Malaria Transmission and the Impact of Control Efforts in Southern and Central Africa

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Statement of Compliance

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

- U.S. Code of Federal Regulations applicable to clinical studies (45 CFR 46)
- ICH GCP E6
- Completion of Human Subjects Protection Training
- NIH 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 trial 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:

Signed: _____ Date: _____

Name
Title

Table of Contents

List of Abbreviations

AE	Adverse Event
CFR	Code of Federal Regulations
CIOMS	Council for International Organizations of Medical Sciences
CRF	Case Report Form
DMID	Division of Microbiology and Infectious Diseases, NIAID, NIH, DHHS
DSMB	Data and Safety Monitoring Board
FWA	Federal-Wide Assurance
GCP	Good Clinical Practice
ICF	Informed Consent Form
ICH	International Conference on Harmonisation
IEC	Independent or Institutional Ethics Committee
IRB	Institutional Review Board
ISM	Independent Safety Monitor
JAMA	Journal of the American Medical Association
MOP	Manual of Procedures
N	Number (typically refers to subjects)
NEJM	New England Journal of Medicine
NIAID	National Institute of Allergy and Infectious Diseases, NIH, DHHS
NIH	National Institutes of Health
OCRA	Office of Clinical Research Affairs, DMID, NIAID, NIH, DHHS
OHRP	Office for Human Research Protections
ORA	Office of Regulatory Affairs, DMID, NIAID, NIH, DHHS
PI	Principal Investigator
SAE	Serious Adverse Event
SMC	Safety Monitoring Committee
SOP	Standard Operating Procedure
WHO	World Health Organization

Protocol Summary

Title: Malaria Transmission and the Impact of Control Efforts in Southern and Central Africa

Population: Study participants will include hospitalized patients with malaria, clinic outpatients with malaria, and residents of selected households. Children and adults, both male and female, are eligible for study participation.

Number of Sites: Four study sites: Choma and Nchelenge Districts in Zambia and Mutasa District in Zimbabwe, and Haute-Katanga Province, Democratic Republic of Congo

Study Duration: Seven years

Subject Duration: Subject duration will range from a single study visit to follow-up for up to two years.

Objectives:

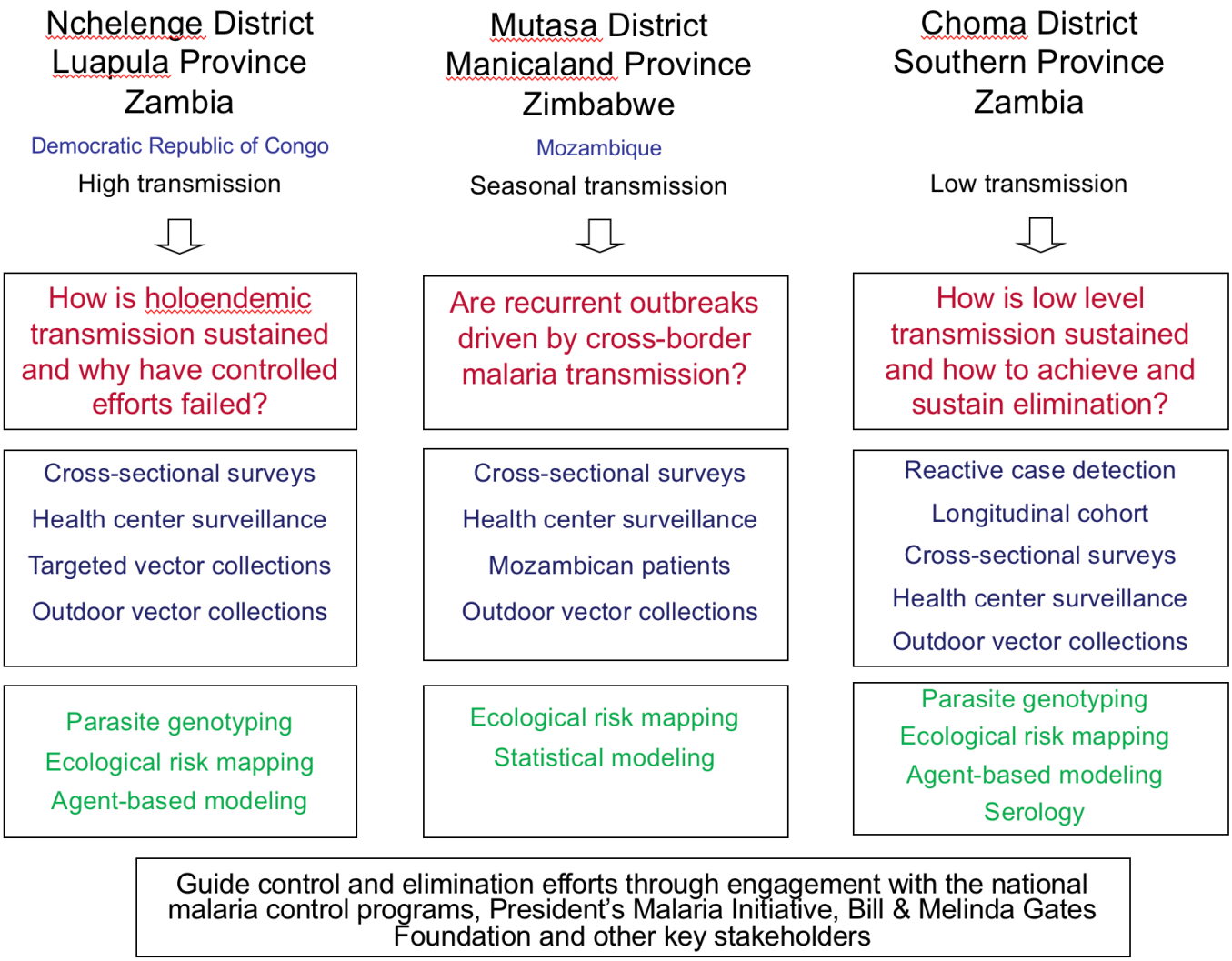
Objective 1: Conduct integrated research on malaria epidemiology, vector biology and parasite genomics in settings of low, seasonal and hyper-endemic perennial transmission in southern and central Africa.

Objective 2: Inform national malaria control programs and partners in Southern and Central Africa to enable development of locally-adapted control strategies based on integrated studies of malaria epidemiology, vector biology and parasite genomics.

Objective 2: Further promote training and capacity building at research institutions in Zambia, Zimbabwe and the Democratic Republic of Congo.

Protocol Summary

Schematic of Study Design:



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2 BACKGROUND INFORMATION AND SCIENTIFIC RATIONALE

2.1 Background Information

Malaria remains a major public health problem in much of southern and central Africa. Achieving and sustaining malaria control and elimination in this region of sub-Saharan Africa is critical to shrinking the malaria map and achieving long-term malaria elimination goals. Through integrated studies of malaria epidemiology and vector bionomics in three different transmission settings in Zambia and Zimbabwe, we developed detailed understanding of the drivers of malaria transmission at each site, identified key barriers to control and elimination, and informed policy decisions to improve control strategies. We will build upon the infrastructure, expertise and relationships developed by the Southern Africa ICEMR over the past seven years to further advance malaria control and elimination in a critical geographic expanse, extending from a setting of highly seasonal malaria transmission in eastern Zimbabwe on the border with Mozambique, through the pre-elimination setting in southern Zambia, to the high transmission settings in northern Zambia and southeastern Democratic Republic of Congo (DRC). This region is the focus of intense malaria control and elimination efforts, including those of the President's Malaria Initiative, United States Agency for International Development, Bill & Melinda Gates Foundation and the Global Fund to Fight AIDS, Tuberculosis and Malaria as well as numerous smaller regional initiatives such as Elimination 8. No other research group in southern Africa has studied the barriers to control and elimination in such depth, duration and across such a range of transmission settings. The theme of the Southern and Central Africa ICEMR is to address critical research questions on barriers to malaria control and elimination in Southern and Central Africa, specifically explanations for continued malaria in high, moderate and low transmission settings despite current public health interventions.

2.2 Scientific Rationale

The Southern and Central Africa ICEMR will conduct integrated research on malaria epidemiology, vector biology and parasite genetics by addressing the aims of the three research projects. Research Area A Epidemiology will be conducted in three settings with seasonal and hyper-endemic malaria transmission and incorporates several research activities, including: 1) research and impact analysis to assess the optimal combination of public health interventions to reduce the burden of disease due to malaria; 2) assessment of the degree of parasite population diversity and structure and its relevance to interventions; and 3) multidisciplinary research to identify, validate, evaluate, and optimize interventional tools and strategies. Research Area B Transmission I will focus on intractable malaria vector populations and residual transmission at the field sites in southern and northern Zambia and also addresses

several research activities, including: 1) research on vector biology and ecology; 2) impact of vector management and control strategies on disease transmission; and potentially 3) evaluation of novel transmission reduction strategies. Research Area C Transmission II will be conducted at a low transmission, pre-elimination setting in Choma District, Southern Province, Zambia and will focus on barriers to malaria elimination, addressing: 1) study of asymptomatic carriers and their contribution to disease transmission; and 2) assessment and evaluation of monitoring tools and surveillance methods when malaria transmission is at low levels.

2.3 Potential Risks and Benefits

2.3.1 Potential Risks

Potential risks to subjects include the risks of finger prick blood samples and loss of confidentiality. Finger prick blood sampling is a minimally invasive procedure, but participants may experience mild discomfort during finger prick. Staff will be trained to conduct finger pricks in a manner that minimizes discomfort. Hardcopies of data will be stored in secure laboratories and offices, and electronic data will be stored in password protected computers.

Participants may feel uncomfortable with a team visiting their home. In some communities, individuals are stigmatized for participation in research studies. To minimize this discomfort, permission to visit the household will be sought from headmen and head of households. Participants can refuse participation at any time without consequence.

Although the real risk is minimal, some people perceive a risk or do not care for the odor associated with the permethrin spray catches (PSC). In addition, people must stay outside of their home temporarily. To minimize this inconvenience, PSCs will be planned in advance and individual households notified. Staff will vent the homes as soon as soon as the collection in complete to dissipate the odor associated with the spray.

2.3.2 Known Potential Benefits

Persons with malaria confirmed by positive rapid diagnostic test may be identified during the community surveys and will be offered treatment with antimalarial drugs provided through the local health system.

An immediate benefit from the PSC is knockdown and elimination of a significant proportion of the biting arthropods treated homes. Although this benefit is short-term due to the rapid dissipation and short longevity of the permethrin, individual home-owners are often pleased with this result.

3 OBJECTIVES

Research Area A: Barriers to Malaria Control in Southern and Central Africa

Aim 1: Identify evolving temporal and spatial patterns and drivers of parasite prevalence, incidence and vector densities and guide targeted intervention strategies in moderate to high malaria transmission settings in Southern and Central Africa.

Aim 2: Identify persistent and emerging barriers to malaria control in moderate to high malaria transmission settings in Southern and Central Africa, including insecticide resistance, antimalarial drug resistance and cross-border malaria transmission.

Aim 3: Evaluate and optimize combinations of vector control and drug-based malaria control interventions in moderate to high malaria transmission settings in Southern and Central Africa in collaboration with implementing partners.

Research Area B: Intractable Malaria Vector Populations and Residual Transmission in Southern and Central Africa

Aim 1: Define and extrapolate risk of vector exposure in the context of ongoing programmatic control at a high transmission setting along the northern Zambia/Democratic Republic of Congo border and at a low transmission setting in southern Zambia.

Aim 2: Identify entomological barriers to vector control in both low and high transmission settings in Zambia.

Aim 3: Utilize changes in parasite genetic diversity in vector mosquitoes as an indicator of successful vector control and reduction of transmission in high transmission settings in Zambia and the DRC.

Research Area C: Achieving, Sustaining and Documenting Malaria Elimination in Southern Province, Zambia

Aim 1: Optimize active and passive surveillance strategies to identify and characterize residual foci of transmission in a low transmission setting in Southern Province, Zambia.

Aim 2: Determine the risk of imported malaria and the contribution of the asymptomatic reservoir to focal transmission in a pre-elimination setting in Southern Province, Zambia.

Aim 3: Quantify the role of secondary vectors in sustaining malaria transmission in a pre-elimination setting in Southern Province, Zambia.

Aim 4: Assess the utility of serosurveillance for monitoring and documenting elimination in a pre-elimination setting in Southern Province, Zambia.

4 STUDY DESIGN

Study sites

Nchelenge District, Zambia: Nchelenge District is located in northern Zambia in the marshlands of Luapula Province along Lake Mweru and shares an international border with the Democratic Republic of Congo. Luapula Province has had the highest malaria prevalence in children younger than five years in Zambia. Nchelenge District covers an area of 4,090 km2 and has an estimated population of 150,000. The district is at an altitude of approximately 800 meters above sea level in a habitat characterized as marsh, with a single rainy season from November through April, followed by a dry season from May to October. *Anopheles funestus* is the major vector with *An. gambiae* playing a secondary role. LLINs and IRS programs have been implemented in Nchelenge District since 2006 and 2007, respectively, with targeted IRS conducted with pirimiphos-methyl since 2014 and universal LLIN distribution in 2017. The catchment area is heterogeneous, with a densely populated urban area along the lake and farmland located further inland. However, the population is mobile, traveling between the lake for the fishing season and inland for farming, with a fishing ban in effect from December 1st to March 1st.

Haut-Katanga District, Democratic Republic of Congo: Haut-Katanga District is in Katanga Province, located in the southeastern edge of the DRC on the border with Zambia. The district is at an altitude of approximately 1,700 meters above sea level and the inhabitants are mostly fisherman and subsistence farmers. The study areas of Kilwa and Kashobwe share an international border with Zambia along Lake Mweru and the Luapula River. These sites have hyperendemic malaria transmission with minimal vector control interventions. LLINs were distributed in 2016 but IRS has not been conducted.

Mutasa District, Zimbabwe: Mutasa District is located in eastern Zimbabwe in Manicaland Province, bordering Manica Province in Mozambique. Manicaland Province has had a disproportionally high burden of malaria in Zimbabwe. Mutasa District covers an area of 622 km2 and has an estimated population of 170,000, consisting mostly of agricultural laborers. Elevation varies dramatically across the district, from 600 m in the river valleys to 2,500 m in the inland mountain areas. Transmission of *P. falciparum* is highly seasonal, with peaks during the rainy season from November to April. The Southern Africa ICEMR demonstrated that *An. funestus* s.s. is the major malaria vector in Mutasa District, contrary to the prevailing belief that *An. gambiae* was the major vector, and showed high levels of pyrethroid resistance. As a consequence, seasonal IRS with pirimiphos-methyl was started in 2014.

Choma District, Zambia: The catchment area of Macha Hospital is located in Choma District, Southern Province, Zambia. Macha Hospital is located approximately 70 km from the nearest town of Choma on a plateau at an altitude of approximately 1,100 meters above sea level and in a habitat characterized as Miombo woodland. There is a single rainy season from approximately

November through April, followed by a cool, dry season from April to August and a hot, dry season from August to November. The catchment area is populated by traditional villagers living in small scattered homesteads. *Anopheles arabiensis* was established by our group as the primary vector responsible for malaria transmission, which peaks during the rainy season. The Southern Province of Zambia historically had hyperendemic *P. falciparum* transmission but the parasite prevalence and number of hospitalizations for malaria declined dramatically over the past decade and a half. The number of pediatric hospitalizations for malaria declined approximately 97% over the past 15 years and the parasite prevalence by active case detection decreased from 8% in 2007 to 1% or less in 2010 to 2013 (active case detection by the Southern Africa ICEMR was discontinued after 2013 because of the low prevalence). Passive case detection consisting of weekly reports of suspected and confirmed cases of malaria from 14 rural health centers was started in 2008 and is on-going, with the data collated and analyzed by the Southern Africa ICEMR. A slight increase in cases was observed in 2016, raising concerns about resurgence. ACTs were introduced as first-line anti-malarial therapy in Zambia in 2002 and into the study area in 2004. Approximately 7,000 LLINs were distributed in the whole Choma District in 2013 and 160,000 in 2014. The area is targeted for malaria elimination and the National Malaria Elimination Program implemented reactive test-and-treat to improve surveillance and decrease the parasite reservoir.

Overview of study designs

Community-based surveys: This study design will include longitudinal (repeat visits) and cross-sectional (single visits) surveys of selected households.

Clinic-based surveillance for symptomatic malaria: The study design will consist of surveillance of children and adults seeking out-patient care at a clinic or health center with confirmed malaria, defined as signs and symptoms of malaria confirmed by rapid diagnostic test (RDT) to document changes in the incidence and clinical patterns of malaria. A questionnaire will be administered, and a blood sample collected from some patients. Used, positive RDTs will also be collected from some health centers for parasite genotyping.

Hospital-based surveillance for symptomatic malaria: The study design will consist of surveillance of children and adults hospitalized with malaria to document changes in the incidence and clinical patterns of severe malaria.

Mosquito collections: Mosquito collections will be conducted in the households of those participating in the longitudinal and cross-sectional community-based surveys. Additional mosquito collections will take place in various ecological settings outside of human habitations, including around animal shelters and potential breeding sites.

Parasite collections: Parasites will be obtained from blood samples collected from symptomatic individuals at hospitals and clinics as well as from asymptomatic individuals participating in the longitudinal and cross-sectional community-based surveys.

Aim-specific study designs

Research Area A: Barriers to Malaria Control in Southern and Central Africa

Aim 1: Identify evolving temporal and spatial patterns and drivers of parasite prevalence, incidence and vector densities and guide targeted intervention strategies in moderate to high malaria transmission settings in Southern and Central Africa.

The study design is a prospective, observational study consisting of serial cross-sectional surveys using a two-stage sampling strategy based on satellite images. Passive case detection consists of weekly SMS text messages (Nchelenge District, Zambia and Kilwa and Kashobwe, DRC) or monthly reports of counts of confirmed and suspected malaria cases from district health centers (Mutasa District, Zimbabwe). Active surveillance will take place monthly in Nchelenge District, monthly in Mutasa District along the border with Mozambique, and biannually in the DRC. Passive case detection will consist of reported numbers of confirmed and suspected malaria cases from all 11 health centers in Nchelenge District, Zambia, 20 health centers in Kilwa, DRC, 8 health centers in Kashobwe, DRC, and 43 health centers in Mutasa District, Zimbabwe.

Aim 2: Identify persistent and emerging barriers to malaria control in moderate to high malaria transmission settings in Southern and Central Africa, including insecticide resistance, antimalarial drug resistance and cross-border malaria transmission.

The study design for Aim 2 will have three components: 1) serial cross-sectional mosquito collections for measurement of insecticide resistance in Nchelenge District, Zambia; 2) serial cross-sectional dried blood spot collections through active and passive case detection for drug-resistance genotyping of parasite DNA in Zambia, Zimbabwe, and eastern DRC; and 3) prospective studies using parasite genetics to assess potential cross-border malaria transmission at the international borders of Zambia and the DRC and Zimbabwe and Mozambique.

Aim 3: Evaluate and optimize combinations of vector control and drug-based malaria control interventions in moderate to high malaria transmission settings in Southern and Central Africa in collaboration with implementing partners.

The study design will consist of pre-post studies of the impact of control interventions based on active case detection through serial cross-sectional surveys and passive case detection

consisting health center reports of confirmed and suspected malaria cases. Critical to valid inferences regarding impact evaluation in the absence of cluster randomized trials will be models that account for concurrent interventions and confounding factors such as changes in temperature and rainfall.

Research Area B: Intractable Malaria Vector Populations and Residual Transmission in Southern and Central Africa

This project will combine field collections of mosquitoes and questionnaire data from residents in households in Nchelenge District, Zambia, Haut-Katanga District, DRC and Choma District, Zambia, semi-field studies in an established facility in Choma District, and genetic studies of vectors led by the Parasite Genetics Core at the University of North Carolina and University of Massachusetts, USA. As described in 'Research Area A: Epidemiology' and 'Research Area C: Transmission II', collections of mosquitoes will be made from households and from animal shelters using CDC light traps. The total numbers of vectors will be outcomes reported under Research Areas A and C, additional data derived from these collections and complementary sampling methods will be used for detailed bionomic and spatio-temporal studies of mosquitoes in this research area. As appropriate, vector data will be linked and analyzed with epidemiological and parasite data collected from the same households for Research Areas A and C to provide a composite understanding of malaria transmission and how these components interact.

Aim 1: Define and extrapolate risk of vector exposure in the context of ongoing programmatic control at a high transmission setting along the northern Zambia/Democratic Republic of Congo border and at a low transmission setting in southern Zambia.

The study design is a prospective, observational study. In northern Zambia, the studies will consist of serial cross-sectional surveys conducted several times per year. In the DRC cross-sectional studies will be conducted in the wet and dry seasons. In southern Zambia, households identified through the reactive test-and-treat program will be visited at days 0, 30 and 90. In the longitudinal cohort, study households will be selected within a defined catchment area of Mapanza Rural Health Center.

Aim 2: Identify entomological barriers to vector control in both low and high transmission settings in Zambia.

Study methods under this aim will include both field, semi-field and laboratory assays. Field collections will build on those carried out under Aim 1, with the addition of collections targeted to locations where human exposure is anticipated based on questionnaire data. Data generated will determine mosquito dynamics, their association with interventions and describe behaviors of recognized and potential secondary vectors. Laboratory studies will assess vector competence

of identified secondary vectors by conducting infectious feeds using laboratory cultures of *Plasmodium falciparum.* Population heterogeneity of vector exposure within the community will be quantified based on coincident human and vector activity in the field, and individual variation in attractiveness of mosquitoes will be assessed in an exposure-free assay in a semi-field setting.

Specimens collected under Aim 1 will provide some of the samples required for Aim 2 and so will employ the same prospective, observational study design in Nchelenge and Choma Districts. Using the questionnaire data from Aim 1 to determine where people spend time after sunset prior to sleeping and before dawn after waking, more intensive mosquito collections will be targeted to those locations in Choma District. Collections of larvae and adults of secondary vectors will be conducted in areas where the surveillance data from Aim 1 indicates their presence. These will be used for subsequent infectious feeds for determination of vector competence in the laboratory. The comparative attractiveness to mosquitoes of pairs of individuals with differing probabilities of malaria infection residing in Choma District will be completed using exposure-free assays in the semi-field system at Macha Research Trust.

The semi-field system in Macha is a large net screen-walled and plastic-roofed greenhouse constructed on a concrete slab, similar to those established in Tanzania for mosquito research. The semi-field system provides a more realistic environment than a laboratory within which to study mosquito behavior and responses to interventions. Climatic conditions match those of the surrounding environment and the large area of the semi-field system permits mosquitoes to demonstrate behaviors such as mating and dispersal that may be altered in the confinement of small laboratory cages. The semi-field system allows for quantifiable studies on large numbers of vectors in a contained area, which would otherwise be expensive and logistically challenging or impossible in field studies. As the mosquitoes originate from colonized stocks, the semi-field system allows for foraging studies of vectors with minimal risk to personnel.

Aim 3: Utilize changes in parasite genetic diversity in vector mosquitoes as an indicator of successful vector control and reduction of transmission in high transmission settings in Zambia and the DRC.

Aim 3 will use *P. falciparum*-positive *An. funestus* identified in Aim 1. Mosquitoes will originate from CDC light traps set within sleeping structures and next to animal enclosures from households sampled in Nchelenge District, northern Zambia and Haut-Katanga District, DRC.

Research Area C: Achieving, Sustaining and Documenting Malaria Elimination in Southern Province, Zambia

Aim 1: Optimize active and passive surveillance strategies to identify and characterize residual foci of transmission in a low transmission setting in Southern Province, Zambia.

We will use both active and passive case detection to identify residual foci of transmission in the catchment area of Macha Hospital. Active case detection initially will be linked to the reactive screen-and-treat and passive case detection will consist of weekly SMS reports from all 14 health centers in the catchment area. The study population consists of residents of the index case households and neighboring households within 250 meters of the index case household.

The reactive test-and-treat study design will end enrollment in March 2018 and complete the 90-day follow-up visits in June 2018. We will return to the cross-sectional surveys of randomly selected households during April, May and June to characterize changes in baseline parasite prevalence from 2013 when we last conducted cross-sectional surveys (2007-2013). These cross-sectional surveys will also be use examine changes in age-specific seroprevalence to address Aim 4.

In July 2018, the study design will change to a prospective, longitudinal study consisting of repeated household surveys in a subset of households in the catchment area of Mapanza Rural Health Center. The longitudinal cohort will provide data to address all four aims of this research area. Enrolled households will be visited monthly for a minimum of one year but up to two years, for a maximum of 24 study visits. The first study visit will include a household census to enumerate all household residents and their age, date of birth and sex. The study duration will be contingent on the willingness of the household to continue participation beyond one year. Informed consent will be obtained at the initial study visit. At each study visit, household members will be administered a survey questionnaire and a fingerprick blood sample will be collected to prepare a DBS card for parasite genotyping and a capillary blood tube for serology. Tympanic temperature will be recorded and those with a temperature of 38°C or higher will have a malaria rapid diagnostic test (RDT) performed. Participants with a positive RDT will be offered treatment with artemether-lumefantrine according to Zambian national guidelines. Pregnant women with a positive RDT will be taken to Mapanza Rural Health Center for care and treatment. A subset of enrolled households will have indoor and outdoor mosquito traps set the night prior to the household visit.

To identify all *P. falciparum* infections in the longitudinal cohort, we will establish passive case detection at Mapanza Rural Health Center. All individuals presenting to Mapanza Rural Health Center with confirmed malaria by RDT will be eligible for enrollment, regardless of whether they are participants in the longitudinal cohort study. This will simplify procedures at the health center, ensure that we do not miss participants in the longitudinal cohort, and provide additional information on parasite genotypes from individuals seeking care at the rural health center. Study ID cards, including the participant's name and study ID number, will be given to participants in the longitudinal cohort and they will be asked to present their card should they seek care at Mapanza Rural Health Center. Written consent will be obtained to collect demographic information, history of illness and a fingerprick blood DBS sample for parasite genotyping. Used, positive RDTs labeled with date and age will also be collected from Mapanza Rural Health

Center. Health care workers at Mapanza Rural Health Center will ask those with confirmed malaria who agree to be in the study whether they have a study ID card.

An SMS text-based system for recording weekly number of suspected and confirmed malaria cases at 14 health centers in the catchment area of Macha Hospital was established by MRT and JHMRI in 2008 will continue.

Aim 2: Determine the risk of imported malaria and the contribution of the asymptomatic reservoir to focal transmission in a pre-elimination setting in Southern Province, Zambia.

The study design will consist of active and passive case detection as described under Aim 1. Monthly sampling of participants enrolled in a longitudinal cohort will enable characterization of chains of transmission using novel parasite genotyping methods and distinguish imported from indigenous parasite strains.

Aim 3: Quantify the role of secondary vectors in sustaining malaria transmission in a pre-elimination setting in Southern Province, Zambia.

The study design will consist of active and passive case detection as described under Aim 1 and in Research Area B. Mosquitoes will be collected within and around households recruited for active surveillance through the reactive test-and-treat strategy and longitudinal cohort. Repeated sampling of vectors, both indoors and outdoors, offers an opportunity to link epidemiological and entomological data to draw inferences regarding the dynamic, relative roles of primary and secondary vectors.

Aim 4: Assess the utility of serosurveillance for monitoring and documenting elimination in a pre-elimination setting in Southern Province, Zambia.

The study design will be cross-sectional surveys of 25 randomly selected households per month for April, May and June 2018 followed by the longitudinal cohort study described under Aim 1. The random selection of households is done using high-resolution satellite imagery. Longitudinal sampling also will enhance our understanding of serological markers of recent infection, with the goal of identifying *P. falciparum* antigens that permit accurate timing of infection in cross-sectional serological surveys.

5 Study Population

5.1 Selection of the Study Population

We will monitor changes in the epidemiology of malaria in three study populations at each of the three sites: 1) hospitalized children and adults with symptomatic malaria (through a record review only); 2) children and adults seeking outpatient care with symptomatic malaria; and 3) symptomatic and asymptomatic children and adults residing in randomly selected households. Symptomatic malaria will be defined as a febrile illness with parasitemia confirmed by RDT or microscopy. Asymptomatic malaria will be defined as parasitemia confirmed by RDT or microscopy without fever. Study populations will be enrolled through community-based surveys and at outpatient facilities and hospitals in three different epidemiological settings: 1) Choma District, Southern Province, Zambia; 2) Nchelenge District, Luapula Province, Zambia; and 3) Mutasa District, Manicaland Province Zimbabwe. The size and shape of the study areas in each of the three study districts were selected based on household density, expected malaria transmission patterns, logistics (e.g. transportation of field staff and specimens) and the cost of satellite imagery.

5.2 Inclusion/Exclusion Criteria

Cross-sectional household surveys

Inclusion criteria

1. resident of selected household
2. willing and able to provide consent or have a parent or guardian who is willing and able to provide consent
3. persons of all ages are eligible for enrollment
4. pregnant and breastfeeding women are eligible for enrollment

Exclusion criteria

1. non-resident of selected households (i.e. visitor)

Longitudinal household surveys in southern Zambia

Inclusion criteria

Household surveys

1. Resident or temporary visitor of a selected household aged 3 months or older, including women who are pregnant or breastfeeding
2. Willing and able to provide consent or have a parent or guardian who is willing and able to provide consent

Clinic-based surveillance for symptomatic malaria

Inclusion criteria

1. Evaluated at Mapanza Rural Health Center
2. Malaria confirmed by RDT
3. 3 months of age or older
4. Willing and able to provide written consent or have a caretaker, parent or guardian who is willing and able to provide written consent

Exclusion criteria

Household surveys

1. Younger than 3 months of age
2. Severe illness, other than severe malaria, precluding participation, as judged by family member or study staff

Clinic-based surveillance for symptomatic malaria

1. Younger than 3 months of age
2. Severe illness, other than severe malaria, precluding participation, as judged by clinical officer or nurse

Outpatient malaria

Inclusion criteria

1. present to clinic or hospital within the study area and have confirmed malaria
2. willing and able to provide consent or have a caretaker who is willing and able to provide consent
3. persons of all ages are eligible for enrollment
4. pregnant and breastfeeding women are eligible for enrollment

Exclusion criteria

1. severe illness precluding participation, as determined by the attending physician or clinical officer

6 STUDY PROCEDURES/EVALUATIONS

6.1 Study Procedures

Research Area A: Barriers to Malaria Control in Southern and Central Africa

Aim 1: Identify evolving temporal and spatial patterns and drivers of parasite prevalence, incidence and vector densities and guide targeted intervention strategies in moderate to high malaria transmission settings in Southern and Central Africa.

Active case detection: *Random sampling of households*: For the cross-sectional surveys, we will use high resolution satellite images of the study areas to establish the sampling frame and select study households. Households will be enumerated manually by placing a marker on the centroid of each potential residence, creating an attribute table containing unique identifiers and geographic coordinates for each household. *Community mobilization*: Community mobilization activities will be conducted to inform local leaders and the community about study objectives and activities and disseminate findings. These activities will be conducted by locally trained field supervisors at the start of the study and periodically throughout the study period. *Field Teams*: Field teams will consist of a field supervisor and 3-4 interviewers. Field teams will identify the study household using household coordinates and global positioning system (GPS) devices. *Day 1 visit*: Study visits will be conducted over two days. During the first visit, permission for study participation will be obtained from the head of the household. The household will be informed that study staff will return the following day to administer questionnaires and obtain blood specimens. Field staff will obtain oral consent to set up overnight light traps. *Mosquito CDC light trap collection*: Field staff will ensure that everyone sleeping inside the selected house will be protected by a LLIN on the sampling night. Additional LLINs will be supplied if needed. A CDC light trap will be placed at the foot end of the beds/sleeping areas, just outside of the LLIN. The trap is intended to collect mosquitoes coming into the house in search of the human hosts that are protected by LLINs and will be run from 1800 hrs to 0600hrs the next morning. *Day 2 visit*: CDC light traps will be dismantled, and mosquitoes stored for return to the field station and laboratory. After written informed consent is obtained from eligible adults and parental permission from caregivers of eligible children, a standardized questionnaire will be administered to each participant. Standardized, field tested survey instruments developed over the past six years will be used to collect information from each adult study participant or caretaker for children younger than 16 years of age on demographic characteristics, travel history and migration, general knowledge of malaria (transmission, prevention, and treatment), malaria treatment history and health seeking behavior, use of LLINs and IRS, and socio-economic indicators. Data will be captured and stored on Android-based tablets using ODK software and will be uploaded daily onto a server and/or downloaded onto a computer hard

drive. *Specimen collection*: After completing the questionnaire, a study staff member will collect finger-prick blood specimens from enrolled children and adults. Blood will be spotted onto an approved RDT that detects *P. falciparum* HRP-2 and spotted onto filter paper for molecular assays.

Passive case detection: To continue to track malaria cases at health centers, we will use an established system in Nchelenge District, Zambia whereby the 11 district health centers report weekly by SMS text message the number of confirmed and suspected malaria cases. This system will be established at the study sites in the DRC. In Mutasa District, we will rely on data from the health management information system (HMIS) of the Ministry of Health and Child Care that routinely collects weekly malaria-related data from 43 health facilities in Mutasa District. Indicators include the number of positive RDTs, patients clinically diagnosed with malaria, and malaria deaths, stratified by younger and older than 5 years.

Aim 2: Identify persistent and emerging barriers to malaria control in moderate to high malaria transmission settings in Southern and Central Africa, including insecticide resistance, antimalarial drug resistance and cross-border malaria transmission.

Insecticide resistance testing: Insecticide resistance testing will be performed as previously reported for Nchelenge District, Zambia and Mutasa District, Zimbabwe. Briefly, indoor resting mosquitoes will be collected in selected households using aspirators. WHO susceptibility tests, synergist tests and resistance intensity tests will be conducted on wild females and their progeny.

Drug resistance surveillance: We will conduct molecular surveillance for drug resistance in the context of active and passive case detection. Our focus will be on assessing the prevalence/frequency of mutations in genes representing the greatest impediment to control: K13 (kelch) that confers resistance to artemisinin; dhps and dhfr that confer resistance to sulfadoxine-pyrimethamine used in IPTp; pfcrt and pfmdr1 associated with amodiaquine resistance (artesunate-amodiaquine is the first-line antimalarial in the DRC).

Parasite genotyping: Parasite diversity and dispersal will be assessed at two border regions, along the DRC-Zambia border and along the Zimbabwe-Mozambique border. Dried blood spot samples from RDT positive individuals will be obtained from two regions in Nchelenge District, along the lakeside and further inland, and as well in two regions in DRC, Kilwa and Kashobwe. Along the Zimbabwe-Mozambique border, dried blood spots will be collected in Mutasa District, Zimbabwe from individuals with confirmed malaria presenting to health centers along the border and who self-report recent travel to Mozambique or not. Parasite isolates will be genotyped using molecular inversion probes by the Parasite Genetics Core. This approach allows high resolution genotyping of polyclonal infections which are common in these regions as well as recovery of haplotype sequences for downstream analysis. The molecular inversion probes will be used to estimate degree of genetic relatedness and to infer the degree of parasite spatial

population structure between parasite populations as well as parasite temporal stability. These estimates will allow us to make inferences about the extent of cross-border transmission.

We will also use dried blood spots collected during the 2018 Malaria Indicator Survey in Zambia to characterize parasite genotypes from a nationally representative sample. Antimalarial resistance markers will be identified, and a national parasite genotyping map will be generated to better identify sources and sinks of parasite transmission in Zambia.

Aim 3: Evaluate and optimize combinations of vector control and drug-based malaria control interventions in moderate to high malaria transmission settings in Southern and Central Africa in collaboration with implementing partners.

The study procedures for Aim 3 will be those described under Aim 1 for active and passive surveillance. We will leverage the long, time series to establish robust estimates of parasite prevalence, incidence and mosquito counts and their seasonal and secular variation prior to the interventions. Through active surveillance, we will monitor interventions such as LLIN ownership and use to account for the potential impact of multiple, concurrent interventions. We will parameterize an agent-based model to assess the optimal package of interventions for Nchelenge District, Zambia.

Research Area B: Intractable Malaria Vector Populations and Residual Transmission in Southern and Central Africa

Aim 1: Define and extrapolate risk of vector exposure in the context of ongoing programmatic control at a high transmission setting along the northern Zambia/Democratic Republic of Congo border and at a low transmission setting in southern Zambia.

Household selection: The sampling strategy for Research Area B will follow those described for Research Areas A and C.

Mosquito CDC light trap collection: CDC light traps will be placed in houses as well as outdoors next to household-associated animal enclosures. In Nchelenge District, for each enrolled household a single trap will be set within one sleeping structure and one next the main animal enclosure of that household. In Haut-Katanga Province, traps will be set within one sleeping structure per selected household. In Choma District, one trap will be set in the index house and one next to an animal shed, as well as in houses and next to animal shelters in up to three neighboring households within 140 m of the index household for the reactive case detection.

For indoor collections, field staff will ensure that everyone sleeping inside the selected house will be protected by an LLIN on the sampling night. If enough LLINs to cover all sleeping areas are not available, additional LLINs will be supplied. A CDC light trap will be placed at 1.5 meters

above the floor at the foot end of the beds/sleeping areas, just outside of the LLIN. For outdoor collections, traps will be hung next to animal enclosures at 1.5 meters from the ground. All traps will be run from 18:00 to 06:00 and collected the following day. In Choma District, for those households revisited at 30 days and at 90 days after the initial visit during reactive test and treat, light traps will be set in the same indoor and outdoor locations for these selected households to monitor changes over time.

Household questionnaires: Questionnaires will be administered electronically to consenting participants using Android tablets with ODK software to collect information on household structure, wealth indices, presence and use of vector control tools including ITNs, and other personal malaria protection methods. Questions to capture the amount of time and activities spent outdoors in evening hours will be asked to capture the potential for vector exposure.

Aim 2: Identify entomological barriers to vector control in both low and high transmission settings in Zambia.

Assessment of coincident intervention coverage and vector presence: As well as mosquito abundance, the questionnaire data on intervention coverage and reports from local District control officers will be used to generate maps of intervention coverage which will be overlaid with the spatio-temporal distribution of vectors. This will be used to identify areas where intervention coverage is sub-optimal and so determine human populations at increased risk of exposure in Nchelenge and Choma Districts.

Assessment of residual transmission: The data collected from interviews conducted in Choma District under Aim 1 on human activity in the early evening through dawn will be used to identify localities for more intensive mosquito collections. Light traps and UV traps will be set close to where people gather.

For a subset of localities, human landing collections to estimate foraging rates and other trapping techniques such as barrier screens to determine mosquito activity and flight paths will be conducted from 18:00 to 06:00. During these studies, the number of community residents at that locality by hour will be recorded to estimate hourly exposure. Details on human landing catches will be provided in an amendment to this protocol.

Study of secondary vectors: Specimens from all collections will be identified and blood feeding rates, host preference, infectivity and entomological inoculation rates (EIRs) determined. Species that are found with *Plasmodium* parasites will undergo genetic sequencing and phylogenetic analyses to confirm identities. Associating behavioral traits and human exposure of secondary vectors will allow assessment of their contribution to malaria transmission. Vector competence will be confirmed by infective feeds of wild caught mosquitoes in the laboratory.

Differential attractiveness of individuals: Healthy adult male volunteers will be recruited from consenting households in Choma District visited as part of the longitudinal cohort, selecting households with high vector counts and *P. falciparum* infections ('hot homes') as well as a similar number of households with low vector densities and where no infected individuals were found ('cold homes'). A history of malaria and current health will be recorded for all consenting males over the age of 20. A single healthy individual will be recruited from each household, selecting those reporting a history of multiple malaria episodes from 'hot homes' and those with the fewest episodes from 'cold homes'. 'Hot' and 'cold' volunteers will be paired and requested to sleep overnight in separate tents located at Macha Research Trust from which human odors will be pumped via ducting into traps within the semi-field system. Uninfected colony mosquitoes will be released into the semi-field system on each night of the experiment and allowed to disperse. The proportion of released mosquitoes caught in respective traps will be assessed each morning. During these exposure-free assays, breath and skin odor from headspace piped into the field cage will be actively sampled using sorbent cartridges containing either TENAX-TA or Sulficarb sorbents (Markes International, USA). Cartridges will capture a broad spectrum of volatile organic compounds present in the breath and skin odor to detect candidate compounds mediating enhanced mosquito attraction to humans. Thermal desorption-gas chromatography/mass spectrometry (TD-GC/MS) analysis of the volatile organic compounds will be carried out at Johns Hopkins University. After completion of the assays, blood will be collected from consenting individuals for malaria testing by RDT, microscopy and for collection of dried blood spots on filter paper for subsequent PCR detection of malaria infection. These data will be used retrospectively to determine whether differential attractiveness of individuals correlates with *Plasmodium* infections. RDT-positive individuals will be offered treatment according to national guidelines.

Aim 3: Utilize changes in parasite genetic diversity in vector mosquitoes as an indicator of successful vector control and reduction of transmission in high transmission settings in Zambia and the DRC.

Mosquito collection and parasite assay procedures are outlined in Aim 1. *P. falciparum*-positive *An. funestus* identified in Aim 1 will be further processed by deep sequencing to identify *var2CSA* variants and diversity in each infected mosquito. Briefly, deep sequencing at the *var2CSA* locus will provide high resolution of variants in a given infection by enhanced sensitivity for minority SNPs, and enable to estimate degree of genetic relatedness which will be compared to the human data derived from Research Area A.

Research Area C: Achieving, Sustaining and Documenting Malaria Elimination in Southern Province, Zambia

Aim 1: Optimize active and passive surveillance strategies to identify and characterize residual foci of transmission in a low transmission setting in Southern Province, Zambia.

Active case detection linked to reactive test-and-treat: When a symptomatic individual presents to a health center or health post and malaria is confirmed by RDT, the health care worker is instructed to go to the index case household and test all individuals residing within 140 meters of the index case household with a malaria RDT and treat those who are positive with artemether/lumefantrine (Coartem®). The health care worker sends an SMS text message to the study team when an index case is identified. The study team accompanies the health care worker when he or she conducts the reactive case detection, usually within one week of diagnosis. All households within 250 meters of an index case are eligible for participation in the study. Study procedures at the selected households include explanation of the study to the head of household, written informed consent from adult participants and from caregivers of children, administration of the study questionnaire that includes travel history, and collection of a finger prick blood sample as a dried blood spot on Whatman 903 sample collection cards and for a *Pf*HRP2-based RDT. In March 2016 the Southern Africa ICEMR added two additional study visits at 30 and 90 days after the initial visit, and these repeated visits will be continued. The purpose of adding follow-up visits is: 1) some individuals screened at the initial visit may have early infection, below the limit of detection by malaria RDT or PCR, but may become positive at a subsequent visit; 2) ongoing, local transmission may occur despite reactive case detection; and 3) to identify whether asymptomatic individuals who are RDT negative but PCR positive become symptomatic and RDT positive or clear infection. These repeat visits will help further assess the effectiveness of reactive screen-and-treat and the optimal timing.

Cross-sectional serosurveys: We will conduct cross-sectional serosurvey for the months of April, May and June 2018 as we did from 2010 to 2013 under the Southern Africa ICEMR. We will use high resolution satellite images of the study areas to randomly select 30 study households per month for a total of 90 households.

Study visits will be conducted over two days. During the first visit, permission for study participation will be obtained from the head of the household. The household will be informed that study staff will return the following day to administer questionnaires and obtain specimens. Field staff will obtain verbal consent to set up overnight light traps. Field staff will ensure that everyone sleeping inside the selected house will be protected by an LLIN on the sampling night. If enough LLINs to cover all sleeping areas are not available, additional LLINs will be supplied. A CDC light trap will be placed near the ‘head’ of one of the beds/sleeping areas, just outside of the LLIN. The trap is intended to collect mosquitoes coming into the house in search of the human hosts that are protected by LLIN.

Data and sample collection will be conducted on the second day. After written informed consent has been obtained from eligible adults and parents of eligible children, a standardized questionnaire will be administered to each participant. A trained interviewer will read each question aloud. Participants will be allotted time to respond to each question and may refuse to respond to any question for any reason. It will take approximately 15 minutes to administer the

questionnaire to each individual. Standardized, field tested survey instruments will be used to collect information from each adult study participant (or caretaker for children younger than 16 years of age) on demographic characteristics, general knowledge of malaria (transmission, prevention, and treatment), malaria treatment history and health seeking behavior, recent travel history, use of ITNs and IRS, and socio-economic indicators. Data will be captured and stored on Android phones using ODK software and will be uploaded onto a server or downloaded onto a computer hard drive. After completing the questionnaire, study staff will collect finger-prick blood specimens from enrolled children and adults. The middle or ring finger will be cleaned with a sterile alcohol pad and lanced using an auto-lancet. Blood will be: 1) spotted onto rapid diagnostic test cassettes that detect *P. falciparum* HRP-2; 2) collected in a capillary tube for serology; and 3) spotted onto filter paper for parasite detection by PCR and parasite genotyping.

A trained nurse will measure and document the temperature of study participants using an oral thermometer and will weigh all RDT-positive participants. The weight will be used to determine the appropriate dose of antimalarial drug. Individuals who are RDT-positive will be offered treatment with ACT by study staff with drugs provided by the Ministry of Health in Zambia or Zimbabwe. Quinine (plus clindamycin if available) will be offered to pregnant women estimated to be in the first trimester of pregnancy, according to guidelines of the World Health Organization (WHO) and Ministry of Health in Zambia and Zimbabwe. Pregnant women will be identified by self-report. If an individual has signs and symptoms of severe malaria, or other illness requiring urgent evaluation, transport to a local health center or hospital will be provided.

Longitudinal cohort: The study procedures for the longitudinal cohort will be similar to those for the cross-sectional serosurveys, with the several exceptions: 1) consent will only be obtained on the initial study visit for a particular individual; 2) the questionnaire will be shortened to collect only information relevant to the prior month (e.g. travel history and history of malaria); 3) RDTs will only be performed on participants with a temperature of 38^{o}C or higher; 4) additional mosquito collections will be made in a subsample of households dependent on the epidemiology, including spray catches and outdoor collections.

Passive case detection: Weekly counts of suspected and RDT confirmed cases are sent via SMS from 14 health centers serving the catchment of Macha Hospital to the Southern Africa ICEMR investigators at Macha Research Trust. The location of each health center will be mapped and the catchment population estimated by both distance from the health center and reported health center utilization from prior household surveillance. Ecological features surrounding each health center will be obtained from satellite imagery and remote sensing. These 14 health centers will be asked to store all positive RDTs. The age and sex of the patient, date and whether or not they traveled within the past month will be indicated on the RDT. The RDT will then be stored in sealed in plastic bags with desiccant until retrieved by study staff. At the laboratory at Macha Research Trust, the RDTs will be stored at -20^{o}C until DNA extraction and parasite genotyping is performed using a 24 SNP barcode. Parasites may also be genotyped using molecular inversion probes.

Aim 2: Determine the risk of imported malaria and the contribution of the asymptomatic reservoir to focal transmission in a pre-elimination setting in Southern Province, Zambia.

Active case detection will be as described for Aim 1. *Passive case detection*: When symptomatic individuals are confirmed to have malaria by RDT at eight health centers within ~25 km of Macha Hospital, the study team is notified by text message and travels to the health center where participants are enrolled. Written informed consent from adult participants and from caregivers of children is obtained, a study questionnaire that includes travel history is administered, and a finger prick blood sample is collected as a dried blood spot on Whatman 903 sample collection cards and for a *Pf*HRP2 RDT.

We will use travel histories and parasite genetic approaches to clarify how transmission is maintained and whether infected travelers and asymptomatic carriers contribute to transmission. First, we will compare genetic relatedness in a Bayesian phylogeny framework of index cases to secondary cases identified within a 140 m radius to assess whether parasites in secondary cases are genetically related to the index case as a result of local transmission. If a malaria infection is found in someone with a reported travel history, we will attempt to obtain parasite samples from that site, to genetically confirm importation Next, we will compare the population of symptomatic cases to the population of asymptomatic cases. Although our previous results based on 24 barcodes suggest that chronically asymptomatic infected reservoir are not contributing substantially to on-going transmission (see above), our analysis was limited due to the high frequency of polyclonal infections observed in study participants. The limitations of SNP barcoding assay can now be overcome using our novel approach based on deep sequencing of the *var2csa* locus and other hypervariable loci will enable us to resolve all haplotypes in a given infection, and thus measure individual- and population level variation, and improve phylogenetic resolution for understanding transmission chains. Our goal is to understand whether parasite populations from symptomatic and asymptomatic people are intermixed or genetically distinct. Intermixed populations would suggest that the asymptomatic reservoir contributes regularly to on-going transmission. Distinct population structure would indicate that asymptomatic individuals do not contribute substantially to sustaining low-level transmission.

Rural Health Center parasite barcoding and genotyping. Fourteen rural health centers (RHCs) provide weekly summary reports of malaria cases via SMS text messages to our partners at Macha Research Trust (MRT). These reports contain the number of malaria rapid diagnostic tests (RDTs) that were used and the number that were positive, stratified by those younger than and older 5 years of age. This passive case detection system is used to monitor spatial and temporal changes in the burden of malaria. We will collect the positive RDTs from these RHCs for extraction of *Plasmodium falciparum* DNA for parasite genetic analyses. Testing for malaria using an RDT will continue as indicated by Zambia's national policy at the fourteen RHCs. In the event that an RDT is positive, health care workers from the fourteen RHCs will record the date, patient age in years and sex, and whether the individual reported travel outside Southern

Province during the prior month by denoting '+' for yes or '-' for no. This information will be directly written on the RDT cassette. No personal identifiers will be recorded. The health care worker will place the cassette in a Ziploc bag with a desiccant for storage. The health care worker will notify the MRT field team by text message that one or more positive RDTs are available. After transport to the MRT laboratory, each RDT will be assigned a unique identification number. The RDT will be stored at the MRT laboratory until DNA extraction and genotyping. We optimized DNA extraction procedures from spiked RTDs in collaboration with our partners from the University of North Carolina. Parasite genoptying will consist of a 24 SNP barcode that has been used at the MRT for prior malaria studies. We will create parasite genotype maps of RHC catchment areas and use this information to infer local transmission networks and parasite importations.

Aim 3: Quantify the role of secondary vectors in sustaining malaria transmission in a pre-elimination setting in Southern Province, Zambia.

Mosquito collections will be conducted concurrently with screening of individuals from households identified as part of the reactive test-and-treat strategies in Choma District, as described in Aim 1. Once notified of an index case, the study team will accompany the health care worker to the home for notification and geocoding of households, from which maps will be generated to allow for identification of households within 140 m of the index case. The following day, CDC light traps will be set in all sleeping structures within consenting index case households and next to animal enclosures within the index case household. Traps will also be set indoors and outdoors in consenting neighboring households within 140 m of the index household. For indoor collections, field staff will ensure that everyone sleeping inside the selected house is protected by an LLIN on the sampling night. Additional LLINs will be supplied if needed. A CDC light trap will be placed at 1.5 m above the floor at the foot end of the beds or sleeping areas, just outside of the LLIN. For outdoor collections, animal enclosures within the household will be identified and light traps hung next to the shelter at 1.5 m above the ground. Light traps will be run from 18:00 to 06:00 and will be collected the next day. Traps will be taken to the laboratory at Macha Research Trust, and the specimens killed by freezing and stored dry on silica until molecular analysis. For those households revisited at 30 days and at 90 days after the initial visit, light traps will be set in the same locations to monitor changes over time.

The study questionnaire administered at each household as described under Aim 1, will collect data on household factors such as construction, house occupancy and presence and use of vector control tools, which can be used to develop risk models for mosquito exposure. Additional data will be collected at the time of trap setting on possession of domestic animals.

Aim 4: Assess the utility of serosurveillance for monitoring and documenting elimination in a pre-elimination setting in Southern Province, Zambia.

Households will be randomly selected from satellite images for participation in the longitudinal cohort. In addition, households between the 140 and 250 m radii from active case detection linked to reactive test-and-treat will be included. In addition, households in 140 to 250 m radius from active case detection linked to reactive test-and-treat will be included. All residents of selected households are eligible to participate. As described in Aim 1, after informed consent and administration of a questionnaire, a dried blood spot will be collected.

6.2 Laboratory Evaluations

6.2.1 Laboratory Evaluations/Assays

Research Area A: Barriers to Malaria Control in Southern and Central Africa

Aim 1: Identify evolving temporal and spatial patterns and drivers of parasite prevalence, incidence and vector densities and guide targeted intervention strategies in moderate to high malaria transmission settings in Southern and Central Africa.

Quantitative PCR: After DNA extraction, *P. falciparum*, *P. malariae* and *pfhrp* genes will be detected by q-PCR using specific primer sets at Macha Research Trust as previously reported by the Southern Africa ICEMR. Primers for species-specific *cytb* will be used to detect *P. falciparum* and *P. malariae*, and primers for the detection of the *pfhrp* gene were designed to amplify the highly conserved secretary leader of *pfhrp*. The primers were designed to confirm the presence of *pfhrp2* or *pfhrp3* in a single reaction, with expected product sizes of 278 base pairs and 259 base pairs for *pfhrp2* and *pfhrp3*. Five µL of reaction mix containing 5 µL of SYBR® Green PCR Master Mix (ThermoFisher) and 400 nM primer will be added in duplicate to a 96-well plate along with 5 µL of ten-fold diluted DNA extract. Standards will be generated by serial dilution of genomic DNA. Ten-fold dilutions of laboratory-cultured 3D7 genomic DNA will be used as standards for *P. falciparum* and *pfhrp* gene detection, whereas *P. malariae* genomic DNA from a single infected individual will be used as a standard for detection of *P. malariae.* Reactions will be run in a StepOnePlus™ RealTime PCR System (Applied Biosystems). Absolute quantification will be used to determine gene copy numbers for the *P. falciparum* and *pfhrp* q-PCR assays based on the standard curve generated from the 3D7 dilutions.

Entomological laboratory procedures: Mosquitoes will be killed by freezing and sorted by genus and enumerated. Anophelines will be identified morphologically to sex and species using standard morphological keys and visually scored as blood fed. Samples will be stored dry on silica until processed for molecular analyses. *Molecular identification of mosquitoes*: A temporally and spatially representative subset of mosquitoes will undergo molecular confirmation of identities. DNA from mosquito abdomens will be extracted using a modified salt extraction method and PCR used for identification of *An. funestus* or *An. gambiae* sibling species, with amplicons visualized on 2.5% agarose gels. Those specimens for which no

amplification product is observed will be assessed with a PCR targeting the anopheline ribosomal DNA intergenic spacer 2 (ITS2), modified by Das et. a.l, to identify other species of anophelines. For all specimens found to be harboring malaria parasites, sequencing of the ITS2 and CO1 regions will be done to confirm morphological and PCR identifications. *Blood meal analysis*: All samples that undergo molecular species identification will also be analyzed by PCR for presence and identification of host blood using a multiplex PCR targeting the cytochrome b region of mitochondrial DNA and also a more sensitive PCR and restriction fragment length polymorphism (RFLP) assay. These data will be used to calculate blood feeding rates and the human blood index. *Detection of sporozoite positive mosquitoes and calculation of EIRs*: The head and thoraces of female anopheline mosquitoes will be homogenized and ELISAs run to detect *P. falciparum* circumsporozoite antigens. Homogenates of samples shown to be positive will be boiled for ten minutes to denature cross-reacting antigens which may result in false positives and re-analyzed using the same ELISA. Positive samples will also undergo PCR to detect *P. falciparum* from abdominal DNA extractions. Foraging rates will be calculated as the mean number of vectors caught per trap/night, and this will be multiplied by the proportion of mosquitoes found to be sporozoite positive by standard ELISA methods to estimate the EIR.

Aim 2: Identify persistent and emerging barriers to malaria control in moderate to high malaria transmission settings in Southern and Central Africa, including insecticide resistance, antimalarial drug resistance and cross-border malaria transmission.

Insecticide resistance assays: WHO insecticide resistance tests will be used to assess the susceptibility of wild-caught female anopheline mosquitoes to WHO diagnostic doses of pyrethroids (0.05% deltamethrin and 0.05% lambda-cyhalothrin), carbamates (0.1% bendiocarb and 0.1% propoxur), organochlorines (4.0% DDT and 4.0% dieldrin) and organophosphates (5.0% malathion, 1.0% fenitrothion and 0.25% pirimiphos-methyl). Each exposure will consist of ~25 randomly selected mosquitoes for the seven insecticides plus controls. Mortality will be recorded 24 hours post-exposure. A proportion of the mosquitoes will be brought to the laboratory of Maureen Coetzee at the University of Witwatersrand. First generation adults will be used for synergist and resistance intensity assays. Synergist assays will be performed by exposing mosquitoes to 4% piperonyl butoxide (PBO), an inhibitor of monooxygenases, for 1 h prior to exposing them to deltamethrin and bendiocarb. For resistance intensity assays, female anopheline mosquitoes will be exposed to 0.05% deltamethrin, 0.1% bendiocarb and 0.05% lambda-cyhalothrin treated papers continuously for 8 hours with knockdown recorded at various times.

Drug resistance genotyping: We will use a combination of approaches to increase our understanding of the impact of drug resistance on malaria control and elimination. The goals of these studies are to: 1) quantify the presence of mutations with the highest potential for impacting malaria control based upon drugs used for artemisinin-combination therapy (ACT) and IPTp at the field sites [Pfmdr1 (86Y, 184F, 1034C, 1042D, 1246Y), Pfcrt (76T, codon 72-76 haplotype), Pfdhfr (108N, 51I, 59R, 164L), Pfdhps (437G, 540E, 581G), K13 (propeller domain

SNPs)]; 2) assess the presence of additional mutations involved in resistance [Pfnhe1 (tandem repeats), Pfmrp1 (191H, 437S), Pfap2-mu (160N/T), and Pfubp1 (1528D)]; 3) explore mutations associated with artemisinin resistance backbones [fd (ferredoxin), arps10 (apicoplast ribosomal protein S10), mdr2 (multidrug resistance protein 2) and crt (chloroquine resistance transporter)]; and 4) conduct screens for novel mutations and changes in parasite populations which may impact drug efficacy using PoolSeq. Next generation sequencing will be done at the Parasite Genetics Core while other assays will be performed at the site laboratories or the Johns Hopkins Malaria Research Institute. Drug resistance testing will begin with assessment of resistance allele or haplotype frequency in the population using a novel pooled deep sequencing approach developed by the Parasite Genetics Core to rapidly assess the frequency of these alleles. Samples will undergo individual DNA extraction and pools will be created from the extracted DNA prior to deep sequencing. This method was validated against single infection Sanger sequencing, providing nearly identical results. To ensure high quality estimates of allele frequencies and provide data that is harmonized with sister ICEMR sites in Uganda for combined analysis, a proportion of pools will be assessed by individual Sanger sequencing. Amplicons will be bidirectionally sequenced using ABI Big Dye Terminator chemistry (Applied Biosystems). Chromatograms will be generated using Sequencher v4.8 (Gene Codes) and reads will be scored by visual inspection. We will use MalHaploFreq to convert allele prevalence into frequencies after incorporation of the multiplicity of infection, as determined by genotypes for merozoite surface protein 1 (*msp1*) and 2 (*msp2*) and defined as the largest number of either *msp1* or *msp2* alleles.

Several lessons have been learned from genomic studies of drug resistance in Asia: 1) artemisinin resistance can develop with multiple genetic origins; and 2) multiple different mutations can lead to changes in resistance to artemisinins and partner drugs. Both raise concerns for development of de novo resistance to ACTs in Africa. The Parasite Genetics Core previously explored mutations among African parasite isolates using a pooled deep sequencing approach and identified an array of mutations in the K13 propeller. However, few of these mutations were shared with Asian parasites, raising questions about whether these African K13 mutations are involved in artemisinin resistance. One of these mutations has now been proposed as a resistance determinant. Additional characterization of K13 alleles and selection pressures is critical in Southern and Central Africa to assess the risk of de novo development of artemisinin resistance.

To investigate temporal stability of parasites we will sequence dried blood spots from 2017-2024 to include PoolSeq spanning 7-year period. PoolSeq allows assessment of genome-wide selection in parasites using pooled DNA, significantly reducing the costs associated with scans for selection, and has already been successfully used to study malaria. We will normalize input DNA from each sample by quantifying the amount of parasite DNA using real-time PCR and generate a pool from each site for each year (with a minimum of 50 isolates per site). These will be hybrid-captured, preserving allele frequencies in the captured DNA, and deep sequenced. This approach will allow: 1) assessment of changes in K13 alleles or alleles associated with the

artemisinin resistance backbone; and 2) identify changes in allele frequencies of other alleles in the genome, potentially leading to the identification of novel resistance mechanisms associated with artemisinins.

Parasite genotyping: We will explore parasite genetic diversity at the individual- and population level using a novel amplicon deep sequencing approach and molecular inversion probes. For strain typing, DNA will be extracted from dried blood spots using a previously described Chelex extraction method. Following DNA extraction, we will amplify 500 bp of the ID1-DBL2x region of *var2CSA* using PCR and visualize on a 1% agarose gel to confirm. Invitrogen PureLink Quick PCR Purification Kit will be utilized for PCR cleanup and PicoGreen will be used to quantify amplicon concentrations in each sample. We will use the NEBNext® Ultra™ DNA Library Prep Kit for Illumina® to ligate unique barcodes to each sample. Equimolar amounts of barcoded amplicons will be pooled into a final library which will be quantified using the Agilent 2200 TapeStation screen tape assay. The library will be deep sequenced using the Illumina MiSeq to generate 300 bp forward and reverse reads. Parasite samples from elsewhere in the DRC and from Malawi currently stored in the Parasite Genetics Core will be used as controls. Based upon previous studies that indicate parasites from diverse geographical regions range from approximately 60-95% identity at the *var2CSA* locus, we conservatively estimate that highly related parasites will be 90% identical at the nucleotide sequence level (sd 10), and more distantly related parasites will be 85% identical (sd 10). Under these parameters, we will need to sequence 63 parasites from each population to achieve 80% power, α=.05. The sequencing of this highly polymorphic marker will enable us to assess the genetic relatedness of parasites from Nchelenge District and the DRC and from Zimbabwe and Mozambique and determine the extent of parasite interbreeding and partitioning in space, in light of the substantial cross-border human population movements.

Aim 3: Evaluate and optimize combinations of vector control and drug-based malaria control interventions in moderate to high malaria transmission settings in Southern and Central Africa in collaboration with implementing partners.

Parasite genotyping: As described for Aim 2.

Research Area B: Intractable Malaria Vector Populations and Residual Transmission in Southern and Central Africa

Aim 1: Define and extrapolate risk of vector exposure in the context of ongoing programmatic control at a high transmission setting along the northern Zambia/Democratic Republic of Congo border and at a low transmission setting in southern Zambia.

All mosquitoes collected will be killed by freezing and sorted by genus. Counts of culicine mosquitoes will be recorded. All anophelines will be enumerated, identified to sex and species

using standard morphological keys and visually scored as blood fed. This will be done at the Tropical Disease Research Centre (TDRC), Ndola for Nchelenge District and DRC samples, and at Macha Research Trust (MRT) for Choma District samples. Samples will be stored dry on silica before conducting molecular analyses at Macha Research Trust.

Molecular identification of mosquitoes: A temporally and spatially representative subset of the collections will undergo molecular confirmation of identities. DNA from mosquito abdomens will be extracted using a modified salt extraction method, and PCR used for identification of *An. funestus* or *An. gambiae* sibling species. For specimens for which no amplification product is observed following these primary vector assays, a PCR targeting the anopheline ribosomal DNA intergenic spacer 2 (ITS2) and modified by Das et al. to identify other anophelines will be used. Sequencing of regions of ITS2 and CO1 will be done to confirm identifications for all specimens found to be harboring malaria parasites.

Blood meal analysis: All samples that undergo molecular species identification, will also be analyzed by PCR for presence and identification of host blood using a multiplex PCR targeting the *cytb* region of mtDNA and also a more sensitive PCR and restriction fragment length polymorphism (RFLP) assay. These data will be used to calculate blood feeding rates and the human blood index (proportion of blood feds that fed on humans) of anophelines caught.

Detection of sporozoite positive mosquitoes and calculation of EIRs: The head and thoraces of female anopheline mosquitoes will be homogenized and ELISAs run to detect *P. falciparum* circumsporozoite antigens. Homogenates of samples that are shown to be positive, will be boiled for ten minutes to denature any cross-reacting antigens which may result in false positives and re-analyzed using the same ELISA. Positive samples will also undergo PCR to detect *P. falciparum* from abdominal DNA extractions. Foraging rates will be calculated as the mean number of vectors caught per trap/night, and this will be multiplied by the proportion of mosquitoes found to be sporozoite positive by standard ELISA methods to estimate EIRs.

Aim 2: Identify entomological barriers to vector control in both low and high transmission settings in Zambia.

Mosquitoes collected from the variety of trapping methods will be processed for species identity, blood meal analyses, determination of infection rates and EIR. Identities of potential secondary vectors will be confirmed by sequencing techniques. For those species suspected as serving as secondary vectors, collections of resting adults and larvae within Choma District will be brought to the insectary at Macha Research Trust. Wild caught or emerging adults will be blood fed and allowed to oviposit in individual cups to produce a F1 generation. The identity of the individual ovipositing females will be confirmed molecularly. Cultured laboratory strains of chloroquine sensitive *P. falciparum* (NF54) will be used for infectious feeds of F1 females. Up to 50 female mosquitoes aged 2-7 days post emergence will be placed into several cups and starved of glucose. Warmed infected blood will be fed to mosquitoes via a membrane feeder placed on the

top of the cup. After feeding, fully fed mosquitoes will be carefully transferred into two double netted cages designed to prevent escape and which do not require internal manipulation. Glucose will be offered ad libitum through the cage top. After 6-9 days, mosquitoes in one cage will be killed for dissections to determine oocyst counts. The remaining mosquitoes will be killed after 14 days for circumsporozoite ELISAs to detect sporozoites and qPCR to confirm infection rates in mosquitoes.

Sorbent canisters containing odors trapped from volunteers will be sent to Johns Hopkins Bloomberg School of Public Health for TD-GC/MS analysis. Using this approach individual volatiles collected onto sorbent tubes will be thermally desorbed onto a GC column, and constituent volatiles identified using electron ionization-mass spectrometry. For confirmation of identity, mass spectra and retention times of detected compounds will be compared with those of commercially available standards.

Blood samples from volunteers for the attraction assays will be used to make thick and thin smears for detection of parasites, species determination and parasitemia estimates at MRT. DNA will be extracted from DBS using a chelex method and qPCR carried out as described under Research Area A. Briefly, primers for species-specific *cytb* will be used to detect *P. falciparum* and *P. malariae*. Quantification will be estimated by comparison of these products to a serial dilution of genomic DNA or plasmid. Reactions will be run in StepOnePlus™ RealTime PCR System (Applied Biosystems). Absolute quantification will be used to determine gene copy numbers for *P. falciparum* based on the standard curve generated from the 3D7 dilutions. To detect *P. falciparum* gametocyte mRNA, RNA will be extracted from DBS, cDNA will be made, and nested PCR will be performed to detect *Pfs25* transcripts. One circle of a 50 µL blood spot will be excised, placed in a 1.5 ml tube and the sample homogenized using Qiashredder (Qiagen). RNA will be recovered using RNeasy (Qiagen) and in-column DNAase digestion will be performed to remove genomic DNA. Prepared samples will be used for cDNA preparation using Omniscript RT Kit (Qiagen).

Aim 3: Utilize changes in parasite genetic diversity in vector mosquitoes as an indicator of successful vector control and reduction of transmission in high transmission settings in Zambia and the DRC.

Parasite genotyping: We will use a novel genotyping approach to explore parasite genetic diversity within (α)- and between (β) –individual mosquitoes. For strain typing, DNA will be extracted from mosquitoes using a previously described Chelex extraction method. Following DNA extraction, individual samples will be deep sequencing at the *var2csa* locus and using molecular inversion probes. Use of uniquely indexed PCR primers will allow us to retain individual mosquito level data in the context of pooled sequencing. The methods described here are identical for those used to evaluate genetic diversity of parasites from human samples in Research Area A and will enable us to draw inferences based on our observations in both human and vector populations.

Research Area C: Achieving, Sustaining and Documenting Malaria Elimination in Southern Province, Zambia

Aim 1: Optimize active and passive surveillance strategies to identify and characterize residual foci of transmission in a low transmission setting in Southern Province, Zambia.

Infection with *P. falciparum* will be determined using both RDTs and qPCR. Dried blood spots collected from study participants enrolled in active case detection linked to reactive test-and-treat will be used to detect *Plasmodium* DNA. *Quantitative PCR*: After DNA extraction, *P. falciparum*, *P. malariae* and *pfhrp* genes will be detected by q-PCR using specific primer sets at Macha Research Trust. Primers for species-specific *cytb* will be used to detect *P. falciparum* and *P. malariae*. 18S rRNA primers will be used to confirm species identification. Five µL of reaction mix containing 5 µL of 2x iQ™ SYBR® Green Supermix (Bio-Rad) and 400 nM primer will be added in duplicate to a 384-well plate along with 5 µL of ten-fold diluted DNA extract. Ten-fold dilutions of laboratory-cultured 3D7 genomic DNA will be used as standards for *P. falciparum* and *pfhrp* gene detection, whereas *P. malariae* genomic DNA from a single infected individual will be used as a standard for detection of *P. malariae.* Reactions will be run in a Bio-Rad CFX384™ real time thermo cycler. Absolute quantification will be used to determine gene copy numbers for the *P. falciparum* and *pfhrp* q-PCR assays based on the standard curve generated from the 3D7 dilutions.

Aim 2: Determine the risk of imported malaria and the contribution of the asymptomatic reservoir to focal transmission in a pre-elimination setting in Southern Province, Zambia.

Parasite genetics: DNA will be extracted from dried blood spots collected from study participants using a previously described Chelex extraction method. Following DNA extraction, 500 bp of the ID1-DBL2x region of *var2csa* will be PCR amplified using uniquely indexed primers, called MIDs, for multiplexing. Use of MID tags will ensure that we capture individual level data. Invitrogen PureLink Quick PCR Purification Kit will be used for PCR cleanup and PicoGreen will be used to quantify DNA concentrations in each sample. We will use the NEBNext® Ultra™ DNA Library Prep Kit for Illumina® for library preparation. Equal amounts of ID1-DBL2x amplicon from each sample will be pooled into a final library and quantified using the Agilent 2200 TapeStation screen tape assay. The library will be deep sequenced using the Illumina MiSeq to generate 300 bp forward and reverse reads. A recently developed parasite genotyping method using molecular inversion probes will also be used.

Aim 3: Quantify the role of secondary vectors in sustaining malaria transmission in a pre-elimination setting in Southern Province, Zambia.

Mosquitos will be identified morphologically to sex and anopheline species using standard morphological keys and visually scored as blood fed and enumerated. Anopheline specimens will undergo molecular confirmation of identities. DNA from mosquito abdomens will be extracted using a modified salt extraction method and PCR used for identification of *An. funestus* or *An. gambiae* sibling species, with amplicons visualized on agarose gels. Those specimens for which no amplification product is observed following either *An. funestus* or *An. gambiae* PCR, will be assessed with a PCR targeting the anopheline ribosomal DNA intergenic spacer 2 (ITS2, modified by Das et al.) to identify other species of anophelines, including potential secondary vectors.

Aim 4: Assess the utility of serosurveillance for monitoring and documenting elimination in a pre-elimination setting in Southern Province, Zambia.

Antibodies to whole asexual parasite (NF54) lysate will be measured by enzyme immunoassay using serum from capillary blood samples as previously reported by the Southern Arica ICEMR. Briefly, whole *P. falciparum* asexual stage antigens will be coated on Immulon 2HB flat-bottomed 96-well plates (Thermo, Rochester, New York) and incubated, washed and blocked with 5% skim milk. Eluted samples will be plated in triplicate and incubated with peroxidase-labeled goat anti-human IgG (KPL, Inc., Gaithersburg, Maryland) followed by ABTS solution (KPL). Absorbance will be measured at 405 nm using a plate reader (Biotek ELx800UV) and IgG levels will be expressed as the optical density. A threshold optical density (OD) value of 0.57 was previously established to distinguish seronegative from seropositive individuals based on the mean OD value plus three standard deviations from filter paper spotted with serum from ten individuals never exposed to malaria. Individuals with OD values above this threshold will be defined as seropositive.

6.2.2 Specimen Collection, Preparation, Handling and Shipping

6.2.2.1 Instructions for Specimen Preparation, Handling, and Storage

Specimen Collection, Preparation and Handling of Human Blood

Sample processing and storage will be standardized across the field sites. For parasite detection and genotyping, 5 to 10 spots of approximately 50 µL of blood will be collected on Whatman® 3MM filter paper cards. The cards will be dried overnight and stored individually in sealed plastic bags with a desiccant at -20^{o}C. All specimens will be labeled with a unique subject-specific study identification number and the date of collection. Specimen storage and tracking will be managed with FreezerWorks software at Macha Research Trust.

Specimen Collection, Preparation and Handling of Mosquitoes

Mosquitoes will be processed similarly regardless of collection method (light trap or spray catch). Following collection, specimens will be kept cool and returned to the local laboratory to be killed by freezing at -20^{o}C. Mosquitoes will be identified onsite by morphology and individually stored in numbered and site/date-coded microfuge tubes containing a small quantity of silica gel and cotton to desiccate and stabilize the specimen. Specimens will be split into head/thorax and abdomen, and parasite genomic DNA will be extracted separately from each half by modified salt extraction.16 DNA will only be extracted from abdomens if a blood meal is visible.

6.2.2.2 Specimen Shipment

The capacity to perform sophisticated parasite and vector genomic studies currently does not exist in Zambia or Zimbabwe, and these assays will be performed in the United States. Specimens shipped to the Johns Hopkins Bloomberg School of Public Health or the University of North Carolina will be dried blood spots, parasite DNA, and dried mosquitoes. Specimens are expected to be shipped several times per year for genomic assays. Mosquitoes also will be shipped periodically to the University of the Witwatersrand, Johannesburg, South Africa for assays of insecticide resistance.

When samples are to be shipped, material transfer agreements will be sought through the Medical Research Council of Zimbabwe and the Ministry of Health and National Health Research Authority in Zambia, with full justification of the need for samples to be shipped out of the country.

7 STATISTICAL CONSIDERATIONS

7.1 Study Outcome Measures

Research Area A: Barriers to Malaria Control in Southern and Central Africa

Aim 1: Identify evolving temporal and spatial patterns and drivers of parasite prevalence, incidence and vector densities and guide targeted intervention strategies in moderate to high malaria transmission settings in Southern and Central Africa.

The primary outcomes will be parasite prevalence as measured by RDT and qPCR and numbers of female vector mosquitoes captured per night by indoor CDC lights traps through active case detection, and estimated malaria incidence through passive case detection using reported or calculated health center catchment areas as denominators. The parasite prevalence, monthly incidence and vector counts will be used to track temporal and spatial patterns in malaria transmission, which will be compared within and between study sites and transmission settings. Additional descriptive entomological outcomes include blood feeding rates, human blood index, and entomological inoculation rates, and further studies related to vector bionomics are described in Project B Transmission I.

Aim 2: Identify persistent and emerging barriers to malaria control in moderate to high malaria transmission settings in Southern and Central Africa, including insecticide resistance, antimalarial drug resistance and cross-border malaria transmission.

The outcome measures include: 1) prevalence and mechanisms of insecticide resistance in Nchelenge District, Zambia; 2) prevalence and associations of antimalarial drug resistance genotypes; and 3) estimates of degree of parasite genetic relatedness and association between genetic diversity and their sampling locations in Nchelenge District, Zambia and eastern DRC and in residents of both Zimbabwe and Mozambique with confirmed malaria at health centers in Mutasa District.

Aim 3: Evaluate and optimize combinations of vector control and drug-based malaria control interventions in moderate to high malaria transmission settings in Southern and Central Africa in collaboration with implementing partners.

The primary outcome measures will be changes in parasite prevalence, incidence and mosquito counts before and after interventions such as IRS or mass drug administration campaigns. Additionally, to assess the efficacy of various control programs, we will compare the frequency

of polyclonal infections and unique *var2CSA* variants between regions in which interventions were implemented or not, as well as within the same region pre and post intervention.

Research Area B: Intractable Malaria Vector Populations and Residual Transmission in Southern and Central Africa

Aim 1: Define and extrapolate risk of vector exposure in the context of ongoing programmatic control at a high transmission setting along the northern Zambia/Democratic Republic of Congo border and at a low transmission setting in southern Zambia.

The primary outcomes will be mean household vector counts from indoor collections and mean catch of vectors from outdoor collections. These will be used to create risk models and maps, for studying associations with remotely sensed and climatic data, and to assess the impact of control. Questionnaire data collected at households will be used for the quantification of time spent in the evenings and night outside of bed nets prior to by different age groups. This will be used to guide the location complementary collection tools for mosquitoes and be used to model human-vector exposure. Secondary outcomes will be species composition both from indoor and outdoor collections, blood feeding rates and host preference of various species, infectivity rates of female anophelines caught and determination of EIR.

Aim 2: Identify entomological barriers to vector control in both low and high transmission settings in Zambia.

The primary outcome is to identify barriers to vector control. Spatial and temporal patterns of malaria vector mosquitoes and entomological risk maps created under Aim 1, will be overlaid with presence of vector control to identify whether the targeting of interventions is appropriate. The mean trap catch from collections targeted to where people are most active between the hours of dusk and dawn will be overlaid with human activity by age groups to quantify human-vector exposure and determine the extent of protection offered by use of nets. To assess the role of secondary vectors, counts of mosquito species by collection type will be determined, and foraging rates, host preferences, and infectivity with *P. falciparum* used to assess their role in transmission. Following experimental *P. falciparum* infections of field collected mosquitoes in the laboratory, the proportion of mosquitoes found with oocysts and sporozoites will be used to assess competence of species. Outcomes for the mosquito attraction study will be the relative proportion of mosquitoes attracted to one person or bait vs. another in a choice experiment.

Aim 3: Utilize changes in parasite genetic diversity in vector mosquitoes as an indicator of successful vector control and reduction of transmission in high transmission settings in Zambia and the DRC.

The identity and relative frequency of *var2CSA* sequence and molecular inversion probe variants will be the primary outcome variables. These will be processed and analyzed identically

to variants identified from human samples in Research Area A. The data will be therefore directly comparable within and across Research Areas. Diversity of *var2CSA* variants will be compared spatially, from within and outside intervention zones (i.e. IRS sprayed vs. unsprayed), and temporally, from year to year and season to season.

Research Area C: Achieving, Sustaining and Documenting Malaria Elimination in Southern Province, Zambia

Aim 1: Optimize active and passive surveillance strategies to identify and characterize residual foci of transmission in a low transmission setting in Southern Province, Zambia.

Residual foci of transmission will be identified through active and passive case detection and their frequency, magnitude and spatial and ecological characteristics will be measured and mapped.

Aim 2: Determine the risk of imported malaria and the contribution of the asymptomatic reservoir to focal transmission in a pre-elimination setting in Southern Province, Zambia.

Outcome variables will include the frequency of imported malaria cases, the proportion of secondary cases related to the index case, and the proportion of active case detection cases which represent the asymptomatic reservoir. Additionally, we will test for and estimate the degree of association between phylogeny and the pattern of the spatial structure of the parasite genotypes and infer phylogenetic relationships between symptomatic and asymptomatic parasite populations.

Aim 3: Quantify the role of secondary vectors in sustaining malaria transmission in a pre-elimination setting in Southern Province, Zambia.

The primary outcome will be household vector counts from indoor mosquito collections and mean catch of vectors from outdoor collections.

Aim 4: Assess the utility of serosurveillance for monitoring and documenting elimination in a pre-elimination setting in Southern Province, Zambia.

Our primary interest is to identify changes in age-specific seroprevalence and to estimate seroconversion rates as transmission declines and elimination goals are achieved.

7.2 Sample Size Considerations

Community-based cross-sectional surveys

We anticipate we will enroll approximately 600 individuals per year over seven years in Nchelenge District, 400 individuals per year in Mutasa District, and 1000 per year in the Democratic Republic of Congo. For the cross-sectional serosurveys in Choma District, we plan to enroll approximately 30 households per month, comprising approximately 150 residents per month, for three months. The total sample size is expected to be 450 participants. For the longitudinal cohort in Choma District, we plan to include 160 households with approximately five residents per household for a total of 800 participants followed for up to two years. As transient visitors to selected households will also be included, the sample size might be slightly higher.

7.3 Participant Enrollment and Follow-Up

Community-Based Surveys

Participants in the cross-sectional surveys will be enrolled once. Participants in the longitudinal cohort in Choma District will be followed monthly for up to two years, for a maximum of 24 study visits.

Health Center Surveillance

We will record the number of malaria cases at each participating clinic in each study area.

At Mapanza Rural Health Center, we will enroll individuals with confirmed malaria.

Hospital Surveillance

We will record the number of hospitalized malaria cases at St. Paul's Hospital in Nchelenge District to assess changes in the incidence of severe malaria as malaria transmission decreases. This will entail review of medical records and will not involve contact with patients. Personal identifiers will not be collected.

7.4 Analysis Plan

Research Area A: Barriers to Malaria Control in Southern and Central Africa

Aim 1: Identify evolving temporal and spatial patterns and drivers of parasite prevalence, incidence and vector densities and guide targeted intervention strategies in moderate to high malaria transmission settings in Southern and Central Africa.

Statistical models: Several epidemiological metrics will be used to monitor and evaluate malaria control interventions including: parasite prevalence determined by serial cross-sectional household surveys; mosquito counts through CDC light traps and malaria incidence determined by passive case detection. A quasi-experimental design will be used to allow changes in these metrics to be evaluated by: 1) comparing the average metric values pre-intervention period to the observed value post-intervention to obtain a relative percent change; 2) using time-series analyses to compare observed post-intervention values to predicted post-intervention values, assuming a continuation of pre-intervention trends (estimated counterfactual). The impact of interventions on the metrics will be estimated by computing risk ratios with corresponding 95% confidence intervals. In both cases, models will adjust for clustering (in health facilities for the passive case detection and in households for the active case detection and mosquito counts) and possible confounding of the intervention's effect by environmental variables and seasonality.

Parasite genotyping: MiSeq raw sequences will be filtered for quality control and clustered according to the SeekDeep bioinformatics pipeline. SeekDeep de-multiplexes by barcode and MID tag and compares individual- and population level diversity based on k-mer distances (http://baileylab.umassmed.edu/SeekDeep/). The final output yields consensus sequences and their relative frequencies for each individual. We anticipate that lower polyclonal infections and fewer unique variants will be observed in sprayed regions compared with unsprayed regions, reflecting declined transmission and reduced opportunities for recombination at the var2CSA locus.

Aim 2: Identify persistent and emerging barriers to malaria control in moderate to high malaria transmission settings in Southern and Central Africa, including insecticide resistance, antimalarial drug resistance and cross-border malaria transmission.

Insecticide resistance: The proportion of mosquitoes that die at 24 hours after exposure to each insecticide will be calculated with 95% confidence intervals. Resistance intensity assay data will be compared using one-way ANOVA and variations in mortality in the synergist assay will be tested by chi-square.

Drug resistance genotyping: Differences in the relative distributions of resistance markers will be analyzed: 1) temporally within a given site; and 2) geographically among different sites with special attention paid to between-site variation in, e.g., transmission intensity, prevalence of polyclonal infections, control interventions, malaria vector species, choice of first-line antimalarial agent, malaria- and mosquito-specific serologic results, rainfall and other weather variables, and cross-border movement. We will investigate potential factors that are protective and predictive of the emergence of drug resistance by using pair-wise comparisons to compute differences in proportions and applying linear models to test our hypothesis that changes in the distribution of molecular markers of drug resistance will vary both through time, and among geographic and other epidemiologic features.

Parasite genotyping: MiSeq raw sequences will be filtered for quality control and clustered according to the SeekDeep bioinformatics pipeline. SeekDeep de-multiplexes by barcode, and compares α and β-diversity (see Parasite Genetics Core) based on k-mer distances (http://baileylab.umassmed.edu/SeekDeep/). The final output will yield consensus sequences and their relative frequencies for each individual. *var2CSA* consensus sequences will be further used to infer the degree of parasite spatial population structure within a Bayesian framework implemented in BEAST. We will test for association between phylogenetic relationships of the parasites and their sampling locations, using Bayesian Tip-association Significance (BaTS). We will also use multivariate approaches to test signature of spatial clustering (i.e. principal component analysis and discriminant analysis of principal components of pairwise distance matrices implemented in R-statistical environment). To further characterize the movement of parasite haplotypes, Mantel tests will be performed. Mantel tests will allow us to understand what environmental networks acts as corridors to gene flow among malaria parasites in the field site.

Aim 3: Evaluate and optimize combinations of vector control and drug-based malaria control interventions in moderate to high malaria transmission settings in Southern and Central Africa in collaboration with implementing partners.

Measures of weather and climate: Weather patterns will be analyzed with respect to El Nino and global warming to assess the impact of interventions. MODIS (USGS) linked with precipitation data from The Climate Hazards Group Infrared Precipitation with Stations (CHIRPS), a collaboration between University of California, Santa Barbara's Climate Hazards Group and USGS's Earth Resources Observation and Science (EROS) that links rainfall data observed from space with more than three decades of rainfall data collected at ground stations worldwide. Precipitation data will be validated by weather monitors from the Global Surface Summary of the Day (GSOD, National Oceanic and Atmospheric Administration).

Parasite genetics: To evaluate the efficacy of IRS in Nchelenge District, parasites from individuals randomly sampled from sprayed and unsprayed grids will be deep sequenced at the *var2CSA* locus and using molecular inversion probes. Similarly, to evaluate the efficacy of a mass drug administration, we will randomly sample individuals from grids before and after the campaign. Analysis of parasites extracted from these samples will enable us to assess the effect of the intervention on the frequency of polyclonal infections and number of unique *var2CSA* variants circulating.

Temporal trends: To evaluate temporal trends in parasite prevalence as measured by RDT and qPCR, logit-link, log binomial regression models based on the generalized estimating equation (GEE) approach will be implemented. The proposed GEE approach accounts for the correlation between repeated measures on the same individual. Age, sex, wealth index and other potential variables of interest detected during the univariate analyses will be considered for the adjusted

models. Crude and adjusted results from the log-binomial regression models will be expressed as prevalence rate ratios (RR). Using the passive case detection data from health centers and estimates of the population size in each catchment area, monthly malaria case counts at each health center will be standardized as rates per 1,000 population. To evaluate changes in malaria incidence rates and vector counts, random effects Poisson regression models with adjustments for health center level clustering will be used. In the event of over-dispersion beyond that generated by random effects Poisson, negative binomial regression models, also with random effects for center-level clustering, will be used. To account for possible confounding, additional regression analyses will adjust for seasonality and environmental variables such as temperature, rainfall, elevation and proximity to different order streams. Time-varying environmental variables will be treated with time lags ranging up to 3 months to account for possible lag time in their effect on the outcome. Results will be expressed as incidence rate ratios with 95% confidence intervals.

Spatial risk models: To generate, refine and extend the spatial risk models, environmental variables will be generated for each study area and integrated into ArcGIS version 10.3 (ESRI ArcGIS, Redlands, CA). A digital elevation model (DEM) with 90-meter resolution will be obtained from the Shuttle Radar Topography Mission (SRTM) version 3 for each study area, in which each pixel represents a 90-meter average elevation around each pixel's center. The DEM will be processed in ERDAS Imagine 2015 software (Hexagon Geospatial, Norcross, Georgia) and imported into ArcGIS. The ArcHydro Tools module of ArcGIS, based on elevation and degree of slope derived from the SRTM image to determine water flow direction and accumulation, will be used to build stream networks with corresponding Strahler stream classification. The Strahler classification assigns ordered values 1, 2, 3, etc. based upon the hierarchy of tributaries, with the beginning of each stream or river segment a first order or category 1 stream. Category 2 streams are formed when two category 1 streams come together, category 3 streams formed when two category 2 streams come together and so on. Population density will be calculated as the sum of enumerated structures located within 500 meters of a study household. The distance from each enrolled household to the nearest road, nearest health facility and specific geographical features (e.g. international borders and Lake Mweru) will also be calculated. The normalized difference vegetation index (NDVI) was used to assess ground cover. NDVI is derived from LandSat 8 OLI (Operational LandImager) and Moderate Resolution Imaging Spectroradiometer (MODIS) from the US Geological Survey (USGS) Land Processes Distributed Active Archive Center (LP DAAC). Values for NDVI range from -1 to +1. Negative values represent bodies of water, values near zero represent asphalt, and increasing values correspond to increasing abundance of actively photosynthesizing vegetation or "greenness". Rainfall data from a weather-monitoring tool will be used to generate a variable for season, including historical data and the HOBO Micro Station (Onset Computer Corporation, Bourne, MA). The HOBO Micro Station is a four-sensor data logger designed to take measurements of rainfall, temperature and relative humidity, and wind speed at hourly intervals. One HOBO Micro Station was placed in Nchelenge District, Zambia and two in Mutasa District, Zimbabwe at low and high elevation.

Research Area B: Intractable Malaria Vector Populations and Residual Transmission in Southern and Central Africa

Aim 1: Define and extrapolate risk of vector exposure in the context of ongoing programmatic control at a high transmission setting along the northern Zambia/Democratic Republic of Congo border and at a low transmission setting in southern Zambia.

Vector risk maps: Risk maps will be generated for household catches for the two major vectors, *An. funestus* and *An. gambiae* as described above for Research Area A. SaTScanTM software using Bernoulli and Poisson spatial models will be used to detect clusters of the presence of vector mosquitoes, and high vector counts for both indoor and outdoor catches. Clusters can overlap spatially but will be restricted to having no cluster centers inside other clusters. Statistically significant clusters will be mapped in ArcGIS; spatial overlap and common ecological and household features of detected clusters between seasons will be assessed. Where vectors are present for indoor and outdoor catches a Poisson spatial model will be used to detect clusters of high and low vector counts. The same restrictions and mapping procedures will be conducted as the Bernoulli spatial model.

Aim 2: Identify entomological barriers to vector control in both low and high transmission settings in Zambia.

Intervention coverage: The number of ITNs provided to each RHC for community distribution by the district control officer will be collected and coverage of IRS will be collected from implementing partners. Coverage levels of ITNs will be estimated for each RHC catchment area using the number provided for the RHC catchment population and the number of households within each RHC catchment population. These coverage levels will be compared to the self-reported ITN ownership and use, and IRS reports captured through active field surveys. Coverage levels maps will be created as spatial-area level choropleth maps. The cluster detection maps created in Aim 1 will be overlaid on intervention coverage maps to determine the percent of the distribution of interventions within identified clusters of vectors. This will be done with the clusters of vector presence and the clusters of high vector counts.

Vector exposure: To determine whether certain sectors of the community are at greater risk of exposure to malaria vectors than others, the mean catch of vectors trapped from each collection will be used to calculate man biting rates for each hour. The potential exposure of individuals to these vectors will then be estimated using individual community responses to the behavioral questionnaires. Human exposure to vectors and the true protective efficacy of bed nets will be calculated using previously described methods.

Attractiveness of individuals: The mean response rate of mosquitoes to each volunteers in the dual-choice assays will be calculated as the mean proportion (%) of released females captured

in each trap following each assay of paired ‘hot’ and ‘cold’ individuals. Mean catch sizes for ‘hot’ and ‘cold’ volunteers will be estimated. Independent effects of ‘hot/cold’ individuals, temperature, humidity and wind speed on catch size will be tested using a generalized linear model with log link function and negative binomial distribution. If differences in attractiveness between these two treatment groups are observed, GC arrays (heat maps) constructed from TD-GC/MS data will be used to visualize changes in the patterns of volatiles produced by individuals from ‘hot’ and ‘cold’ households. Principal component analyses will be further used to identify individual volatile organic compounds or clusters of volatiles associated with human attractiveness to mosquitoes or malaria infection.

Aim 3: Utilize changes in parasite genetic diversity in vector mosquitoes as an indicator of successful vector control and reduction of transmission in high transmission settings in Zambia and the DRC.

MiSeq raw sequences will be filtered for quality control and clustered according to the SeekDeep bioinformatics pipeline. SeekDeep de-multiplexes by barcode and compares α and β-diversity in mosquito based on k-mer distances. The output yields var2CSA variant consensus sequences and their relative frequencies for each mosquito. We anticipate that lower polyclonal infections and fewer unique variants will be observed in mosquitoes from areas where control interventions (IRS or ITNs) have had greater impact on parasite transmission, reflecting declined transmission and reduced opportunities for recombination at the var2CSA locus in the mosquito midgut. Polyclonal infections may reduce at a slower rate if interventions are sustained for multiple years or seasons, or alternatively with adverse environmental conditions such as drought that may reduce transmission. var2CSA consensus sequences will be additionally used to infer the degree if genetic relatedness within a Bayesian framework implemented in BEAST. We will test for association between phylogenetic relationships of the parasites and their sampling locations, using Bayesian Tip-association Significance (BaTS). We will also use multivariate approaches to further test signature of level-population spatial clustering (i.e. principal component analysis and discriminant analysis of principal components of pairwise distance matrices implemented in R).

Research Area C: Achieving, Sustaining and Documenting Malaria Elimination in Southern Province, Zambia

Aim 1: Optimize active and passive surveillance strategies to identify and characterize residual foci of transmission in a low transmission setting in Southern Province, Zambia.

Spatial cluster detection: SaTScan™ software using a Bernoulli spatial model will be used to detect clusters of index cases and clusters of cases in neighboring households. Clusters can overlap spatially but will be restricted to having no cluster centroids inside other clusters. The maximum cluster size will be set to be less than or equal to 25% of the total population at risk to

capture the micro-epidemiology of malaria transmission. Statistically significant clusters will be mapped in ArcGIS and spatial overlap and common ecological features of detected clusters between seasons will be assessed. The cross K-function will be estimated to determine the degree to which malaria cases in neighboring households spatially cluster around index case households, relative to what would be expected under the hypotheses of spatial independence. Cross K-function results will also provide an estimate of the distance from which index case households and neighboring cases become spatially independent.

Patterns of passively detected cases through health center reports: The population served by each of the 14 health centers will be estimated in GIS by calculating contiguous, distance-based Thiessen (Voronoi) polygons and determining the number of household structures within each polygon with satellite imagery and estimates of the distribution of household size. Malaria incidence per household will be calculated and mapped for each season. Spatial patterns in malaria incidence will be explored by comparing the incidence in each health center and their first and second order nearest neighbors. Common ecological features among the populations served by each of the health centers will be compared to determine areas at elevated risk for clinical malaria.

Aim 2: Determine the risk of imported malaria and the contribution of the asymptomatic reservoir to focal transmission in a pre-elimination setting in Southern Province, Zambia.

Parasite genetics: MiSeq raw sequences will be filtered for quality control, de-multiplexed by MID tags and clustered using the k-mer distances implemented in the SeekDeep bioinformatics pipeline (http://baileylab.umassmed.edu/SeekDeep/). Our first analysis will focus on comparing index cases to secondary cases and assessing genetic relatedness. We will compare percent nucleotide identity of *var2CSA* consensus sequences calculated with SeekDeep from individual index and secondary cases to test for genetic relatedness. Parasites which are 80% or more identical will be considered clones; parasites less than 70% identical will be considered distinct clones. To confirm importation, we will relate the identified parasite variants to reported travel history if available, and compare our data to controls from Malawi and the DRC. Sensitivity analyses will be conducted using different cut-offs. Cases with a recent travel history who present clones which are less than 60% identical by nucleotide sequence will be considered imported cases. Secondary cases unrelated to the index case with no history of recent travel will be considered the asymptomatic reservoir. We will calculate the frequency which secondary cases are related to the index case, as well as the frequency of imported cases and the frequency of asymptomatic reservoir cases. *var2csa* consensus sequences will be further used to test for association between phylogenetic relationships of the parasites and their sampling locations, using Bayesian Tip-association Significance (BaTS). Additionally, we will perform linear regression to test for an association between percent identity between index and secondary cases and the geographic distance from the index household after controlling for environmental factors, mosquito abundance, and socio-demographic characteristics. Our second analysis will focus on assessing whether the parasite populations from symptomatic and

asymptomatic cases are intermixing. *var2csa* consensus sequences will be used to infer the degree of parasite population structure within a Bayesian framework implemented in BEAST. We will also use multivariate approaches (i.e. principal component analysis and discriminant analysis of principal components-DAPC of pairwise distance matrices implemented in R-statistical environment) to further test signature of genetic clustering, and specifically whether genetic variation is partitioned into a between- group and a within-group component. DAPC yields synthetic variables which maximize genetic differentiation between groups (pre-defined groups, symptomatic vs asymptomatic cases) while overlooking within-group variation

Aim 3: Quantify the role of secondary vectors in sustaining malaria transmission in a pre-elimination setting in Southern Province, Zambia.

The association of household features with vector counts will be modeled using a multivariable, zero inflated negative binomial regression model with a log-link. Semivariogram plots based on regression standardized residuals will be used to determine residual spatial variation. Seasonal maps of vector presence will be generated. As described under Aim 1, SaTScan™ software using a Bernoulli spatial model will be used to detect clusters of the presence of vector mosquitoes for both indoor and outdoor catches. Clusters can overlap spatially but will be restricted to having no cluster centers inside other clusters. Statistically significant clusters will be mapped in ArcGIS, and spatial overlap and common ecological and household features of detected clusters between seasons will be assessed.

Aim 4: Assess the utility of serosurveillance for monitoring and documenting elimination in a pre-elimination setting in Southern Province, Zambia.

Estimated seroconversion rates will be obtained by fitting reverse catalytic model to the observed seroprevalence. With these models, the mean annual rate of conversion to seropositive, λ, and the mean annual rate of reversion from seropositive to seronegative, ρ, will be estimated by using standard maximum likelihood and assuming a binomial error distribution. Previous analysis showed that antibody responses to whole parasite lysates are long lived, and therefore we expect reversion rates to be close to 0. We will focus on fitting multiple models to explore if λ changes throughout the study period by allowing time-varying λs and will compare competing models using likelihood ratio tests.

8 SUBJECT CONFIDENTIALITY

All samples and records will be assigned matching unique code numbers (refer to Section 6.2). Samples and records will be identified by code number only, and documentation linking those code numbers with specific individuals will be stored in limited access, locked file cabinets or electronic databases in the offices of the site research team. There would be no direct identifiers, e.g. - subject name, address, social security, etc. will not be available to the research staff. All subjects' data and samples will be encoded before the samples are to be used by the research team, participation investigators/study teams either at the site or upon shipment to the USA as well as the sponsor and their agents.

This confidentiality is extended to cover testing of biological samples and parasite genetic tests in addition to the clinical information relating to participating subjects.

No information concerning the study (study protocol, documentation) or the data will be released to any unauthorized third party without prior written approval of the local IRB/IECs and of the sponsor.

The clinical study site will permit access to all documents and records that may require inspection by the sponsor or its authorized representatives, including but not limited to, medical records (office, clinic or hospital) and pharmacy records for the subjects in this study.

8.1 Future Use of Stored Specimens

Specimens and data collected from participants who provide specific informed consent agreeing to future use of specimens and identifiable data will be stored for the duration of the study but a biorepository will not be created. The duration of the study is expected to be ten years, including three years after the end of the current grant funding. Specimens will either be destroyed or transferred to another study. Specimens will be labeled with a unique identification number (as above) and will be stored at secure limited-access project laboratories (Macha Research Trust for Choma District, Tropical Diseases Research Centre for Nchelenge District and Biomedical Research and Training Institute for Mutasa District). Blood samples, mosquitoes and *Plasmodium* parasites will be stored at -80^{o}C in freezers with back-up generators. Samples shipped to the Johns Hopkins Bloomberg School of Public Health, the University of North Carolina, and the University of the Witwatersrand will be stored in -80^{o}C freezers in limited-access secure laboratories.

9 INFORMED CONSENT PROCESS

Community-based surveys

The informed consent process for the community-based surveys will consist of two phases: permission from local community leaders and individual written informed consent. Consent will be obtained in a hierarchical manner according to the communal nature of the consent process within these communities. Verbal permission from the chief of the study areas will be sought through a meeting with senior study staff. Verbal permission from the headman responsible for the study households will then be sought, also through meetings with senior study staff.

For individual informed consent, study field staff will visit households over two days. All staff with participant contact will be trained in human subjects research. The visit on the first day will involve informing participants of the next day's visit and collecting the mosquito specimens using spray catches or light traps. Verbal permission for the mosquito collections will be obtained from the head of the household or responsible adult household member. On the second day, written informed consent will be obtained from each study participant. Consent will be obtained from individuals 16 years of age or older. Consent forms will be translated into local languages and read to the participants and caretakers in a language understandable by them. Participants and caretakers will provide consent by signing their name or leaving a thumbprint. A copy of the consent form will be provided to the participant or caretaker.

Hospital and clinic-based studies

For the hospital and clinic-based surveillance, we will collect routine, unidentifiable surveillance data for each confirmed case of malaria (date, age, sex, village and how diagnosis was confirmed) from the medical records. Used, positive RDTs will be collected from 14 rural health centers in the catchment area of Macha Mission Hospital in Choma District and will be labeled with the age and sex of the patient, the date, and whether the individual traveled outside the district within the past month. We will not collect names and consent will be waived by the IRBs.

9.1 Informed Consent/Assent Process (in Case of a Minor or Others Unable to Consent for Themselves)

Consent from parents or guardians will be obtained for children younger than 16 years of age. For the clinic-based studies, children who are age 7 years of age or older will be asked to provide assent in addition to the parental or guardian consent. Assent will be sought from these children as there is no prospect of direct benefit for the child and antimalarial treatment will be provided as part of clinical care. Assent will not be obtained as part of the community-based surveys because the study procedures are not greater than minimal risk and there is a prospect of direct benefit as some study children may be diagnosed with malaria and offered treatment.