access to prompt and appropriate therapy for all illnesses using standard guidelines according to the Ugandan Ministry of Health [39]. Participants will be asked to seek all medical care for themselves and their child/children at the study clinic and to avoid the use of any other anti-malarial medication outside of the clinic. The study clinic will be open every day from 8.00 am to 5:00 pm. Visits to the clinic will be classified as either scheduled routine visits or unscheduled non-routine visits.

6.1.3.1 Clinic visits

Routine visits: These will be visits conducted at the study clinic once every 4 weeks for the duration of the observation period to ensure protocol compliance and obtain regularly scheduled clinical and blood samples. At each visit, a standardized evaluation will be done and a standard case record form will be completed. Participants or parents/guardians will be asked about visits to outside health facilities, use of any anti-malarial medications outside the study protocol, LLIN usage, and updates to vaccination history. The study protocol will be reinforced with discussion regarding the need to come to the study clinic promptly upon the onset of any illness and to avoid use of outside anti-malarial medications. Each routine visit, participants will have blood collected by finger prick/heel stick if they are under 6 months of age, or venepuncture aged 6 months and older. For venipuncture,2.5-15 mL of blood will be collected based on the participant's weight as specified in Table 3. Blood collected will be used to perform a thick blood smear, measure hemoglobin levels (every 12 weeks), collect filter paper samples, for immunologic assessments, and to store blood for various studies. In selected participants, a subset of the blood collected may be used as negative controls for studies of non-malarial pathogens and/or for experimental methods aimed at detecting malaria parasites in the blood including detection of gametocytes. These tests will be for research purposes only and will not affect patient care. If venipuncture is refused at the point of any individual collection time, a finger prick blood sample will be requested and the participant will still be able to continue participation in study.Study participants found to have a fever (tympanic temperature $\geq$ 38.0°C) or history of fever in the previous 24 hours at the time of their routine visit will have their thick smear read urgently. If the thick blood smear is positive, the patient will be diagnosed with malaria and managed as described in section 6.1.3.2 below. If the thick blood smear is negative, the patient will be managed as described in section 6.1.3.3 below. Study subjects who do not present to the clinic for their scheduled routine visits will be visited at home and requested to come to the study clinic as soon as possible. Participants and/or guardians will be requested to bring any documents (including a vaccine card) where their vaccinations and dates are written down, to the final routine visit.

Non-routine visits: Non-routine visits will be defined as any unscheduled visits to the study clinic. All patients presenting for a non-routine visit will undergo a standardized clinical history and physical examination. Subjects found to have a fever (tympanic temperature $\geq$ 38.0°C) or history of fever in the previous 24 hours will have blood obtained by finger-prick (< 1 mL) for an urgent blood smear and filter paper sample collection. If the thick blood smear is positive, the patient will be diagnosed with malaria and managed as described in section 6.1.3.2 below. If the thick blood smear is negative, the patient will be managed as described in section 6.1.3.3 below. Regardless of the outcome of the

thick blood smear, participants presenting with fever, history of fever, and/or a maculopapular rash may have a nasal and/or oral swab and 2ml of blood collected and stored for identification of non-malarial pathogens and/or for experimental methods aimed at detecting malaria parasites in the blood including detection of gametocytes. These tests will be performed for research purposes only and will not affect patient care. Patients who do not have fever and are not suspected to have malaria will receive standard-of-care treatment as per local treatment guidelines. Follow-up non-routine visits for a previously diagnosed illness will be scheduled as per the clinicians' discretion. During follow-up visits, blood smears will be collected and prepared per the discretion of the study clinician and managed as described above.

Follow-up and procedures for additional Immunology studies: Based on sampling performed at monthly routine visits detailed above, a subset of participants will be enrolled into an immunology sub-cohort. This sub-cohort will be prospectively followed to allow for in-depth peripheral blood sampling across symptomatic and asymptomatic infections over the course of the observation period, in order to compare the immune trajectories of paired symptomatic and asymptomatic infections in the same individuals. For symptomatic malaria infections, blood will be drawn as above via phlebotomy on the day study participants are diagnosed with symptomatic malaria. Subjects will be treated for malaria as described in section 6.1.3.2 of the protocol, and asked to return to clinic 7 and 14 days later for follow-up blood draws as outlined in Table 3 and completion of a standardized evaluation and filling out a case record form as described above. For participants with asymptomatic parasitemia (either diagnosed by microscopy or qPCR at the time of routine visits), subjects will be asked to return to clinic 14 days later for follow-up blood draws as outlined in Table 3 and completion of a standardized evaluation and filling out a case record form as described above. Asymptomatic parasitemia will not be treated as per Uganda Ministry of Health guidelines. A subset of participants presenting with non-malarial febrile illness may be used as negative controls and will undergo phlebotomy and be asked to return to clinic 7 and 14 days later for follow-up blood draws as outlined in Table 3 and completion of a standardized evaluation and filling out a case record form as described above.

Overnight travel and the risk of malaria infection:
At the time of all visits to the study clinic, study participants or their parents/guardians will be asked whether they spent at least one night outside their homes since the last clinic visit. If they did, a detailed questionnaire (Appendix B) will be administered to capture data on where they travelled to, the number of nights they spent away, and malaria control interventions used, if any.

6.1.3.2 Malaria diagnosis and treatment

Patients with suspected malaria, defined as fever (tympanic temperature $\geq$ 38.0°C) or history of fever in the previous 24 hours will undergo a standardized evaluation as described above. Those found to have malaria based on presence of fever and a positive thick smear will have blood collected by venipuncture (2.5-10mL depending on the participant's weight as specified in Table 3). If the participant would exceed the maximum volume of blood allowable for a 30-day period outlined in Appendix C, venipuncture will not be done and only a small amount of blood (< 1mL) will be

collected by finger prick / heel stick for a thin smear to determine species and hemoglobin measurement. Collection of this additional blood will not be required if blood was already collected as part of a routine visit.

Malaria will be classified as uncomplicated or complicated based on the following criteria:

Uncomplicated malaria (all of the following):

1. Fever (≥ 38.0°C tympanic) or history of fever in the previous 24 hours
2. Positive thick blood smear
3. Absence of complicated malaria.

Complicated malaria (any of the following):

1. Evidence of severe disease (see Appendix D) with a positive thick blood smear
2. Danger signs in children < 5 years of age (see Appendix D) with a positive thick blood smear

Episodes of malaria will also be classified into the following categories according to the timing of previous malaria episodes for treatment purposes:

1) New episodes of malaria will be defined as any first episode or any episode occurring > 14 days after the diagnosis of a previous episode
2) Treatment failures will be defined as any of the following:
    a. Complicated malaria occurring 1-14 days after the diagnosis of a previous episode
    b. Fever (≥ 38.0°C tympanic) or history of fever in the previous 24 hours with a parasite density ≥ the parasite density of an episode of malaria diagnosed 2 days prior
    c. Fever (≥ 38.0°C tympanic) or history of fever in the previous 24 hours with a parasite density ≥ 25% of the parasite density of an episode of malaria diagnosed 3 days prior
    d. Fever (≥ 38.0°C tympanic) or history of fever in the previous 24 hours with a positive thick blood smear of any parasite density occurring 4-14 days after the diagnosis of a previous episode

All patients diagnosed with new episodes of uncomplicated malaria will be prescribed artemether-lumefantrine (AL), the recommended first-line treatment in Uganda. Patients with complicated malaria or treatment failure following treatment with AL will be prescribed quinine or IV artesunate according to national malaria treatment guidelines. Patients with treatment failure following treatment with quinine or IV artesunate will be treated with quinine plus clindamycin. Pregnant women diagnosed with uncomplicated malaria will be treated with quinine if they are in their 1st trimester and AL if they are in their 2nd or 3rd trimester in accordance with local guidelines. Patients who require inpatient care will be referred for admission at TDH.

On the day malaria is diagnosed, patients will also be prescribed paracetamol (10mg/kg) to take every 8 hours until resolution of fever. For children with anemia (Hb< 10 gm/dL), we will follow the Integrated Management of Childhood Illness (IMCI) guidelines: anemic children will be prescribed iron sulfate (100 mg daily for 2 weeks) and mebendazole (only children > 1 year of age; 250 mg age 1-2 years; 500 mg > 2 years age; no more than every 6 months).

6.1.3.3 Non-malarial illnesses

Patients who are found to have illnesses other than malaria will receive standard-of-care treatment in the study clinic, according to the Uganda Clinical guidelines and algorithms[39], or will be referred for admission at TDH. If a patient is diagnosed with a non-malarial illness at the same time as malaria, treatment of the non-malarial illness will be at the discretion of the physician following the Uganda clinical guidelines administered at the same time as the malaria treatment.

6.1.3.4 Administration of medications and study transport

All medications prescribed for study participants will be provided by the local health facility pharmacy if available or provided by the study team if medications are not available at the local health facility. The study will ensure adequate supplies of antimalarial drugs and appropriate treatment for all diagnosed malaria episodes. Study participants will be reimbursed for all travel to and from the study clinic.

6.1.3.5 Criteria for cohort study discontinuation

Study participants will be prematurely withdrawn from follow-up if they meet any of the following criteria:

1) Permanent movement out of Buteba, Osukuru, or Kayoro parishes
2) Inability to be located for > 4 months
3) Withdrawal of informed consent
4) Inability to comply with the study schedule and procedures

Table 3. Collection of blood/biologic samples

Procedure		Enrolment, Routine, and Immunology visits	Non-routine visits	Malaria diagnosed*
Volume of blood drawn by weight	1 kg	2.5 mL	≤2 ml	2.5 mL
	2-4 kg	5 mL		5 mL
	5-7 kg	5-10 mL		5-10 mL
	8 kg or more	5-15 mL		5-10 mL
Thick blood smear		X	If fever and/or maculopapular rash present	
Thin blood smear		X		X
Dried blood spots collected on filter paper		X	If fever present	X
Hemoglobin measurement		Every 12 weeks		X
Whole blood for parasite culture		Enrolment and Routine only		X
Whole blood for qPCR		X		X

Whole blood or buffy coat for human genetic testing	Enrolment and select Routine		
Plasma for serology	X		X
Nasal and/or oral swab		If fever and/or maculopapular rash present	
Whole blood or RBC for parasite diversity	Samples that are positive for malaria parasites		
Whole blood for gametocyte studies by RT PCR	Selected samples		
Whole blood for cellular immunology assays	Selected samples		
Whole blood for membrane feeding assays	Selected samples		

* If the participant would exceed the maximum volume of blood allowable for a 30-day period outlined in Appendix C, venipuncture will not be done at the time malaria is diagnosed and only a small amount of blood (< 1ml) will be collected by finger prick / heel stick

6.2 Entomological surveillance studies

Entomological surveillance will be conducted in all households of participants enrolled in the cohort studies with the exception of the human landing catches and indoor resting collections which will be conducted in different households and larval collections which will be conducted in productive mosquito breeding sites within the study area. Mosquito pit traps will be deployed in the close proximity (10 to 20 metres) to houses where indoor mosquito collection will be done in order to better understand outdoor mosquito resting behaviour. Indoor resting collections and mosquito pit trap collections will be conducted every two weeks in the non-cohort houses for a duration of up to 6 months from onset of collections. All mosquitoes collected will be transported to our field site laboratory where they will undergo laboratory testing (see section 6.3.12). A summary of the entomological surveillance studies is provided in Table 4 below.

Table 4. Summary of entomological surveillance studies

Type of collection	Number of households	Frequency of collections
CDC light trap	All 80 cohort households	Every 2 weeks
Human landing catches	Round 1: 16 non-cohort households Round 2: 12 non-cohort households	Round 1: Every 4 weeks Round 2: Every 2 weeks
Indoor resting collections	16 non-cohort households	Every 2 weeks
Mosquito pit trap collections	16 non-cohort households (Outdoor mosquito collection)	Every 2 weeks
Mosquito larval collections	Not applicable	Twice a year during rainy seasons

6.2.1 CDC light trap collections

CDC light trap collections will be conducted in all cohort households every 2 weeks for the duration of the observation period. Within each household CDC light trap collection will be done in all rooms where cohort study participants sleep. Miniature CDC light traps (Model 512; John W. Hock Company, Gainesville, Florida, USA) will be positioned 1 m above the floor at the foot end of a bed in all rooms where household members sleep. Traps will be set at 7pm and collected at 7am the following morning. The presence and use of LLINs will be recorded in the cohort households where the light traps are set. A questionnaire will be administered to collect data on behavioural risk factors such as staying outdoors late and leaving the bed early in the morning that may influence the risk of mosquito bites (Appendix B).

6.2.2 Human landing catches

In Round 1, human landing catches will be conducted every 4 weeks in 16 households (8 households within Tororo district and 8 households within Busia district) randomly selected from our enumeration database. In Round 2, human landing catches will be conducted every 2 weeks in 12 households in the Tororo district. This will include the original 8 Tororo households from the previous human landing catch round and an additional 4 Tororo households from randomly chosen our enumeration database that are in locations further from the border. These households will be different from the households participating in the cohort studies as to not interfere with the other entomological surveillance studies. Field workers will approach households in the order they were randomly selected and conduct a door-to-door screening to identify households that might be interested in participating. Written informed consent from the head of household or an adult household representative will be obtained by the field worker prior to conducting the landing catches. Residents not home during the initial contact will be re-visited up to 3 occasions over a 6-week period before eliminating them from our sample selection process. In Round 1, four different houses will be sampled each night, at least 300m apart, for four consecutive nights. Thus, all 16 households will be sampled in the same week over each 4 week time interval. In Round 2, four households will be sampled each night for three consecutive nights, ensuring all 12 households are sampled in the same week over each 2 week time interval.

6.2.2.1 Recruitment of field workers for human landing catches

The criteria for recruitment will include: (1) willingness to take chemoprophylaxis for malaria, (2) willingness to abstain from alcohol during working hours, and (3) no significant past medical history. Field workers will be recruited and screened for the eligibility criteria by study supervisors. Field workers will be paid for their work and provided malaria chemoprophylaxis, including mefloquine (250mg tab orally once weekly) or doxycycline (100mg tab orally each day). The risks and benefits of each treatment option will be explained to the field workers, and they will be allowed to choose their preferred treatment. Field workers will also be offered medical treatment for any illness that develops during the period of their employment.

6.2.2.2 Methods for human landing catches

At each house two field workers will be stationed outdoors 10m from the house, and two will be stationed indoors. Outdoor and indoor collections will be conducted from 6pm to 8am. Field workers

will collect mosquitoes landing on their exposed legs using aspirators and torchlight. Field workers will collect mosquitoes for 50 minutes, with a 10 minute break each hour. Mosquitoes will be collected at hourly intervals through the night and processed the following morning. Two additional field workers will be present to provide relief when the other four tire during the night. This will require 6 field workers/house, or a total of 24 field workers per night, plus one supervisor, at each site.

During Round 2, the field workers will perform human behavioral observations in the human landing catch households. This is a method for identifying during each observation hour of the night whether household members are 1) inside the home and under a bednet, 2) inside and not under a bednet, 3) outside the home but inside the compound (~10m from the home) or 4) outside the compound . To achieve this, the field workers will conduct a short survey at the beginning of the night to identify which people plan to sleep in the household that night and which rooms have bednets. Then, during the 10 minute break each hour, the indoor and outdoor field workers will confer and perform a brief observational survey to count the number of people in each of the four locations specified above. The field workers indoors will note which household members are indoors and attempt to determine whether or not each is under a bednet. Intrusions of privacy will be kept to a minimum. If household members are not readily visible (e.g. a door is closed) then the field worker will count the household members in that room as indoors and note whether or not a bednet is present over the sleeping areas in the room. Field workers will not open closed doors or invade private sleeping spaces/rooms.

6.2.3 Mosquito larval collections

Mosquito larval collections will be done in areas with productive mosquito breeding sites within the study area targeting the two annual rainy seasons. Purposeful sampling will be done to maximize collection of the aquatic stages of mosquitoes using a350-mL dipper (Clarke Mosquito Control Products, Roselle, IL). At each site, ~10 dips will be made in places likely to harbour mosquito larvae, such as around tufts of submerged vegetation or substrate, edges of water bodies, and around floating debris. In extensive water bodies, dipping will be carried out over a 100-m walk. Anopheline larvae will be raised to adults for phenotypic resistance testing.

6.2.4 Mosquito resting collections

To enhance the collection of blood fed mosquitoes, indoor resting collections will be conducted every 2 weeks in16 households (8 households within Tororo district and 8 households within Busia district) for a duration of up to 6 months. Mosquitoes will be collected in all rooms where household members sleep by standard resting catches. Between 6:00 and 8:00am, up to two entomologists will spend ~30 minutes per house searching for resting anopheline mosquitoes which will be aspirated by InsectaZooka/prokopack (field aspirator). All mosquitoes collected will be transferred to paper cups and transported to our field site insectary where they will undergo laboratory testing. In addition to the listed mosquito sampling methods, we shall utilize outdoor mosquito pit traps within 10 to 20 meters of the households where indoor mosquito collections will be done. Mosquito pit traps are artificial pit shelters, 5 to 6 ft deep, dug under natural shade so that their openings (4 to 5 x 3 to 4ft) are shaded from above. A suitable cover is placed partially over the pit trap entrance. About

2ft from the bottom of the pit trap, small cavities, about 30 cm deep are dug horizontally from each of the four sides of the pit. Mosquitoes are collected from these small cavities or from the wall of the pit itself. The pit is encircled with a thorn or fence enclosure to prevent small animals or young children from falling into them or having them used as toilets (Muirhead-Thomson 1958).

6.2.5 WHO standard cone bioassays and insecticide quantification on wall surfaces

6.2.5.1 Rationale: In response to the higher than expected malaria burden in Tororo district following implementation of clothianidin + deltamethrin based indoor residual spraying, standard WHO cone bioassays on indoor wall surfaces have been proposed to monitor insecticide residual efficacy on different wall surfaces. The wall bioassays will be conducted to monitor the most recent rounds of IRS implemented in March 2022 and March 2023. The study will only screen 60 houses currently followed under the PRISM Border cohort in Tororo (where IRS is being implemented). These target houses will be approached for written consent and those providing consent will undergo cone bioassays and wall sample collections for insecticide quantification using HPLC as described below.

6.2.5.2 Cone bioassays: Cone bioassays on wall surfaces will be conducted following the implementation of the latest rounds of IRS in March 2022 and March 2023 on different wall surfaces such as plastered and painted, plain brick and mud-walled using a standardized WHO protocol.[56] The study will use wall surfaces available in the 60 cohort houses with sampling conducted every 1 to 2 months for a period of up to 12 months after each round of IRS i.e. until March 2024. The sampling periods will follow the latest rounds of IRS and will be dependent on the availability of resources.

Following standard WHO methodology, cones will be placed at heights of 0.5 m, 1.0 m, and 1.5 m above the floor. Cones lined with self-adhesive tape will be fixed on the sprayed walls for the assay. The control cone will be affixed on a wall lined with a paperboard with adhesive on an unsprayed surface. Two- to five-day-old female mosquitoes will be used for the tests. Susceptible *An. gambiae s.s.* Kisumu strain mosquitoes will be introduced into the plastic cones in batches of 10 and left exposed on the sprayed surface for 30 minutes at different heights. When available, larvae of *An. gambiae s.l./An. funestus* will be collected in the sprayed district and reared to adults. As an alternative, when larval collections are not feasible, adult blood-fed female Anopheles collected alive from resting and human landing collections may be used to rear adult progeny mosquitoes for use in wall cone assays. The wild mosquitoes will be exposed to the sprayed walls following the same protocol. Numbers of mosquitoes knocked down at the 30th minute mark will be recorded. At the end of the 30-minute exposure period, the mosquitoes will be carefully collected and transferred to paper cups and provided with 10% sugar solution soaked on cotton wool pads placed on top of the paper cups covered with netting with final mortality recorded after 24 hours holding period post-exposure and/or dependent on manufacturer recommendations. Tests for the airborne effect of the active ingredient will be conducted with mosquitoes placed inside a mosquito net cage and hung 10 cm away from the sprayed wall surface at a height of 1.0 m above the floor with knock-down recorded at 30 mins and 60 mins after exposure. The mosquitoes will then be transferred into clean paper cups and kept for a 24-hour holding period or according to manufacturer recommendations with the percentage mortality calculated for each house and recorded. *An. gambiae s.s.* (Kisumu strain) and wild caught *An. gambiae s.l. An. funestus* will be used for quality and decay rate, and fumigant effect assessment.

6.2.5.3 High-performance liquid chromatography (HPLC) for insecticide quantification: To quantify the amount of insecticide on the surface of the walls, samples will be taken adjacent to where the cone assays are conducted.57 Samples will be obtained using adhesive strips with four glue dots on each strip. The strips will be stuck to Whatman No1 filter paper to avoid self-folding and stored at 4°C prior to HPLC analysis. In accordance with lab standard protocols57 the four glue dots will be individually removed from the backing using a hole-punch (radius 0.365 cm2). Insecticide will be extracted using acetone with the inclusion of dicyclohexyl phthalate (DCP) as an internal standard. Following sonication and evaporation to dry-ness, samples will be re-suspended in acetonitrile and subject to high-performance liquid chromatography (HPLC) analysis on an Ultimate 3000 UV detector (Dionex) and analysed with Dionex Chromeleon software. The quantities of each insecticide per sample will be calculated with respect to standard curves established with known concentrations of authenticated standards (PESTANAL®, Sigma-Aldrich, UK) and corrected by internal standard (DCP). Final insecticide content in g/m2 will be estimated using a 15% surface active ingredient extraction efficiency estimation.

6.3 Laboratory methods

6.3.1 Microscopy

Thick and thin blood smears will be prepared for microscopy using blood samples obtained from finger-prick/heel-stick or venipuncture. New glass slides, frosted at one end, will be used to make the thick and thin blood smears. Thick blood smears will be made by placing a drop of blood in the middle of the slide. An applicator stick will be used to spread the blood into a spot of approximately 1 cm in diameter. Thin blood smears will be made by placing a drop of blood near the end of the slide and creating a feathered edge using a coverslip. Blood smears will be dried on a slide tray, in a dust-free environment. Thick and thin blood smears will be stained with 2% Giemsa for 30 minutes. Thick smears will be evaluated for the presence of asexual and sexual (gametocytes) parasites. Parasite densities will be calculated from thick blood smears by counting the number of asexual and sexual parasites, respectively, per 200 leukocytes (or per 500, if the count is less than 10 parasites per 200 leukocytes), assuming a leukocyte count of 8,000/μl. A thick blood smear will be considered negative when the examination of 100 high power fields does not reveal parasites. When malaria is diagnosed, thin smears will be used for species identification based on standard morphology criteria. For quality control, all slides will be read by a second microscopist and a third reviewer will settle any discrepant readings. All microscopy work will be done at the study clinic in TDH. Microscopy slides will be stored in TDH or a central laboratory in Kampala for up to 10 years.

6.3.2 Dried blood spots collected on filter paper

Each time a thick blood smear is obtained; blood will also be collected onto filter paper. Samples will be collected by venipuncture or by finger-prick/heel-stick sampling. Filter paper (Whatman no 1, Whatman 3MM; Whatman, Maidstone, UK) will be pre-cut into individual squares and stapled to a thick card which will serve as its cover. Blood spots will be collected onto the filter paper in volumes of approximately 25 μl per blood spot (4 blood spots per sample). Filter paper samples will be labelled with the sample's bar codes on the covering cardboard and will be allowed to dry at ambient temperature and relative humidity before closing the card over the filter paper (like closing a matchbook). Filter paper samples will be transported from the field in zip lock bags and placed into a stock card filter paper box for final storage at ambient temperature or at -20^{o}C with a

desiccant. Filter paper samples may be stored for up to 10 years in TDH or a central laboratory in Kampala. Molecular studies will include the extraction of DNA and/or RNA from filter paper, followed by characterization of parasite and host nucleic acid sequences. Future molecular studies will be performed only for research purposes and will have no impact on the clinical management of study participants.

6.3.3 Hemoglobin measurement

Hemoglobin will be measured from fingerprick or venipuncture blood samples using a portable spectrophotometer (HemoCue, Angelholm, Sweden).

6.3.4 Parasite culture and determination of ex vivo drug sensitivity

Blood collected by venipuncture on the day malaria is diagnosed in selected subjects will be used for parasite culture at our parasitology laboratory located at TDH. Parasites will be cultured following standard protocols. In brief, erythrocytes will be separated from plasma by centrifugation and removal of the supernatant and buffy coat, and the infected erythrocytes will then be cultured in RPMI medium supplemented with human serum or Albumax serum substitute. Cultured parasites will be evaluated for in vitro drug sensitivity, molecular characteristics, and other features to characterize antimalarial drug resistance and other aspects of malaria. Information from the parasitology studies will have no impact on patient care. To measure ex vivo drug sensitivity, fresh isolates collected from patients presenting with falciparum malaria and control laboratory strains of P. falciparum will be incubated with 3-fold serial dilutions of drugs of interest in 96-well culture plates under standard culture conditions for 72 hours[40]. Samples will then undergo freeze-thaw, aliquots will be incubated with SYBR green for 1 hour, and fluorescence (485 nm excitation; 530 nm emission) will be measured. IC50s will be derived by plotting percent growth against log drug concentration and fitting the data by variable slope, sigmoidal curve fit in Prism 6.0 (GraphPad Software, San Diego, CA). For some drugs modifications of this protocol will be utilized, e.g. using the ring survival assay to measure sensitivity to dihydroartemisinin[41].

6.3.5 Assessment of *P. falciparum* genotypes associated with drug resistance

We will use methods that are standard in our laboratories to evaluate sequences of interest in isolates from patients with malaria or malarial parasitemia. *P. falciparum* DNA will be extracted from filter paper bloodspots using Chelex-100, genes of interest will be amplified with nested PCR, and polymorphisms of interest will be characterized using a ligase detection reaction-fluorescent microsphere assay, all as previously described [42]. Resistance can also be associated with gene amplification, and for relevant genes (*pfmdr1, plasmepsin2)*copy number will be determined using real-time PCR assays. In some cases other methods will be used to determine *P. falciparum* gene sequences, including standard Sanger sequencing after amplification of genes of interest, deep sequencing to evaluate multiple genes in a multiplex platform, or whole genome sequencing.

6.3.6 Quantitative PCR

Quantitative PCR (qPCR) will be performed at the time of enrollment, at each routine visit (every 4 weeks), and when malaria is diagnosed. At each of these time points, 200 µL of whole blood will be used and DNA will be extracted using Qiagen spin columns or by related methods. Extraction products will be tested for the presence and quantity of *P. falciparum* DNA via a highly sensitive

qPCR assay targeting the multicopy conserved *var* gene acidic terminal sequence [43] or other Plasmodium-specific sequences. This assay is now routinely performed by our group and demonstrates sensitivity and reproducible quantification to 0.1 parasites/µL. These assays will be performed at our laboratories at TDH, where qPCR capacity is already established. In addition, experimental parasite detection methods, including methods specifically developed to detect gametocytes, may be performed on a subset of fresh blood or stored samples. These methods will not replace qPCR but be performed in addition to qPCR, with qPCR remaining the gold standard detection method.

6.3.7 Human genetic testing

Using whole blood collected at the time of enrolment, we will perform detailed characterization of the genome sequence of each cohort study participant. For the purposes of determining the origin of mosquito blood meals, a panel of microsatellites will be typed in all study participants and a subset of blood fed mosquitoes so that fed mosquitoes can be matched to the person bitten. A more comprehensive evaluation of host genetics will also be performed to determine how host genetics influence malaria susceptibility. As an initial approach we will apply a genome-wide approach, e.g. using 2.5 million single nucleotide polymorphisms (Omni chip) using cohort data to identify new loci that explain genetic variation in protection from establishment of infection and from the consequences of blood stage infection. As an alternative approach, human genomes will be characterized by more comprehensive genomic approaches, such as whole exome or whole genome sequencing. Sequencing will be by Illumina or other deep sequencing methodologies, benefitting from continued improvements with this technology. Genomic variants will be characterized from sequence alignments using standard informatic tools. Due to the need to perform these complex and expensive assays at centers with established protocols for high throughput testing and with high volumes of samples, human genetic testing will be performed by laboratories at LSHTM, UCSF, or at other reference centers depending on the best available technology within funding constraints at the time of testing.

6.3.8 Serology

Antibody profiles will be generated using high throughput screening of plasma, evaluating responses to a large number of analytes. We have produced a large panel of recombinant proteins that will be used for measuring serologic profiles. Up to 50 proteins can be analyzed at one time using the Magpix platform, which we have established in Uganda. In addition, the Drakeley lab (LSHTM) has recently established a state-of-the-art high throughput microarray platform (Arrayjet) which includes a protein microarray inkjet printer, laser scanner and robotic liquid handling platform for serologic screening of cohort samples. With this system, only picogram quantities of antigen are required for each array, such that our existing expression systems can generate sufficient quantities of antigens for many thousands of slides, each containing hundreds or thousands of proteins. During this project, selected plasma samples will be probed via Magpix and protein arrays, followed by standard data normalization procedures to obtain antibody responses to a large panel of proteins. In addition to Magpix and the Arrayjet platforms, available in Uganda and LSHTM respectively, complementary approaches may include measurement of antibodies by other platforms, including other microarray platforms using crude extract to evaluate a much larger number of analytes, phage display to evaluate responses to the entire parasite proteome, enzyme linked immunosorbent assays (ELISA),

and functional assays. Serologic assays will be performed in Uganda, LSHTM, UCSF, and/or in laboratories of collaborators depending on the specific assay and availability of necessary equipment and expertise.

6.3.9 Parasite diversity

We will characterize the diversity of infecting *P. falciparum* by characterizing sequences of highly diverse parasite proteins. These will most likely utilize sequencing-based methods for characterizing parasites, including deep sequencing of select, highly polymorphic genetic loci, potentially including RNA transcripts of parasite expressed variable genes such as PfEMP1. Due to the need for high-throughput genomics cores to process laboratory samples and produce and analyze next-generation sequence data, these assays will be performed at core facilities at UCSF or in the laboratories of collaborators or commercial facilities where such work is routine.

6.3.10 Molecular gametocyte testing

In selected samples, an aliquot of blood will be stored in RNA preserving buffers for parasite and gametocyte detection, quantification and characterization. Parasite RNA will be extracted from whole blood samples using a commercial kit and stored in EDTA tubes for subsequent quantification of gametocytes based on detection of Pfs25 (female gametocytes), PF3D7_1469900 (male gametocytes) and PF14_0367 mRNA (total gametocytes) [44]and potentially other markers if better ones are identified. In addition, multiplicity of infection will be determined by characterization of the diversity of the parasite proteins as described in section 6.3.8 in blood fractions after gametocyte separation by MACS magnetic columns [45,46].We have adapted this technique to prevent gametocyte activation and generate populations enriched in or depleted for gametocytes.

6.3.11 Additional immunologic assays

Venipuncture blood samples at selected times will be collected into RNA preservation media or standard blood collection tubes and used for cellular immunology studies. Following collection, blood will be transported to our immunology laboratory located on the campus of Tororo District Hospital. Whole blood will be assayed for absolute cell counts, utilized in innate immune stimulation assays, and/or stored in RNA preservation media. Remaining whole blood will be separated into plasma and peripheral blood mononuclear cells (PBMCs), following standard protocols. Plasma will be removed promptly and frozen for future immunologic studies, which may include measurement of malaria-specific antibody responses, plasma cytokines, metabolomics, and other features of the host immune response. PBMCs will be isolated by Ficoll centrifugation and counted, and cells not used immediately for assays will be cryopreserved in liquid nitrogen to maintain viability. Fresh PBMCs will be used to evaluate the malaria-specific cellular immune response by flow cytometry, ELISPOT, surface phenotyping and/or intracellular cytokine staining, and other assays of immune function in our research laboratory in Tororo on the campus of Tororo District Hospital. Additional immunologic assays will be performed in research laboratories at the Central Public Health Laboratories in Butabika. For assays requiring advanced instrumentation not available in Uganda (e.g. RNA sequencing, epigenetic profiling, time of flight mass cytometry), cryopreserved samples will be shipped to the London School of Tropical Medicine and Hygiene, the University of California San Francisco or Stanford University.

Information obtained from immunology studies will have no impact on patient care. A subset of samples used in membrane feeding assays will be used for a functional assessment of transmission reducing immunity in the standard membrane feeding assay. This work will be performed at Radboud umc. For this, 90 µL of purified plasma IgG is added to freeze-dried foetal calf sera (FCS) and diluted into human serum containing active complement. This antibody/serum mix is added to mature *P. falciparum* gametocytes (NF54HT-GFP-Luc), and offered to 3-5 day old female *Anopheles stephensi* (Sind-Kasur Nijmegen strain) mosquitoes in the membrane feeding apparatus. Mosquito infection status is determined based on bioluminescence as previously described and compared to control mosquitoes that were offered gametocytes without test IgG[47]. Transmission reducing activity (TRA) is calculated as the percent difference in luminescence intensity for test and control mosquitoes. Antibody responses to gametocyte antigens associated with TRA will be determined by microarray, ELISA or Luminex. Specifically, titers of antibodies against gametocyte antigens Pfs48/45 and Pfs230 will be determined by established methodologies and related to functional TRA in the standard membrane feeding assay on cultured gametocytes and mosquito infection rates in membrane feeding assays conducted in the field in participants naturally infected with malaria[48].

6.3.12 Membrane feeding assays

Membrane feeding assays will first be performed on selected participants with no antimalarial use in the prior 2 weeks and parasites detected by qPCR. For these individuals, venous blood will be used for direct membrane feeding assays(DMFA) to determine infectivity to female *Anopheles* mosquitoes. Such blood samples may be drawn from participants when an episode of uncomplicated malaria is diagnosed or at the time of a routine visit if the participant was found to have asymptomatic parasitemia at the time of their last clinic visit. The DMFA will use locally reared *Anopheles* mosquitoes in an experimental membrane feeders set-up. 3mL of blood will be drawn from selected study participants in heparinized tubes. Approximately half of this blood sample will be immediately transferred for membrane feeding. The remaining blood will be used for serum replacement membrane feeding where the sample is centrifuged to replace autologous plasma with malaria-naïve control serum [15] where the sample is centrifuged to replace autologous plasma with malaria-naïve control serum.

Mosquitoes will be allowed to feed on the venous blood sample for approximately15 minutes. After this, mosquitoes will be kept in our insectary on glucose and dissected 7-10days after feeding. The target temperature in the insectary is 27-29°C with ~70% humidity. Mosquito midguts will be examined for the presence of parasite developmental stages, oocysts, by two independent microscopists. All oocysts will be PCR confirmed using an established protocol for parasite detection on mosquito guts dissected in mercurochrome[49]. In case mosquito numbers exceed the capacity to dissect and microscopically examine mosquito midguts, mosquitoes will be kept until day 14post-feeding for examination of infection status by circumsporozoite ELISA followed by PCR confirmation[49].These mosquitoes will be kept inside locked incubators for those days beyond day 10 since oocysts may rupture and render mosquitoes infectious to humans.
In a subset study participants, we will compare mosquito infection rates between the locally reared *Anopheles* mosquito colony and field-derived mosquitoes. The latter mosquito population will be raised from field caught larvae or gravid mosquitos, depending on local feasibility assessed through routine entomological monitoring. Identical DMFA procedures will be used and mosquito infection

rates will be compared between paired feeders used for colony or field-derived mosquitoes. DMFA output will depend on local mosquito husbandry, feeding success on membrane and fitness post feeding. Protocols may thus be adapted as needed without affecting the total blood drawn from the participants (3mL).

6.3.13 Direct skin feeding assays

A growing number of clinical trials now use direct skin feeding as assay of choice to assess transmission to mosquitos (clinicaltrials.gov NCT01838902; NCT02511353; NCT01867463). As a result of these trials, conducted according to international standards for Good Clinical Practice, rigorous screening approaches have been developed to ensure mosquito colonies are pathogen-free. Also in Nanoro(Burkina Faso), a mosquito colony is specifically reared for this study with extreme safety precautions to ensure that the mosquitoes are pathogen free. Arboviral infections of anopheles colonies are highly unlikely and even if colonies were infected by (animal) donor blood vertical pathogen transmission would be needed to introduce a health hazard when freshly hatched eggs produce larvae that are raised to adult mosquitoes and used for experiments. Vertical transmission of viral infections in Anophelines has not been established for any human pathogens. Because infection of mosquito colonies with pathogens that form a health hazard for humans is at present a theoretical possibility, sites in Kenya and The Gambia have used *An. gambiaes.s*. colonies for direct skin feeding without prior screening for viral infection (clinicaltrials.gov NCT01838902; NCT02511353). We, however chose to exclude infection of our colony more rigorously to ensure participant safety. For this, we followed procedures of the National Institute of Health (NIH, US) and the Malaria Research & Training Centre in Bamako, Mali (MRTC) in their ongoing trials (NCT0186746). Specifically, we will collect all parent mosquitoes (300 females) that produced an egg batch that forms the foundation of skin feeding experiments. These mosquitoes are screened at the Institut Pasteur of Dakar (Senegal) World Health Organization accredited laboratory for 7 arboviruses including Yellow fever, Dengue, Rift valley, Zika virus. Upon receiving confirmation that the colony is free of viral infections, the batch is kept separate from other mosquito colonies in a separate holding room. The nearest other colony is in a separate room that is separated from the currently used mosquito colony by two sets of double doors with mosquito screening. Our colony for direct skin feeding will be maintained on blood from a donor that has been screened for viral infections using national blood donor standards. We shall use methodologies for skin feeding that are increasingly widely used in African research settings such as the KEMRI-Centers for Disease Control laboratories in Kisumu, Medical Research Council Laboratories in The Gambia and Malaria Research & Training Center in Mali. Direct skin feeding assays will be performed on selected participants aged8 years or older with no antimalarial use in the prior 2 weeks and parasites detected by qPCR. Each time a cohort participant is selected for direct skin feeding assays, they will be required to provide informed consent before proceeding with the procedure (see Section 9.0 for Informed Consent and Assent Process). Two cups of 20 mosquitoes will be applied to the calves of study participants 8-14 years of age (total of 40 mosquitoes) and two cups of 30 mosquitoes will be applied to the calves of study participants 15 years of age or older (total of 60 mosquitoes).Mosquitos will be allowed to feed for exactly 15 minutes after which antihistamine cream will be provided to the participant. Mosquito processing will be performed as described above in the section on membrane feeding.

6.3.14 Entomological laboratory studies

A large number of specimens will be collected using different collection methods. We will implement a systematic process for recording information for each specimen including the following: 1) household ID number and the room where the mosquito was collected, 2) method of collection, 3) date of collection, and 4) serial number of the specimen. Specimens will be placed in labelled Eppendorf tubes, and labels will be linked to a database with the information listed above using a bar-coding system. Identification of anophelines will be based on morphological criteria according to established taxonomic keys[50]. Identification of members of the *An. gambiae* complex will be by PCR[51]. Sporozoites will be identified in individual mosquitoes stored with desiccant using ELISA[52]. The source of mosquito blood meals will be assessed from abdomens squashed on filter paper and identified using an ELISA technique[53]. A subset of blood fed mosquitoes containing human blood will be genotyped to determine which, if any, study participants were bitten, as described in Section 6.3.7. Mosquitos may also be tested using molecular methods for the presence of malaria parasites or other pathogens using nucleic acid amplification tests and/or novel deep sequencing based approaches. A subset of these newer tests may be performed at UCSF or at reference centers depending on availability of technology and funding for additional pathogen detection work.

6.3.15 Insecticide resistance testing

Insecticide susceptibility testing will be conducted using adult mosquitoes. Adult mosquito will come from CDC light trap collections done in the cohort households and mosquito larva raised to adults. Resistance phenotyping will be conducted in line with newly developed WHO guidelines, combining standard WHO tube tests with intensity assays where indicated. Test mosquitoes will be 3-5day old females raised from larval collections or, where necessary, mixed physiological age group females. All three primary vectors *Anopheles gambiae, An. arabiensis* and *An. Funestsus* will be assessed. This resistance phenotyping will give us a broad overview of resistance patterns and likely causal mechanisms. For testing of genetic markers of insecticide resistance, DNA will be extracted from morphologically-identified, desiccated mosquitoes. For each species complex it will be necessary to identify individual mosquitoes down to the species level using standard PCR-based assays. Initially we will screen for resistance associated polymorphisms in *A. gambiae* and *A. arabiensis,* as these are likely the primary vectors in most study locations, but this may change over the course of the study. We will screen for polymorphisms in the key insecticide target-sites, *Vgsc* and *Ace1* together with variants in metabolic resistance genes (*Gste2, Gste4, Cyp4j5,* and *Coeae1d).*Samples will be initially genotyped using published and novel TaqMan SNP assays and other advanced molecular analysis tools such as genome wide association studies (GWAS) and sequencing.

6.3.16 Non-malarial pathogen detection studies

Plasma and nasal/oral swab samples collected during febrile episodes as well as controls collected from healthy individuals will be evaluated for the presence of pathogens potentially causing febrile illness using nucleic acid amplification and deep sequencing based methods. One experimental method involves RNA extraction, cDNA library synthesis, and meta-genomic next-generation sequencing, using a specific informatic pipeline to identify pathogens after filtering out human sequence information. These newer tests may be performed at UCSF, the Chan Zuckerberg Biohub,

or at reference centers depending on availability of technology and funding for additional pathogen detection work.

6.3.17 Label-free malaria scope

Scientist from the Chan-Zuckerberg BioHub in San Francisco are developing an automated, microscopy-based malaria diagnostic tool currently referred to as the "label-free malaria scope". This device operates by imaging fresh blood in liquid form without fixation or staining, and uses machine learning to process images of blood cells and detect malaria parasites and quantify parasite density. Because the blood is in liquid form, it is flowed past the imager to screen large numbers of cells with similar resolution as a thin smear but approaching the throughput of a thick smear. The microscope requires minimal training to use and requires 10 uL of blood collected using microsafe plastic capillaries and dispensed into diluent. 10 uL of the diluted blood is added to a flow cell consumable device and placed on the microscope for imaging. This side project will not require any addition blood draws and will only utilize 10 uL of blood already being collected from cohort study participants at the time of their routine visits to the clinic. We aim to collect raw microscope images from up to 1,000 blood samples collected at the time of routine visit, and assess diagnostic accuracy using results from microscopy and qPCR that is already being done. Diagnostic information from the new system will not be provided to cohort study participants nor affect clinical care, and this side project will not utilize any patient identifying information. The raw data will be provided to scientist at the Chan-Zuckerberg BioHub for further analysis in order to validate and improve the machine learning models for parasite detection.

7.0 STATISTICAL CONSIDERATIONS

The primary outcome measures for this study will be descriptive in nature. A summary of key outcome measures by study type are described in Table 5 below. Associations between variables of interest and key outcome measures by study type are described below.

Table 5. Key outcome measures

Study type	Outcome	Definition
Epidemiology studies	Incidence of malaria	Number of incident episodes of malaria per time of observation. Incident episodes of malaria defined as any treatment for malaria > 14 days after any prior treatment for malaria
	Parasite prevalence	Proportion of study participants with a thick blood smear positive for asexual parasites or parasites detected by qPCR at the time of routine assessment
	Force of infection	Number of new infections with malaria parasites per person per time of observation
	Human biting rate	Number of female anopheles mosquitoes captured per room per night
	Sporozoite rate	Proportion of captured female anopheles mosquitoes that test positive for sporozoites
	Annual entomological inoculation rate	Number of infected bites per person per year (human biting rate x sporozoite rate x 365 days/year)

Transmission studies	Gametocyte prevalence and density	Proportion of study participants with a thick blood smear positive for gametocytes or gametocytes detected by molecular methods at the time of routine assessment. Density of gametocytes and gametocyte sex-ratio.
	Parasite infectivity of mosquito vectors	Proportion of blood fed mosquitoes that become infected with malaria parasites following membrane feeding. Infection intensity in infected mosquitoes.
Immunology studies	Sero-prevalence	Proportion of study participants with antibody responses to key malaria antigens
	Cellular immune responses	Frequency and proportion of immune cells with malaria specific responses
	Transmission reducing immunity	Proportion of study participants with antibody responses to gametocyte antigens Proportion of study participants with functional transmission reducing immunity and the magnitude of this functional response
Resistance studies	Prevalence of molecular markers of antimalarial drug resistance	Proportion of blood or mosquito samples positive for malaria parasites that contain genetic polymorphisms associated with resistance to antimalarial drugs of interest
	Prevalence of molecular markers of Insecticide resistance	Proportion of mosquitoes that contain genetic polymorphisms associated with resistance to insecticides of interest

7.1 Planned statistical analysis for epidemiology studies

7.1.1 Analytical Objective 1: To characterize factors determining the malarial force of infection in an intensively studied cohort in Uganda

For this objective our hypotheses are: 1) The relationship between entomologic measures of exposure and the force of infection (FOI) is steep at low transmission but plateaus at transmission increases due to inefficiencies in transmission; and 2) Host genetic factors impact upon the probability that sporozoite inoculation will result in blood stage infection. In this objective, we will first establish quantitative relationships between entomologic measures of exposure for cohort individuals and the FOI experienced by these individuals, testing the hypothesis that there are biological underpinnings for observed deficiencies in transmission. Next, we will determine what host factors are associated with differences in these expected relationships, including the role of host genetics and acquired immunity on the rate of infection. Findings from these analyses will be fundamental to predicting how changes in EIR will result in changes in the true FOI, and how these changes will affect communities with different genetic backgrounds along different parts of the transmission spectrum and in the setting of different control interventions.

Analysis of Relationship between EIR and Force of Infection: We and others have demonstrated that the relationship between measured entomologic exposure and FOI is nonlinear, with a plateau in the FOI at high levels of exposure. Due to inherent limitations in the methodology of existing studies, it is unclear to what degree this apparent inefficiency in transmission represents a true biological phenomenon versus measurement biases. For example, multiple sporozoite inoculation events may "stack up" but be observed as a single infection, or immune individuals may have low parasite

densities which are not detected, leading to underestimation of the FOI. Designing our study to include frequent sampling, highly sensitive detection of infections, and enumeration of unique clones, will mitigate these measurement biases and allow us to determine whether there are biological causes for the plateau in FOI. Taking into account that despite our enhanced sampling there may still be residual measurement bias, we will use simple mechanistic models to simulate individual infection histories (and hence individual forces of infection) under different assumptions including a) efficient transmission and complete observation, b) efficient transmission and incomplete observation, c) inefficient transmission and complete observation, c) inefficient transmission and incomplete observation. We will fit these models to the data using Approximate Bayesian Computation methods. The best fitting model will be selected based on the posterior probabilities. This approach will allow explicit estimation of parameters related to inefficiency of transmission (e.g. heterogeneous biting, density dependent mosquito feeding) needed to reproduce the observed data, allowing us to test our hypothesis regarding the inefficiency of transmission, measure the magnitude of this effect if present, and identify putative sources of this inefficiency if present. Our cohort study design will also allow us to evaluate whether and to what degree the implementation of different types of control interventions differentially affect this relationship. Putative sources of inefficiency will also be evaluated empirically via analyses of entomologic data, e.g. the relationship between the proportion of blood fed mosquitoes and EIR will be used to estimate the role of density dependent mosquito success, and direct observation of bed net usage for each bed of each house every two weeks will allow us to identify the effect of human behavioral response to mosquito density.

Analysis of Host Factors Affecting the Relationship between Entomologic Measures and Force of Infection: While immunity against blood stage parasites has been clearly demonstrated, it is unclear whether individuals may be naturally protected against the establishment of blood stage infection (pre-erythrocytic immunity). Few accurate data on this question exist due to the challenges collecting relevant data in the context of natural exposure. Our proposed study design will allow us to obtain some of the most precise estimates of pre-erythrocytic immunity available to date. To assess the impact of individual level factors including age, candidate host genetic polymorphisms, and recent changes in exposure on the force of infection experienced by individuals, we will use longitudinal random effects models, allowing for flexible relationships between covariates and the outcome of interest. A key difference between our proposed analysis and previous efforts is that we will be able to explicitly control for heterogeneities in individual (or household) exposure, by including the measured EIR as a covariate. Thus, we should be able to properly isolate the impact of covariates on the FOI, at different transmission levels. While a basic formulation model only includes individual random effects, a natural extension can account for within household clustering as well. We will compare different models including multiple individual level covariates (such as age, host genetic polymorphisms, and recent changes in exposure) and their interactions, and base our final model selection on the Deviance Information Criteria (DIC).To further assess the association between host genetic polymorphisms and FOI we will use a mixed regression framework using emmax / gemma software, which will include covariates of interest. For these analyses, instead of using FOI as the outcome variable, we will use our estimates of the individual random effects (θ_i) to ensure adequate adjustment for other covariates of interest, including EIR. In this approach, the SNP of interest will be included assuming several related genotypic mechanisms (additive, dominant,

recessive, heterozygous advantage and general models) and the minimum p-value reported from these correlated tests. To adjust for the confounding effects of population structure, the models will include principal components representing major ethnic groups, as well as kinship random effects accounting for familially related individuals. Thresholds for statistical significance will be established using permutation approaches. This approach accounts for the correlation between markers and tests. Pathway analyses will be implemented on the p-values using grail software (http://www.broadinstitute.org/mpg/grail). All analyses will be performed using LSHTM-based analytical pipelines, which have been fully tested as part of published and ongoing malaria GWAS (Co-investigator Taane Clark). The LSHTM has the capacity for large-scale computing, including computer clusters consisting of 400 nodes and the Medical Research Council UK funded eMEDlab (>6000 nodes, 3Pb storage, both administered by Taane Clark).

7.1.2 Analytical Objective 2: To determine factors affecting the duration, density, and clinical consequences of blood stage malaria infection in Uganda

For this objective our hypotheses are that age and repeated exposure have independent effects on anti-parasite and anti-disease immunity, wane at different rates in the absence of *P. falciparum* exposure, and are modified by host genetic factors. In this objective, we will focus on the dynamics of blood stage of infection, which are largely determined by the innate and acquired immune response. It is clear that host genetics and acquired immunity both play important roles in the trajectory of a given *P. falciparum* infection once established in the blood. However, most investigations of natural immunity have largely ignored the heterogeneity in exposure and protection which is a feature of malaria, and thus these investigations have been limited by the strong confounding between exposure and immunity. We propose to leverage our detailed cohort study to carefully define clinical phenotypes of immunity, and determine host factors associated with these phenotypes. A unique feature of our study design will be the ability to evaluate changes in immunity in response to dramatic changes in transmission anticipated in our selected cohort.

<u>Measuring Anti-parasite and Anti-disease Immunity:</u> We have previously shown that clinical phenotypes of immunity can be best measured when accounting for an individual's exposure. For this project, we will evaluate immunity in 3 complementary ways, each one building on the prior to provide more detailed information but also requiring more data. It should be noted that any one of these approaches represents a significant advance over most existing analyses. Importantly, all 3 can be measured for individuals at different time points, allowing us to evaluate changes within individuals over time with respect to changes in population level transmission.

1) Overall immunity to malaria will be assessed by modeling the probability of an individual developing clinical malaria in a given observation interval (month), conditional on having been infected. However, a key limitation of these analyses has been an inability to properly define the denominator, i.e. the total number of infections an individual has experienced in a given observation interval due to a) low sensitivity of assays used to detect parasites, therefore underestimating the frequency of asymptomatic parasitemia; b) lack of data on the complexity of infections, therefore underestimating the true number of infections; and c) inability to distinguish whether instances of asymptomatic parasitemia during consecutive months represent one or multiple infections. The approach proposed here addresses these

limitations directly by including more frequent (monthly) sampling, highly sensitive detection methods, and longitudinal genotyping. Thus we will model how the probability of malaria, given infection, changes in individuals as a function of age, time, and other covariates of interest.

2) We will further dissect immunity phenotypes into correlated but distinct manifestations of anti-parasite immunity (observed by control of parasite densities) and anti-disease immunity (observed by the parasite density at which individuals develop fever, sometimes referred to loosely as "tolerance").

3) As a novel, alternative approach designed to take full advantage of the data generated, we will further track the trajectories of individual parasite clones to estimate the time-course, peak density and duration of each individual infection. Since symptomatic infections are immediately treated, this analysis will focus on characterizing the trajectories of asymptomatic infections. While it is acknowledged that we will not directly observe peak density for most infections, and that there will be substantial biological variation and limitations on the number of observations for any given infection, we anticipate that we will be able to capture the key differences between these trajectories and how they are affected by individual host factors. Trajectories of parasite density over time will be approximated by log-normal or gamma distributions with shape parameters fit to the data, accounting for left-censoring due to unobserved infection times and right-censoring when individuals are treated with antimalarials. The peak density and duration of individual clones will then be estimated and used as measurements of immunity, as opposed to evaluating infections at discrete time points. If peak density and duration are highly correlated we will choose one to represent anti-disease immunity; if they vary with respect to host factors then we will evaluate them as separate outcomes, providing additional dimension to the anti-parasite phenotype. In addition to providing measures of immunity, this exercise will be of great value in calibrating within host mechanistic models of infection.

Analysis of Factors Influencing Immunity: Once measures of immunity are calculated for individuals at different time points, we will evaluate associations between host factors, including age, exposure, and host genetics and immunity. Analogous to Objective 1, associations will be evaluated via longitudinal random effects models allowing for flexible relationships between covariates and immunity, accounting for repeated measures within an individual. Due to our study design, we will have the advantage of being able to evaluate exposure via calibrated individual entomologic estimates and via an individual's infection exposure history. The former has the advantage of being completely independent of host immunity, while the latter is a more direct measure of an individual's immune exposure to the parasite, and can evaluate both the rate of exposure to distinct parasites (FOI) as well integrated exposure of antigen load (area under the curve of modelled infection trajectories). Given that exposure is expected to change within our cohort over time, relationships between exposure and immunity will be evaluated between individuals, as well as within individuals over time, including the rate at which immunity waxes or wanes. We will evaluate whether the rate of change is modified by age or estimated cumulative prior exposure (i.e. whether there is a statistical interaction). Associations between host genetic polymorphisms and immune phenotypes, including the rate at which they change over time, will be evaluated as described in detail in Objective 1.

Analysis of Consequences of Infection on Hemoglobin Levels: Data from our previous cohort studies suggests that even submicroscopic, asymptomatic parasitemia is associated with lower hemoglobin in children. It is difficult to determine the meaning of this association in our previous data sets due to potential confounding between exposure, immunity, and anemia. Therefore, using carefully defined measures of exposure and immunity as described above, we will evaluate the consequences of the frequency and duration of infection on hemoglobin levels, as well as how anti-disease and anti-parasite immunity may modify these relationships, in an analogous fashion to the analyses above. These evaluations will provide important data on potential benefits of interventions designed to reduce intensity of exposure to blood stage parasitemia, including chemoprevention.

7.2 Planned statistical analysis for transmission studies

7.2.1 Analytical Objective 1: To characterize factors associated with gametocyte production

We will determine the impact of intrinsic and extrinsic factors on gametocyte commitment and maturation using state-of-the art molecular diagnostics and fractionation of parasite populations. To assess the impact of individual level factors on gametocyte commitment and production, we will first quantify total parasite densities and relative proportions of the total parasite population that are immature and mature gametocytes. These indicators of gametocyte production will be related to individual level host factors (including age, candidate host genetic polymorphisms, symptomatic status, immune status and hemoglobin concentration) and infection factors (number of parasite clones, duration of infection, recent infection with additional parasite clones) using longitudinal random effects models, allowing for flexible relationships between covariates and the outcome of interest. We will develop a predictive model for factors determining the presence of gametocytes using multivariate regression, comparing different models including multiple individual level covariates and their interactions. We will base our final model selection on the Deviance Information Criteria.

7.2.2 Analytical Objective 2. To evaluate the impacts of human, and parasite, and mosquito factors on parasite infectivity to mosquito vectors

We will characterize factors that impact upon gametocyte infectivity to mosquitoes. We will use newly developed molecular gametocyte sex-ratio assays to compare different measures of infectivity in colony and wild-caught mosquitoes. Finally, we will evaluate associations between human and parasite factors on parasite infectivity as measured via membrane feeding assays. To assess the impact of human host factors and parasite factors on mosquito infection rates, we will first accurately quantify gametocyte concentration as major determinant of transmission and relate gametocyte density to mosquito infection rates. Next, we will determine the impact of other factors on this association. Gametocyte densities will be estimated based on quantification of the gametocyte marker Pfs25 mRNA using qRT-PCR which is currently the most sensitive quantitative assay for mature gametocytes. Gametocyte sex-ratio will be based on Pfs25 and P230p/ PF3D7_1469900 transcript levels. The shape of the relationship between gametocyte density and mosquito infection rates will be determined by fitting a range of different functional forms (e.g. linear, power, hyperbolic, sigmoid, and Gompertz functions). A non-linear model of gametocyte

density and mosquito infection rates that we developed previously, and may be adapted based on the best fitted association in the PRISM studies, will be used to assess the effect of covariates (gametocyte sex ratio, mosquito source, serum-replacement versus autologous plasma). The effect of mosquito source (colony versus locally derived) and feeding approach (direct skin feeding versus membrane feeding) will be further quantified by analyzing paired feeding experiments using a random-effects meta-analysis to generated odds ratios for the matched pairs, allowing for a comparison of the overall effectiveness (i.e. the proportion of infected mosquitoes) of assay conditions. Immune responses to sexual stage antigens from micro-array data will examined by ANOVA and linear regression models for associations with gametocyte carriage and transmission reduction. We will use findings on gametocyte dynamics and related mosquito infection rates to calculate the number of infectious days during infections for different age groups and infection dynamics (e.g. microscopic versus submicroscopic; symptomatic versus asymptomatic). Infectivity will be directly estimated and extrapolated to time-points when gametocyte data are available but no feeding was performed; taking into account all relevant covariates. This approach will allow the estimation of the area under the curve of infectivity versus time using a simple trapezoid method and within-host modelling of infection dynamics in relation to transmission, using an established mechanistic model.

7.2.3 Analytical Objective 3. To characterize the human infectious reservoir for malaria

We will characterize the relative contribution of different demographic populations to malaria transmission in the context of malaria control interventions. This information will be critical in planning interventions to sustainably reduce transmission. In order to achieve this objective, we will carry out entomological and epidemiological monitoring to specifically quantify the contact between humans and mosquitoes for different demographic groups over time and space and relate this to their infectivity to mosquitoes. Analyses under Objective 3 will focus on assessing the contribution of different demographic populations (e.g. age groups and microscopically detectable versus submicroscopic infections) to the infectious reservoir. For these analyses individuals are selected for mosquito feeding experiments based on individual-level parasite detection (first phase of membrane feeding experiments) and household-level exposure (second phase of membrane feeding experiments). Incident infections, gametocyte production and infectiousness to mosquitoes will be analysed after stratifying household members into three age strata: <5 years, 6-15 years and >15 years. Similarly, age-dependent mosquito biting rates will be determined after stratifying household members into three age strata: <5 years, 6-15 years and >15 years and linking blood meals in mosquitoes to individual household members. The relative importance of these age strata to mosquito biting will be estimated by fitting different distributions (e.g. negative binomial, zero-inflated negative binomial regression and Poisson) to the mosquito data after adding zeroes for all individuals for whom no blood-fed mosquitoes were collected. After model-fitting, random effect regression models will be used that take into account clustering of observations from the same night and household. The relative attractiveness of children and adults estimated by these statistical approaches will be incorporated in calculations of age-specific contributions to the infectious reservoir. A population-level mosquito exposure score that uses both age specific exposure estimated by mosquito resting collections and the proportion of each age group in the demographic composition will be developed. The contribution of the different ages to transmission will be estimated by using the weighted average of the proportion of infected mosquitoes from the same

stratum in membrane feeding (Objective 2), with the population-level mosquito exposure scores. This analysis will be extended to also determine the contribution of microscopically detectable and submicroscopic infections to the infectious reservoir. This will be achieved by including their age-, site- and time-dependent prevalence in the population (Objective 1), the proportion of infected mosquitoes from these strata in membrane feeding assays (Objective 2) and mosquito exposure in these strata (Objective 3).

7.3 Planned statistical analysis for immunology studies

7.3.1 Analytical Objective 1: To measure associations between malaria specific antibody responses and measures of clinical immunity

Determining Serologic Profiles Associated with Immunity: Having performed careful measurements of immune phenotypes, including how they may change over time within individuals, we will next define antibody profiles associated with these phenotypes. The goal of this analysis will be to define a parsimonious and generalizable panel of antibodies that can accurately measure antimalarial immunity. In addition to being able to account for potential confounding by age and exposure, the ability to confirm that changes in profiles over time within individuals accurately reflect changes in clinical immune phenotypes will be an important validation and make this investigation one of the most comprehensive of its kind. Immunity will be evaluated as a binary outcome, e.g. comparing the top versus bottom quartile of individuals within a given age and exposure category, and as a continuous outcome, taking advantage of the immunity measurements described above, adjusting for age and exposure. To determine which combination of antibody responses provides the most accurate prediction of various immune phenotypes we will perform supervised feature selection via LASSO regression and Random Forests to identify candidate markers, with a numeric restriction on the number of candidates selected. Prediction accuracy for different numbers of candidates will be evaluated by fitting and cross validating flexible data adaptive models, to determine how many markers are needed to provide accurate information.

7.3.2 Analytical Objective 2: To measure associations between malaria specific cellular immune responses and measures of clinical immunity

We will next identify transcriptional, cellular, and metabolomic profiles associated with the clinical phenotypes described in 8.3.1, having adjusted for age and exposure. For these analyses, we will perform both unsupervised analyses to cluster samples/phenotypes using non-negative matrix factorization (NMF) and hierarchical clustering, as well as supervised feature selection via LASSO regression and Random Forests, as described in 8.3.1. Finally, we plan to utilize a “systems immunologic” approach to integrate these orthogonal datasets (i.e. transcriptomics, CYTOF, metabolomics) to construct a multiscale, multifactorial response network (MMRN). This analysis will be one of the largest, and most comprehensive, investigations of the systems immunology of malaria of its kind.

7.3.3 Analytical Objective 3: To measure associations between immunity against gametocyte antigens and transmission efficiency

TRA in the standard membrane feeding assay will be analysed as continuous(%TRA) and binary variable (<10% TRA versus ≥90% TRA) and associated with antibody titers using multivariate linear and logistic regression models. For field-based DMFA, the intensity of *P. falciparum* oocysts per individual mosquito midgut and per participant is used as indicator of transmission efficiency and modelled using a generalized linear mixed effect model accounting for zero inflation and over-dispersion to estimate the effects of anti-Pfs48/45 and Pfs230 antibodies on oocyst intensity assuming a negative binomial distribution of oocysts counts in the mosquito's population and taking into account covariates such as gametocyte and asexual parasite density. An established model capturing the association between gametocyte density and mosquito infection rates will be adapted to incorporate the effects of gametocyte sex-ratio and human immune responses to gametocyte antigens[54].Antibody responses to Pfs48/45, Pfs230 and other gametocyte antigens will also be related to prior parasite and gametocyte exposure.

7.4 Planned statistical analysis for resistance studies

7.4.1 Analytical Objective 1: Impact of parasite resistance-mediating polymorphisms on gametocytogenesis – in vitro studies

We will test the hypothesis that certain parasite resistance genotypes are associated with alterations in gametocytogenesis, a prerequisite to mosquito infection, and therefore malaria transmission, by 3 parallel approaches. First, we will compare polymorphism prevalences in freshly collected parasites with or without gametocytes based on Giemsa-stained smears and based on more sensitive methods evaluating gametocyte RNA by quantitative rtPCR for *pfs25.* Second, we will compare the success of gametocytogenesis during short-term culture between parasites with different genotypes, as assessed at the time of sample collection. Third, we will assess the resistance genotypes of parasites after short-term culture, to test whether parasites with certain polymorphisms are more likely than others to progress in culture to gametocytes. To enable our 3 analyses we will assess the genotypes of parasites at the initiation of culture, after 5 days of standard culture, and after 5 days of culture selective for gametocytes. We predict that certain resistance genotypes will be associated with decreased gametocytogenesis, highlighting an impact of resistance on this aspect of fitness, and informing policy-makers on impacts on malaria transmission.

7.4.2 Analytical Objective 2: Impact of parasite and mosquito genotypes on parasite development in mosquitoes

We will test whether certain parasite and/or mosquito resistance genotypes are associated with alterations in parasite development in mosquitoes. First, we will compare the abilities of parasites with different genotypes to develop to the oocyst and sporozoite stage of *A. gambiae*. Second, we will compare the ability of the same parasite strains to develop in *A. gambiae* with different insecticide resistance genotypes. For the first experiments a colony will be maintained with reduced genetic diversity, relative to wild-type, to minimize genotype/genotype interactions. For the second experiments it may be necessary to augment the colony with progeny of wild caught gravid females to maintain variability at key loci. The colony, currently in its fourth generation, was established from resting females collected from houses in Nagongera, and so reflects local vector genetic composition

to ensure parasite-host compatibility. Blood from subjects with documented *P. falciparum* infection will be collected and fed promptly to blood seeking female mosquitoes in small (15x15x15 cm) cages during the mosquito dark cycle (with adjusted light:dark cycle in the insectary) to maximize infectivity. The presence or absence of parasites identified microscopically will be noted, and infected midguts (oocysts) or heads/thoraces (sporozoites) will be stored in 96% ethanol until extraction of DNA by standard methods and genetic analysis. We will compare rates of successful progression to the oocyst and sporozoite stages by parasites with different resistance genotypes and by identical parasites in mosquitoes with different insecticide resistance genotypes. Statistical analysis will use simple contingency table based analyses and generalized linear models, to permit examination of interaction terms between genotypes and environmental variables (e.g. temperature, humidity) and also vector and parasite genotypes.

8.0 SUBJECT CONFIDENTIALITY AND DATA MANAGEMENT

8.1 Record keeping

All clinical data will be recorded onto standardized case record forms (CRFs) by study personnel. Laboratory data will be recorded in laboratory record books and then transferred to the case record forms by study coordinators, who will review the case record forms daily for completeness and accuracy. Data will be entered directly from CRFs into a computerized database maintained at the study site clinic. All computerized data will be double entered to verify accuracy of entry and the database will be backed up on a daily basis and for quality control. Query programs will be written into the database to limit the entry of incorrect data and ensure entry of data into required fields. All the entered data will later be transferred to the data centre in Kampala.

Participants will be identified by their study identification number on study documents and patient names will not be recorded on the CRFs or entered into the computerized database. All patient record forms will be kept in individual files in a secured filing cabinet in the study clinic. All corrections will be made on case record forms following GCP guidelines by striking through the incorrect entry with a single line and entering the correct information adjacent to it. All corrections will be initialled and dated by the study individual making the correction.

Records for this study will be maintained and stored in compliance with the principles of GCP and regulatory and institutional requirements, and in compliance of the requirements for the protection of confidentiality of participants. Only study personnel members will have access to these records. All forms with participant names will be kept in a limited-access locked cabinet, when not in use. The electronic database access limited to study personnel will be maintained in a secure limited access location. Participants will be identified by their study ID number, and participant names will not be included in databases used for analysis. Authorised representatives of the sponsor, the ethics committee(s) or regulatory bodies may inspect all documents and records required to be maintained by the investigators. The investigators will allow all requested monitoring visits, audits or reviews. Data will be stored for at least 10 years. Anonymized data collected in this study may also be shared with other investigators and/or placed into the public domain via a data repository.

8.2 Data quality assurance and monitoring

All members of the study team will be educated in the study protocol prior to the onset of the study. Knowledge of the study protocol and procedures will be assessed and documented with a post-training questionnaire. The study clinicians will complete CRFs at each patient visit and also review these forms for completeness and accuracy. Study group meetings will be conducted regularly to review the progress of the study, address any difficulties, and provide performance feedback to the members of the study group.

8.3 Confidentiality

All records will be kept as confidential as possible. Study participants will be identified only by a unique identification number. Patient names will not be entered into the computerized database. Study participant case record forms will be kept in individual files in a limited-access secured filing cabinet in the study clinics. Additional records will be kept in laboratory record books, which will be stored in a secure location in the central study laboratory. No individual identities will be used in any reports or publications resulting from the study.

Much of the data to be collected for this study will come from tests and procedures normally done in the routine care of patients. All project staff will be trained on procedures for maintaining confidentiality and asked to sign a pledge of confidentiality.

8.4 Future Use of Stored Specimens

This study will ask all participants patients to sign an informed consent form and specify whether or not they agree to allow their leftover samples to be used for newly identified secondary analyses or future use projects. Samples will be used only for research and will not be sold or used in the production of commercial products. These samples will be identified only by numbers so that they cannot be readily identified with the participant. These samples will be stored indefinitely at one of the universities or research organizations involved in this study including Makerere University, the University of California, San Francisco, the London School of Hygiene and Tropical Medicine, Liverpool School of Tropical Medicine, Radboud Institute for Health Sciences, and Stanford University. Samples may be shared with researchers at other institutions. The information we get from these studies will not affect patient care.

We will code samples using study identification numbers. The link between the study identification number and information collected during the course of this study will be maintained after the study is over. However, the link will only be to data records and will not include personal information such as names and addresses. The information linking the participant to the sample/ data will be maintained in a limited access, secured location.

8.5 Sample Disposition Plan

Mosquitoes: Infected mosquitoes will be killed in 70% ethanol before dissection. After dissection for oocyst detection, study relevant samples are stored and, remaining mosquito parts are segregated in

designated infectious material waste bins. These are later disposed along other health centre infectious waste material. Dead uninfected mosquitoes are incinerated or washed down the sink within the insectary.

Blood Samples: Blood samples will be disposed off according to the sample maintenance and destruction Standard Operation Procedures (SOP).

8.6 Compensation of Research Related Injury

Considering the purely observational nature of our study, there will be no intervention given to the participants, and therefore a minimum risk research study. We shall be offering all routine health care to the participants as per the national guidelines free of change (including transport and medications). Participants will be followed up for all scheduled visits as well as sick visits at a study clinic located in Tororo district hospital. The clinic will be open 7 days a week (including weekends). We anticipate participant risk to be the same as the population wide risk while attending care at a health facility.

Injuries that occur during the attendance of care will be appropriately treated by the study attending physician.

9.0 INFORMED CONSENTAND ASSENT PROCESS

This protocol, all procedures, and consent forms and any subsequent modifications must be reviewed and approved by the IRBs of all the participating institutions. This includes the following IRBs:

1) Makerere University, School of Medicine - Research and Ethical Committee (SOMREC)
2) University of California, San Francisco, Human Research Protection Program & IRB
3) London School of Hygiene and Tropical Medicine Ethics Committee
4) Stanford University, Research Compliance Office

Written informed consent will be requested from adults (18 years or older) and parents/guardians of children (< 18 years of age) who meet the eligibility criteria and assent will be requested from children 8-17 years of age as per Uganda National Council for Science and Technology guidelines. Informed consent must be provided for each participant before any investigational procedures are performed. A list of all informed consent forms that will be utilized as part of this protocol are included in Table 6 below.

Table 6. List of informed consent forms

Type of study	Type of person providing consent
Cross-sectional survey	Parent/guardian of children aged 2-10 years
	Assent for children aged 8-10 years
	Adult participants aged 18 years or older
Main cohort study	Parent/guardian of children aged < 18 years
	Assent for children aged 8-17 years

Future use of biological specimens	Adult participants aged 18 years or older Parent/guardian of children aged < 18 years
Household survey	Adult head of household aged 18 years or older
Human landing catches	Workers Adult head of household aged 18 years or older
Direct skin feeding assays	Adult participants aged 18 years or older Parent/guardian of children aged 8-17 years Assent for children aged 8-17 years
Mosquito resting collections	Adult participants aged 18 years or older
Mosquito wall bio assays	Adult participants aged 18 years or older

Written informed consent and assent forms will be available in all of the local languages prevalent in Tororo and Busia districts (English, Japadhola, Kiswahili, Lusamya and Luganda). Before beginning the informed consent process, study staff should determine if the participant is able to read and/or write. If the adult, child, or parent/guardian is unable to read, a witness should be identified. The witness must be present during the informed consent discussion. Any adult over the age of 18 years who is able to read and write, and who speaks the language that the informed consent discussion is being conducted in, may serve as an impartial witness. The witness may be a family member or any other person the participant agrees to that is not study staff.

Study staff will conduct the informed consent or assent discussion in the appropriate language and a translator will be used if necessary and will include a detailed description of the purpose of the study, study procedures and risks and benefits to participants. Each section of the consent form shall be read to the participant or parent/guardian exactly as it is written either by the study staffor by the translator if the study staffcannot read that language, and then further explained to the participant or parent/guardian if necessary. The translator will also assist with the discussion and assessment of comprehension. In the case that the adult or child is illiterate, he/she will be asked to place a thumb print onto an appropriate space on the consent and an impartial witness, who can read the language used in the informed consent process, will be asked to witness the discussion and sign the consent form. If both a witness and a translator are required for the informed consent process, these should be two separate people.

Prior to protocol version 8.0 (developed in June 2022), all participants enrolled in the main cohort study were consented to be followed for up to 2 years. To increase the scientific output of the study, implementation of protocol version 8.0 will allow for follow-up of all cohort study participants to be extended to March 31st 2024 depending on the availability of resources. Following approval of protocol version 8.0 all active cohort study participants will be re-consented to allow for follow-up to be extended up to March 31st 2024. Cohort study participants enrolled after approval of protocol version 8.0 will be consented using updated forms which specify that follow-up will be up to March 31st 2024.

9.1 Capacity development from the PRISM work

The PRISM studies have and continue to build capacity of young researchers both in Ugandan and internationally. Capacity provided include internship placements, supporting/nesting master's and PhD projects, as well as providing data and samples for different career development fellowships. In the table below are is a summary of trainees attached to the PRISM project.

Table 7: Trainees attached to the PRISM project

Name	Nationality	Training level	University	status
Joaniter Nankabirwa	Ugandan	K43 grant fellowship	N/A	On-going
Melisa Conrad	American	K01 grant fellowship	University of California San Francisco	On-going
Jaffer Okiring	Ugandan	PhD	Makerere University	On-going
Henry Dumba Mawejje	Ugandan	PhD	London School of Hygiene and Tropical Medicine	On-going
Isaac Sewwanyana	Ugandan	PhD	London School of Hygiene and Tropical Medicine	On-going
Simon Peter Kigozi	Ugandan	PhD	London School of Hygiene and Tropical Medicine	On-going
Steven Tukwasibwe	Ugandan	PhD	Cambridge University	On-going
Chiara Andolina	Italian	PhD	Radboud University	On-going
Emmanuel Arinaitwe	Ugandan	PhD	London School of Hygiene and Tropical Medicine	On-going
Alex Musiime	Ugandan	MSc	Makerere University	Completed
Rek John	Ugandan	MSc	London School of Hygiene and Tropical Medicine	Completed
Kayongo Edward	Ugandan	MSc	Makerere University	Completed
Ambrose Oruni	Ugandan	PhD	Liverpool School of Tropical Medicine	On-going

9.2 Dissemination and Publication of Research Findings

The results from this research will be communicated to stakeholders through dissemination meetings and to participants using language-appropriate information sheets. Investigators will present results at relevant conferences and submit manuscript(s) to peer-reviewed journals in accordance with the NIH, UCSF, LSHTM, Stanford University, UNCST, and Makerere University guidelines.

9.3 Community Engagement Plan

At the inception of the study, the team will meet with the local district leadership both technical and political to share study plans.

For the last ten years, we have had an existing Community Advisory Board (CAB) comprised of local political, religious, civil society, study participant representatives and technical personnel that hold meetings quarterly to review study progress and share feedback from the community. We will continue engaging the CAB and the local leadership through-out the study period.

10.0 REFERENCES

1. World Health Organization. World Malria Report 2018, 2018.
2. World Health Organization. World Malaria Report 2015, 2015.
3. Okello PE, Van Bortel W, Byaruhanga AM, et al. Variation in malaria transmission intensity in seven sites throughout Uganda. *Am J Trop Med Hyg* 2006; **75**(2): 219-25.
4. Yeka A, Gasasira A, Mpimbaza A, et al. Malaria in Uganda: challenges to control on the long road to elimination: I. Epidemiology and current control efforts. *Acta Trop* 2012; **121**(3): 184-95.
5. Uganda Bureau of Statistics. Uganda Malaria Indicator Survey 2014-15, 2015.
6. Breman JG, Holloway CN. Malaria surveillance counts. *Am J Trop Med Hyg* 2007; **77**(6 Suppl): 36-47.
7. Cibulskis RE, Bell D, Christophel EM, et al. Estimating trends in the burden of malaria at country level. *Am J Trop Med Hyg* 2007; **77**(6 Suppl): 133-7.
8. World Health Organization. World malaria report 2008, 2008.
9. Tusting LS, Rek JC, Arinaitwe E, et al. Measuring Socioeconomic Inequalities in Relation to Malaria Risk: A Comparison of Metrics in Rural Uganda. *Am J Trop Med Hyg* 2016;**94**(3): 650-8.
10. Wanzirah H, Tusting LS, Arinaitwe E, et al. Mind the gap: house structure and the risk of malaria in Uganda. *PLoS ONE* 2015; **10**(1): e0117396.
11. Kigozi SP, Pindola DK, Smith, DL, et al. Associations between urbanicity and malaria at local scales in Uganda. 2015; **14**: 374.
12. Kamya MR, Arinaitwe E, Wanzira H, et al. Malaria transmission, infection, and disease at three sites with varied transmission intensity in Uganda: implications for malaria control. *Am J Trop Med Hyg* 2015; **92**(5): 903-12.
13. Katureebe A, Zinszer K, Arinaitwe E, et al. Measures of Malaria Burden after Long-Lasting Insecticidal Net Distribution and Indoor Residual Spraying at Three Sites in Uganda: A Prospective Observational Study. *PLoS Med* 2016; **13**(11): e1002167.
14. Rek J, Katrak S, Obasi H, et al. Characterizing microscopic and submicroscopic malaria parasitaemia at three sites with varied transmission intensity in Uganda. *Malar J* 2016; **15**: 470.
15. Bousema T, Dinglasan RR, Morlais I, et al. Mosquito feeding assays to determine the infectiousness of naturally infected Plasmodium falciparum gametocyte carriers. *PLoS ONE* 2012; **7**(8): e42821.
16. Collins KA, Wang CY, Adams M, et al. A controlled human malaria infection model enabling evaluation of transmission-blocking interventions. *J Clin Invest* 2018; **128**(4): 1551-62.
17. Howes RE, Dewi M, Piel FB, et al. Spatial distribution of G6PD deficiency variants across malaria-endemic regions. *Malar J* 2013; **12**: 418.
18. Piel FB, Patil AP, Howes RE, et al. Global distribution of the sickle cell gene and geographical confirmation of the malaria hypothesis. *Nat Commun* 2010; **1**: 104.
19. Gong L, Maiteki-Sebuguzi C, Rosenthal PJ, et al. Evidence for both innate and acquired mechanisms of protection from Plasmodium falciparum in children with sickle cell trait. *Blood* 2012; **119**(16): 3808-14.
20. Gong L, Parikh S, Rosenthal PJ, Greenhouse B. Biochemical and immunological mechanisms by which sickle cell trait protects against malaria. *Malar J* 2013; **12**: 317.
21. Sabchareon A, Burnouf T, Ouattara D, et al. Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. *Am J Trop Med Hyg* 1991; **45**(3): 297-308.

22. Fowkes FJ, Boeuf P, Beeson JG. Immunity to malaria in an era of declining malaria transmission. *Parasitology* 2016; **143**(2): 139-53.
23. Malaria Genomic Epidemiology Network. Reappraisal of known malaria resistance loci in a large multicenter study. *Nat Genet* 2014; **46**(11): 1197-204.
24. Benelli G, Beier JC. Current vector control challenges in the fight against malaria. *Acta Trop* 2017; **174**: 91-6.
25. Hayward R, Saliba KJ, Kirk K. pfmdr1 mutations associated with chloroquine resistance incur a fitness cost in Plasmodium falciparum. *Mol Microbiol* 2005; **55**(4): 1285-95.
26. Ochong E, Tumwebaze PK, Byaruhanga O, Greenhouse B, Rosenthal PJ. Fitness consequences of Plasmodium falciparum pfmdr1 polymorphisms inferred from ex vivo culture of Ugandan parasites. *Antimicrob Agents Chemother* 2013; **57**(9):4245-51.
27. Ord R, Alexander N, Dunyo S, et al. Seasonal carriage of pfcrt and pfmdr1 alleles in Gambian Plasmodium falciparum imply reduced fitness of chloroquine-resistant parasites. *J Infect Dis* 2007; **196**(11): 1613-9.
28. Laufer MK, Thesing PC, Eddington ND, et al. Return of chloroquine antimalarial efficacy in Malawi. *N Engl J Med* 2006; **355**(19): 1959-66.
29. Conrad MD, LeClair N, Arinaitwe E, et al. Comparative impacts over 5 years of artemisinin-based combination therapies on Plasmodium falciparum polymorphisms that modulate drug sensitivity in Ugandan children. *J Infect Dis* 2014; **210**(3): 344-53.
30. Shinondo CJ, Lanners HN, Lowrie RC, Jr., Wiser MF. Effect of pyrimethamine resistance on sporogony in a Plasmodium berghei/Anopheles stephensi model. *Exp Parasitol* 1994; **78**(2): 194-202.
31. Hallett RL, Dunyo S, Ord R, et al. Chloroquine/sulphadoxine-pyrimethamine for gambian children with malaria: transmission to mosquitoes of multidrug-resistant Plasmodium falciparum. *PLoS clinical trials [electronic resource]* 2006; **1**(3): e15.
32. Mharakurwa S, Kumwenda T, Mkulama MA, et al. Malaria antifolate resistance with contrasting Plasmodium falciparum dihydrofolate reductase (DHFR) polymorphisms in humans and Anopheles mosquitoes. *Proc Natl Acad Sci U S A* 2011; **108**(46): 18796-801.
33. Mharakurwa S, Sialumano M, Liu K, Scott A, Thuma P. Selection for chloroquine-sensitive Plasmodium falciparum by wild Anopheles arabiensis in Southern Zambia. *Malar J* 2013; **12**: 453.
34. Alout H, Ndam NT, Sandeu MM, et al. Insecticide resistance alleles affect vector competence of Anopheles gambiae s.s. for Plasmodium falciparum field isolates. *PLoS ONE* 2013; **8**(5): e63849.
35. Kabula B, Tungu P, Rippon EJ, et al. A significant association between deltamethrin resistance, Plasmodium falciparum infection and the Vgsc-1014S resistance mutation in Anopheles gambiae highlights the epidemiological importance of resistance markers. *Malar J* 2016; **15**(1): 289.
36. Okebe J, Bousema T, Affara M, et al. The Gametocytocidal Efficacy of Different Single Doses of Primaquine with Dihydroartemisinin-piperaquine in Asymptomatic Parasite Carriers in The Gambia: A Randomized Controlled Trial. *EBioMedicine* 2016; **13**: 348-55.
37. Smit MR, Ochomo EO, Aljayyoussi G, et al. Human Direct Skin Feeding versus Membrane Feeding to Assess the Mosquitocidal Efficacy of High-Dose Ivermectin (IVERMAL Trial). *Clin Infect Dis* 2018; Epub ahead of print.
38. Four Artemisinin-Based Combinations (4ABC) Study Group. A head-to-head comparison of four artemisinin-based combinations for treating uncomplicated malaria in African children: a randomized trial. *PLoS Med* 2011; **8**(11): e1001119.
39. Uganda Ministry of Health. Uganda Clinical Guidelines, 2016.

40. Rasmussen SA, Ceja FG, Conrad MD, et al. Changing Antimalarial Drug Sensitivities in Uganda. *Antimicrob Agents Chemother* 2017; **61**(12).
41. Cooper RA, Conrad MD, Watson QD, et al. Lack of Artemisinin Resistance in Plasmodium falciparum in Uganda Based on Parasitological and Molecular Assays. *Antimicrob Agents Chemother* 2015; **59**(8): 5061-4.
42. LeClair NP, Conrad MD, Baliraine FN, Nsanzabana C, Nsobya SL, Rosenthal PJ. Optimization of a ligase detection reaction-fluorescent microsphere assay for characterization of resistance-mediating polymorphisms in African samples of Plasmodium falciparum. *Journal of ClinMicro* 2013; **51**(8): 2564-70.
43. Hofmann N, Mwingira F, Shekalaghe S, Robinson LJ, Mueller I, Felger I. Ultra-sensitive detection of Plasmodium falciparum by amplification of multi-copy subtelomeric targets. *PLoS Med* 2015; **12**(3): e1001788.
44. Lasonder E, Rijpma SR, van Schaijk BC, et al. Integrated transcriptomic and proteomic analyses of P. falciparum gametocytes: molecular insight into sex-specific processes and translational repression. *Nucleic Acids Res* 2016; **44**(13): 6087-101.
45. Karl S, David M, Moore L, et al. Enhanced detection of gametocytes by magnetic deposition microscopy predicts higher potential for Plasmodium falciparum transmission. *Malar J* 2008; **7**: 66.
46. Karl S, Davis TM, St-Pierre TG. A comparison of the sensitivities of detection of Plasmodium falciparum gametocytes by magnetic fractionation, thick blood film microscopy, and RT-PCR. *Malar J* 2009; **8**: 98.
47. Stone WJ, Churcher TS, Graumans W, et al. A scalable assessment of Plasmodium falciparum transmission in the standard membrane-feeding assay, using transgenic parasites expressing green fluorescent protein-luciferase. *J Infect Dis* 2014; **210**(9): 1456-63.
48. Jones S, Grignard L, Nebie I, et al. Naturally acquired antibody responses to recombinant Pfs230 and Pfs48/45 transmission blocking vaccine candidates. *J Infect* 2015; **71**(1): 117-27.
49. Stone WJ, Eldering M, van Gemert GJ, et al. The relevance and applicability of oocyst prevalence as a read-out for mosquito feeding assays. *Scientific reports* 2013; **3**: 3418.
50. Kilama M, Smith DL, Hutchinson R, et al. Estimating the annual entomological inoculation rate for Plasmodium falciparum transmitted by Anopheles gambiae s.l. using three sampling methods in three sites in Uganda. *Malar J* 2014; **13**: 111.
51. Scott JA, Brogdon WG, Collins FH. Identification of single specimens of the Anopheles gambiae complex by the polymerase chain reaction. *Am J Trop Med Hyg* 1993; **49**(4): 520-9.
52. Wirtz RA, Duncan JF, Njelesani EK, et al. ELISA method for detecting Plasmodium falciparum circumsporozoite antibody. *Bull World Health Organ* 1989; **67**(5): 535-42.
53. Burkot TR, Goodman WG, DeFoliart GR. Identification of mosquito blood meals by enzyme-linked immunosorbent assay. *Am J Trop Med Hyg* 1981; **30**(6): 1336-41.
54. Churcher TS, Bousema T, Walker M, et al. Predicting mosquito infection from Plasmodium falciparum gametocyte density and estimating the reservoir of infection. *Elife* 2013; **2**: e00626.
55. Muirhead-Thomson, R. C. "A pit shelter for sampling outdoor mosquito populations."*Bulletin of the World Health Organization* 19, no. 6 (1958): 1116.
56. World Health Organization, 2006. Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets (No. WHO/CDS/NTD/WHOPES/GCDPP/2006.3). World Health Organization.

57. Fuseini, G., Ismail, H.M., von Fricken, M.E., Weppelmann, T.A., Smith, J., Ellis Logan, R.A., Oladepo, F., Walker, K.J., Phiri, W.P., Paine, M.J. and García, G.A., 2020. Improving the performance of spray operators through monitoring and evaluation of insecticide concentrations of pirimiphos-methyl during indoor residual spraying for malaria control on Bioko Island. *Malaria journal*, *19*(1), pp.1-8.

Appendix A. HOUSEHOLD SURVEY

The household survey will be administered through a completely paperless QDS software system. However the data dictionary of the questionnaire which provides information on the questions being asked and coding system used is included below.

Ques. No.	Variable Name	Question	Variable Type	Variable Codes
Section 1: Identification				
1	VISDATE	Date of final visit	Date	
2	STARTIME	Start time of interview	String (HH:SS)	
3	INTNUM	Interviewer number	Numeric	
4	AGREE	Are you going to conduct the interview with this household?	Numeric	1 - Yes 2 - No
Section 2: Household Members - this section will be repeated for each household member with variable names of line2, name2, etc (maximum of 32)				
5	LINE1	Please give me the names of the persons who usually live in your household and guests of the household who stayed here last night, starting with the head of the household. Line number (identifier) of household member.	Numeric	
6	NAME1	Household member name	String	
7	RLTSHP1	What is the relationship of (NAME) to the head of the household?	Numeric	01 - Head of household 02 - Wife or Husband 03 - Son or Daughter 04 - son in law or Daughter - in - law 05 - Grandchild 06 - parent 07 - Parent - in - law 08 - Brother or sister 09 - Niece/nephew by blood 10 - niece/nephew by marriage 11 - other relative 12 - Adopted/foster/step child 13 - Not related 98 - Don't know 99 - Skipped
8	SEX1	Is (NAME) male or female?	Numeric	1 - Male 2 - Female 9 - Skipped
9	ANYOTH1	Are there any other persons who usually live in your household or guests of the household who stayed here last night?	Numeric	1 - Yes 2 - No 9 - Skipped
10	ANYCH1	Are there any other small children or infants that are not listed?	Numeric	1 - Yes 2 - No 9 - Skipped
11	ANYDW1	Are there any domestic workers/maids, etc. not listed?	Numeric	1 - Yes 2 – No 9 - Skipped

12	ANYGU1	Are there any other guests that stayed here last night that are not listed?	Numeric	1 - Yes 2 - No 9 - Skipped
Section 2B: Household Members - this section will be repeated for each household member listed in Section 2				
13	LIVHER1	Does (NAME) usually live here?	Numeric	1 - Yes 2 - No 9 - Skipped
14	STYHR1	Did (NAME) stay here last night?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Skipped
15	AGE1	How old is (NAME) (in completed years)? If < 1 enter '0'	Numeric	998 - Don't know 9999 - Skipped
16	ANSW1	Is (NAME) answering the questionnaire?	Numeric	1 - Yes 2 - No 9 - Skipped
17	NUMPEOP	Number of household members	Numeric	99 - Skipped
18	ANSW	You did not specify the line number of the person answering the questionnaire. Refer to the Household Schedule and record the line number of the person answering the questionnaire.	Numeric	1 - Yes 2 - No 99 - Skipped
Section 3: Household Characteristics				
19	SWATER	What is the main source of drinking water for members of your household?	Numeric	10 – borehole 11 - pipe into dwelling 12 - piped into yard/compound 13 - public tap 21 - open well in yard/compound 22 - open public well 31 - protected well in yard/compound 32 - protected public well 41 - protected spring 42 - unprotected spring 43 - river/stream 44 - pond/lake 45 - dam 51 – rainwater 61 - water truck 71 - bottled water 96 – other 99 - Refused to answer/skipped
20	OTHERSCS	Specify other source of water	String	
21	TFACLTY	What kind of toilet facility do members of your household usually use?	Numeric	1 - flush toilet 2 - vip latrine 3 - covered pit latrine no slab 4 - covered pit latrine w/slab 5 - uncovered pit latrine no slab 6 - uncovered pit latrine w/slab 7 - composting toilet 8 - no facility/bush/field 96 - other

				99 - Refused to answer/skipped
22	OTHERFCY	Specify other kind of toilet facilities	String	
23	ELECTIRC	Does your household have... ...Electricity?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
24	RADIO	...Radio?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
25	CASSETTE	…Cassette player?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
26	TV	…Television?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
27	MOBILE	…Mobile phone?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
28	PHONE	…Fixed phone?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
29	FRIDGE	…Refrigerator?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
30	TABLE	…Table?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
31	CHAIR	…Chairs?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped

32	SOFA	…Sofa set?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
33	BED	…Bed?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
34	CUPBOARD	…Cupboard?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
35	CLOCK	…Clock?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
36	FUELTYPE	What type of fuel does your household mainly use for cooking?	Numeric	1 - electricity 2 - lpg/natural gas 3 - biogas 4 - paraffin/kerosene 5 –charcoal 6 – firewood 7 - straw/shrubs/grass 8 - animal dung 95 - no food cooked in household 96 – other 99 - Refused to answer/skipped
37	OTHERFUE	Specify other type of fuel used	String	
38	SENERGY	What is the main source of energy for lighting in the household?	Numeric	1 - electricity 2 - solar 3 - gas 4 - paraffin - hurricane lamp 5 - paraffin - pressure lamp 6 - paraffin - wick lamp 7 - firewood 8 - candles 9 - torch/battery powered lamp 96 - other 99 - Refused to answer/skipped
39	OTHERENG	Specify other source of energy for lighting	String	
40	MMFLOOR	MAIN MATERIAL OF THE FLOOR RECORD OBSERVATION. MARK ONLY ONE.	Numeric	11 - earth or sand 12 - earth and dung 31 - parquet or polished wood 33 - mosaic or tiles 34 - bricks 35 - cement/concrete 36 - stones 96 - other 99 - Skipped

41	OTHERMMF	Specify other material of the floor	String	
42	MMROOF	MAIN MATERIAL OF THE ROOF. RECORD OBSERVATION. MARK ONLY ONE.	Numeric	11-thatched (including papyrus) 12-mud 21-wood/planks 22-iron sheets 23-asbestos 24-tiles 25-tin 26-cement 96-other 99 - Skipped
43	OTHERMMR	Specify other material of the roof	String	
44	MMEWALLS	MAIN MATERIAL OF THE EXTERIOR WALLS. RECORD OBSERVATION. MARK ONLY ONE.	Numeric	11 - thatched/straw 21 - mud and poles 22 - un-burnt bricks 23 - un-burnt bricks with plaster/cement 24 - burnt bricks with mud 31 - cement blocks 32 - stone 33 - timber 34 - burnt bricks with plaster/cement 35 - iron sheets 96 - other 99 - Skipped
45	OTHERMME	Specify other material of the exterior walls	String	
46	HHROOMS	How many rooms in your household are used for sleeping? (INCLUDING ROOMS OUTSIDE THE MAIN DWELLING) If there are 15 or more rooms, enter 15	Numeric	98 - Don't know 99 - Refused to answer/skipped
47	HHSPACES	How many sleeping spaces like mats, mattresses, or beds are available in your household? If there are 25 or more sleeping places, enter 25	Numeric	98 - Don't know 99 - Refused to answer/skipped
48	WATCH	Does any member of your household own or have… …A watch?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
49	BICYCLE	….A bicycle?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
50	SCOOTER	…A motorcycle or motor scooter?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped

51	CART	…An animal-drawn cart?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
52	CAR	…A car or truck?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
53	MBOAT	…A boat with a motor?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
54	NOMBOAT	…A boat without a motor?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
55	BANKACCO	…A bank account?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
56	NUMALAND	How many acres of agricultural land do members of this household own? IF NONE, ENTER '0000' IF 9995 OR MORE, ENTER '9995.0' IF DON'T KNOW, ENTER '9999.8'	Numeric	9999.8 - Don't know (9999 - Don't know when using tablet) 99999 - Refused to answer/skipped
57	DMARKT	How far is it to the nearest market place?If less than 1 km, enter 00If more than 95 km, enter 95If Don't know, enter 98	Numeric	98 - Don't know 99 - Refused to answer/skipped
58	HHMEALS	Now I would like to ask you about the food your household eats. How many meals does your household usually have per day?	Numeric	9 - Refused to answer/skipped
59	HHNUMT	In the past week, on how many days did the household eat meat?	Numeric	9 - Refused to answer/skipped
60	HHPSF	How often in the last year did you have problems in satisfying the food needs of the household?	Numeric	1 - never 2 - seldom 3 - sometimes 4 - often 5 - always 8 - Don't know 9 - Refused to answer/skipped
61	DHFCTY	How far is it to the nearest health facility? If less than 1 km, enter 00 If more than 95 km, enter 95 If Don't know, enter 98	Numeric	98 - Don't know 99 - Refused to answer/skipped

62	MTHFCTY	If you were to go this facility,how would you most likely go there?	Numeric	1 - car/motorcycle 2 - public transport(bus,taxi) 3 - animal/animal cart 4 - walking 5 - bicycle 7 - boat 6 - other 8 - Don't know 9 - Refused to answer/skipped
63	OTHERMTH	Specify other means of transport to the health facility	String	
64	PSPRAY	At any time in the past 12 months, has anyone asked permission to come into your dwelling to spray the interior walls against mosquitoes?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
65	GPSPRAY	Did you grant them permission to spray the interior walls of your dwelling?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
66	RGSPRAY	What was the primary reason that you did not grant permission to spray the interior walls of your dwelling against mosquitoes? DO NOT PROMPT. MARK ONLY ONE.	Numeric	1 - had a sick person/baby 2 - spraying not effective against mosquitoes 3 - spraying chemicals are harmful 4 - someone in the house was pregnant 6 - other 8 - Don't know 9 - Refused to answer/skipped
67	OTHERRGS	Specify other reasons for not granting permission to spray the interior walls of your dwelling against mosquitoes	String	
68	TSPRAY	How many months ago was the dwelling last sprayed? If less than 1 month, enter 0	Numeric	99 - Refused to answer/skipped
69	WSPRAY	Who sprayed the dwelling?	Numeric	1 - government worker/program 2 - private company 3 - NGO 6 - other 8 - don't know 9 - Refused to answer/skipped
70	OTHERWSP	Specify other people who sprayed the dwelling	String	
71	DSPRAY	Did you pay for the dwelling to be sprayed?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
72	PPWALLS	Since the spraying, have the walls in your dwelling been plastered or painted?	Numeric	1 - Yes 2 - No

				8 - Don't know 9 - Refused to answer
73	TPPWALLS	How many months ago were the walls plastered or painted? IF LESS THAN ONE MONTH, RECORD '0' MONTHS AGO.	Numeric	99 - Refused to answer/skipped
74	MSPRAY	In the past 12 months, have you seen or heard any messages about spraying the interior walls of your dwelling against mosquitoes?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
75	MSGA	Where did you hear or see message(s)? PROBE: "ANYWHERE ELSE?" RECORD ALL MENTIONED. ...Radio?	Numeric	1 - Yes 2 - No 98 - Refused to answer/skipped
76	MSGB	...TV?	Numeric	1 - Yes 2 - No 98 - Refused to answer/skipped
77	MSGC	...Newspaper/Leaflet?	Numeric	1 - Yes 2 - No 98 - Refused to answer/skipped
78	MSGD	…Health worker/CMD?	Numeric	1 - Yes 2 - No 98 - Refused to answer/skipped
79	MSGE	…Neighbour/Relative/Friend	Numeric	1 - Yes 2 - No 98 - Refused to answer/skipped
80	MSGF	...Community Leader?	Numeric	1 - Yes 2 - No 98 - Refused to answer/skipped
81	MSGG	…Village public address system	Numeric	1 - Yes 2 - No 98 - Refused to answer/skipped
82	MSGH	...Don't know	Numeric	1 - Yes 2 - No 98 - Refused to answer/skipped
83	MSGI	…Other	Numeric	1 - Yes 2 - No 98 - Refused to answer/skipped
84	OTHERMSG	Specify other	String	
85	AHWKER	Is there a community health worker (community medicine distributor/CMD, village health team/VHT, community own resource person/CORP) who distributes malaria medicines in your village or community?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped

86	AMCHWKER	Does the community health worker currently have malaria medicines available?	Numeric	1 - Yes 2 - No 8 - Don't know 9 - Refused to answer/skipped
87	HHAMNETS	Does your household have any mosquito nets that can be used while sleeping?	Numeric	1 - Yes 2 - No 9 - Refused to answer
88	HNUMNETS	How many mosquito nets does your household have? IF MORE THAN 12, ENTER 13	Numeric	99 - Refused to answer/skipped
Section 4: Bednets All questions in this section will be repeated for each mosquito net in the household				
89	OBS1	May I have a look at (all) the net(s) to establish the brand?	Numeric	1 - Observed 2 - Not observed 9 - Skipped
90	MNTHS1	How many months ago did your household obtain the mosquito net? IF LESS THAN ONE MONTH, WRITE '0'. IF MORE THAN 36 MONTHS AGO RECORD 95 IF NOT SURE RECORD 98	Numeric	95 - More than 36 months ago 98 - Not Sure 99 - Refused to answer/skipped
91	WHERE1	Where did you get the mosquito net from?		1 - Gov't hospital 2 - Gov't health center 3 - Private hospital/clinic 4 - Private pharmacy 5 - Shop 6 - Open market 7 - Hawker 8 - Project/ngo 9 - Campaign/VHT (Community, school, etc.) 10 - Church 11 - Friend/Relative 12 - Village Leader 96 - Other 98 - Does not know 99 - Refused to answer/skipped
92	SPCFRO1	Specify other sources of the mosquito net	String	
93	BRAND1	OBSERVE OR ASK THE BRAND OR TYPE OF MOSQUITO NET.	Numeric	11 - permanet 12 - duranet 13 - interceptor 14 - netprotect 15 - olyset 21 - ko net 22 - kooper net 23 - iconet 24 - safi net 31 - B52 32 - bamboo hut 33 - century 34 - lucky net 35 –Victoria 41 - homemade net 96 - other 98 - Don't know brand

				99 - Refused to answer/skipped
94	OTHERB1	Specify other brands or types of mosquito net	String	
95	SMNET1	Since you got the mosquito net, was it ever soaked or dipped in a liquid to repel mosquitoes or bugs?	Numeric	1 - Yes 2 - No 8 - Not sure 9 - Refused to answer/skipped
96	TSMNET1	How many months ago was the net last soaked or dipped? IF LESS THAN 1 MONTH, RECORD '0'. IF 25 OR MORE MONTHS AGO, RECORD 95 IF NOT SURE, RECORD 98	Numeric	95 - More than 25 months ago 98 - Not Sure 99 - Refused to answer/skipped
97	SLPNET1	Did anyone sleep under this mosquito net last night?	Numeric	1 - Yes 2 - No 8 - Not sure 9 - Refused to answer/skipped
98	NUSED1A	What are some of the reasons why this net was not used? ...Too hot	Numeric	1 - Yes 2 - No 99 - Refused to answer/skipped
99	NUSED1B	...Don't like smell	Numeric	1 - Yes 2 - No 99 - Refused to answer/skipped
100	NUSED1C	...No mosquitoes	Numeric	1 - Yes 2 - No 99 - Refused to answer/skipped
101	NUSED1D	...Net too old/too many holes	Numeric	1 - Yes 2 - No 99 - Refused to answer/skipped
102	NUSED1E	...Net not hung	Numeric	1 - Yes 2 - No 99 - Refused to answer/skipped
103	NUSED1F	...Net too dirty	Numeric	1 - Yes 2 - No 99 - Refused to answer/skipped
104	NUSED1G	...Net no longer kill insects	Numeric	1 - Yes 2 - No 99 - Refused to answer/skipped

105	NUSED1H	...Don't know	Numeric	1 - Yes 2 - No 99 - Refused to answer/skipped
106	NUSED1I	...Other	Numeric	1 - Yes 2 - No 99 - Refused to answer/skipped
107	NTHUNG1	If not hung, why not? PROBE FOR THE MAIN REASON. SELECT ONLY ONE.	Numeric	1 - Nowhere to hang 2 - Don't know how to hang net 3 - No tools to hang net 4 - Shape did not fit 5 - Size did not fit 6 - Don't want to hang 7 - Extra net/Used for Visitor/ Owner not home 96 - other 98 - not sure 99 - Refused to answer/skipped
108	OTHRNT1	Specify other reason why the net was not hung.	String	
109	UNETL1A	Who slept under this mosquito net last night?RECORD THE PERSON'S NAME AND LINE NUMBER FROM THE HOUSEHOLD SCHEDULE Person 1 Line number	Numeric	after this question, repeat Section 4 if there are still more nets in the house (based on HNUMNETS)
110	UNETN1A	Person 1 Name	String	
111	ANY1A	Anyone else sleep under this net last night?	Numeric	1 - Yes 2 - No 9 - Skipped
112	UNETL1B	Person 2 Line number	Numeric	
113	UNETN1B	Person 2 Name	String	
114	ANY1B	Anyone else sleep under this net last night?	Numeric	1 - Yes 2 - No 9 - Skipped
115	UNETL1C	Person 3 Line number	Numeric	
116	UNETN1C	Person 3 Name	String	
117	ANY1C	Anyone else sleep under this net last night?	Numeric	1 - Yes 2 - No 9 - Skipped
118	UNETL1D	Person 4 Line number	Numeric	
119	UNETN1D	Person 4 Name	String	
120	ANY1D	Anyone else sleep under this net last night?	Numeric	1 - Yes 2 - No 9 - Skipped
121	UNETL1E	Person 5 Line number	Numeric	
122	UNETN1E	Person 5 Name	String	
123	STOPTIME	End time of interview	time	

124	VSTATUS	Result of Visit	Numeric	1 - Completed 2 - No household member at home or no competent respondent at home at time of visit 3 - Entire household absent for extended period of time 5 - Refused 6 - Dwelling vacant or address not a dwelling 7 - Dwelling destroyed 8 - Dwelling not found 9 - Other
125	OVSTATUS	Specify other result	String	
126	TOTVISIT	Total number of visits	Numeric	
127	COMMENTS	Interviewer's Comments	String	

Appendix B. OVERNIGHT TRAVEL QUESTIONNAIRE

STUDY ID |___|--|___|___|___| Patient Initials |___|___| Date of Visit |___|___|/|___|___|/|___|___|
Site ID *Last First* *day month year*

TRAVEL FORM	To be completed if there is a history of travel. Please complete one form per trip* in chronological order. Trip number |___| (1 = 1st trip, 2 = 2nd trip, etc.)

* trip defined as one or more consecutive nights spent in a single sub-county outside of a participant's primary residence

Study participant travel history	
Date of first night away	Date: |___|___|/|___|___|/|___|___| *(DD/MM/YY) (Day 1 below)*
Date of last night away	Date: |___|___|/|___|___|/|___|___| *(DD/MM/YY) (Last day below)*
Name of the district and sub-county travelled to	District: _____ Code: |___||___| Sub-county: _____ Code: |___||___||___|
Main reason for travel (circle one answer)	1 = work/trading; 2 = visiting relatives/friends; 3 = funeral rites; 4 = school; 5 = holiday; 6 = conference/workshop/church; 7 = partying/wedding/cultural gathering; 8 = accompanying parents/guardians; 9 = Other_____
Participant's perception of the risk of getting malaria at the place they have travelled to (circle one answer)	1 = No risk; 2 = Low risk; 3 = Medium risk; 4 = High risk; 5 = Unknown

		Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Date (add additional pages if trip more than 7 days)		/ /	/ /	/ /	/ /	/ /	/ /	/ /
Where did the participant stay overnight	1 = Hotel; 2 = Friend/relative's home; 3 = School; 4 = Hospital; 5 = Church; 5 = Camp; 6 = Other_____	|___|	|___|	|___|	|___|	|___|	|___|	|___|
Time participant had diner	1 = before 5pm; 2 = 5 pm to <9 pm; 3 = 9 pm to 12 mid-night; 4 = After mid-night; 5 = did not have diner	|___|	|___|	|___|	|___|	|___|	|___|	|___|
Time study participant went to bed	1 = before 5pm; 2 = 5 pm to <9 pm; 3 = 9 pm to 12 mid-night; 4 = After mid-night; 5 = did not go to bed	|___|	|___|	|___|	|___|	|___|	|___|	|___|
Measures taken by the study participant to prevent malaria during travel *(Indicate all that apply)*	1 = None 2 = Slept under a bednet 3 = Used mosquito repellents 4 = Used mosquito coils 5 = Take antimalarial drugs 6 = Other_____		___
___
___			___
___
___			___
___
___			___
___
___			___
___
___			___
___
___			___
___
___	
What did the study participant do between dinner and going to bed? *(Indicate all that apply)*	1 = Helped with work in the house/hotel 2 = Sat in the gardens discussing 3 = Have a drink with friends 4 = Stay at work 5 = Listen to news/watch TV 6 = Go to bed right away		___
___
___			___
___
___			___
___
___			___
___
___			___
___
___			___
___
___			___
___
___	
Notes:								

Initials: _____

Entered_____ Date___/___/___ Verified_____ Date___/___/___

Appendix C. MAXIMUM ALLOWABLE BLOOD DRAW VOLUMES*

Participant's weight (kg)	Participant's Total volume of blood	Maximum mL in one blood draw (2.5% of total blood volume)	Maximum mL in a 30-day period (5% of total blood volume)
1	100	2.5	5
2	200	5	10
3	240	6	12
4	320	8	16
5	400	10	20
6	480	12	24
7	560	14	28
8	640	16	32
9	720	18	36
10	800	20	40
11-15	880-1200	22-30	44-60
16-20	1280-1600	32-40	64-80
21-25	1680-2000	42-50	84-100
26-30	2080-2400	52-60	104-120
31-35	2480-2800	62-70	124-140
36-40	2880-3200	72-80	144-160
41-45	3280-3600	82-90	164-180
46-50	3680-4000	92-100	184-200
> 50	> 4000	100	200

* Adopted from Seattle Children's Hospital and Regional Medical Center Guidelines

Appendix D. WHO CRITERIA FOR SEVERE MALARIA/DANGER SIGNS

Criteria for severe malaria

- Cerebral malaria - defined as unarousable coma not attributable to any other cause in a patient with falciparum malaria
- Generalized convulsions (≥ 3 convulsions over 24 hours period)
- Severe normocytic anemia (Hb< 5 gm/dL)
- Hypoglycemia
- Metabolic acidosis with respiratory distress
- Fluid and electrolyte disturbances
- Acute renal failure
- Acute pulmonary edema and adult respiratory distress syndrome (ARDS)
- Circulatory collapse, shock, septicemia ("algid malaria")
- Abnormal bleeding
- Jaundice

Danger signs (for children < 5 years of age)

- Less than 3 convulsions over 24 hour period
- Inability to sit up or stand
- Vomiting everything
- Unable to breastfeed or drink
- Lethargy

Appendix E. VACCINATION HISTORY QUESTIONNAIRE

Schedule	Vaccine	Protects Against	Date Given (DD/MM/YY)	Clinician Initials	Data Entry Initials
AT BIRTH	BCG	Tuberculosis	|___|___|/|___|___|/|___|___|		1st Entry _____ 2nd Entry_____
	Polio 0	Polio	|___|___|/|___|___|/|___|___|		1st Entry _____ 2nd Entry_____
AT 6 WEEKS	Polio 1	Polio	|___|___|/|___|___|/|___|___|		1st Entry _____ 2nd Entry_____
	DPT + HepB + Hib 1	Diphtheria/Tetanus/Whooping Cough/Hepatitis B/Haemophilus Influenza b	|___|___|/|___|___|/|___|___|		1st Entry _____ 2nd Entry_____
	PCV 1	Pneumococcal Pneumonia	|___|___|/|___|___|/|___|___|		1st Entry _____ 2nd Entry_____
	Rota 1	Rotavirus Diarrhoea	|___|___|/|___|___|/|___|___|		1st Entry _____ 2nd Entry_____
AT 10 WEEKS	Polio 2	Polio	|___|___|/|___|___|/|___|___|		1st Entry _____ 2nd Entry_____
	DPT + HepB + Hib 2	Diphtheria/Tetanus/Whooping Cough/Hepatitis B/Haemophilus Influenza b	|___|___|/|___|___|/|___|___|		1st Entry _____ 2nd Entry_____
	PCV 2	Pneumococcal Pneumonia	|___|___|/|___|___|/|___|___|		1st Entry _____ 2nd Entry_____
	Rota 2	Rotavirus Diarrhoea	|___|___|/|___|___|/|___|___|		1st Entry _____ 2nd Entry_____
AT 14 WEEKS	Polio 3	Polio	|___|___|/|___|___|/|___|___|		1st Entry _____ 2nd Entry_____
	DPT + HepB + Hib 3	Diphtheria/Tetanus/Whooping Cough/Hepatitis B/HaemophilusInfluenza b	|___|___|/|___|___|/|___|___|		1st Entry _____ 2nd Entry_____
	PCV 3	Pneumococcal Pneumonia	|___|___|/|___|___|/|___|___|		1st Entry _____ 2nd Entry_____
	Rota 3	Rotavirus Diarrhoea	|___|___|/|___|___|/|___|___|		1st Entry _____ 2nd Entry_____
AT 9 MONTHS	Measles	Measles	|___|___|/|___|___|/|___|___|		1st Entry _____ 2nd Entry_____