RANDOMIZED, OPEN-LABEL CONTROLLED TRIAL OF DAILY TRIMETHOPRIM-SULFAMETHOXAZOLE OR WEEKLY CHLOROQUINE AMONG ADULTS ON ANTIRETROVIRAL THERAPY IN MALAWI

DAIDS Protocol Number: DAIDS-ES 10822
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Principal Investigator: Miriam K. Laufer, M.D., M.P.H.

DAIDS Medical Officer: Trinh T. Ly, MD

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Statement of Compliance

The study described in this protocol will be conducted according to current Good Clinical Practices (US 21 CFR Part 50-Protection of Human Subjects and Part 56-Institutional Review Boards, U.S. 45 CFR 46, 21 CFR 312, ICH E6; 62 Federal Register 25691 (1997), the NIH terms of the award, and the applicable rules and regulations of Malawi).

The University of Malawi College of Medicine IRB (FWA000011868) will review and approve the protocol prior to study start. In addition, the University of Maryland will review the study. The Michigan State University IRB will defer to the review and approval process of the University of Maryland. Documentation of the approval by these bodies will be kept in the Principal Investigator's study file.

All key personnel (all individuals responsible for the design and conduct of this study) have completed Human Subjects Protection Training.

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 U.S. federal regulations and ICH guidelines.

Principal Investigator:

Signed: _____ Date: _____

Miriam K. Laufer, M.D., M.P.H.
Associate Professor of Pediatrics

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LIST OF ACRONYMS

ACT	Artemisinin-based combination therapy for malaria
AE	Adverse Event/Adverse Experience
ART	Anti-retroviral therapy
BMP	Blantyre Malaria Project
CFR	Code of Federal Regulations
CICERO	Collaborative Institutional Comprehensive Evaluation of Research Online
CIOMS	Council for International Organizations of Medical Sciences
COMREC	University of Malawi College of Medicine Research and Ethics Committee
CQ	Chloroquine
CRF	Case Record Form
DAIDS	Division of Acquired Immunodeficiency Syndrome
DHFR	Dihydrofolate reductase
DHO	District Health Officer
DHPS	Dihydropteroate synthase
DSMB	Data Safety and Monitoring Board
EAE	Expedited Adverse Event
EC	Ethics Committee
FDA	Food and Drug Administration
FWA	Federal-Wide Assurance
GCP	Good Clinical Practice
ICF	Informed Consent Form
ICH	International Conference on Harmonisation
ICMJE	International Committee of Medical Journal Editors
IDES	Internet Data Entry System
IPT	Intermittent Preventive Therapy
IRB	Institutional Review Board
ISM	Independent Safety Monitor
MLW	Malawi-Liverpool-Wellcome Trust Clinical Research Programme
N	Number (typically refers to subjects)
NIAID	National Institute of Allergy and Infectious Diseases, NIH
NIH	National Institutes of Health
NTS	Non-typhoid *Salmonella*

OHRP	Office for Human Research Protections
PCP	*Pneumocystis jirovecii* pneumonia
PCR	Polymerase Chain Reaction
PfCRT	*Plasmodium falciparum* chloroquine resistance transporter
PD	Protocol deviation
PI	Principal Investigator
PID	Participant identification number
PLHIV	People Living with HIV
PRO	Protocol Registration Office
QA	Quality Assurance
QC	Quality Control
RSC	Regulatory Support Center
SADR	Suspected Adverse Drug Reaction
SAE	Serious Adverse Event/Serious Adverse Experience
SOP	Standard Operating Procedure
SP	Sulfadoxine-pyrimethamine
SPNS	Special Programs of National Significance
TB	Tuberculosis
TS	Trimethoprim-sulfamethoxazole
UNICEF	United Nations Children's Fund
UP	Unanticipated Problem
WHO	World Health Organization

PROTOCOL SUMMARY

Full Title: Randomized, open-label controlled trial of daily trimethoprim-sulfamethoxazole or weekly chloroquine among adults on antiretroviral therapy in Malawi

Short Title: TSCQ Malawi

Clinical Phase: III

Principal Investigator: Miriam K. Laufer, MD, MPH

Sample Size: Up to 1500 (maximum of 500 in each of three study arms)

Population: Malawian adults aged 18 years or older living with HIV in Blantyre, Malawi, Central Africa on TS prophylaxis who have been receiving antiretroviral therapy for $\geq$6 months and have a CD4 count $\geq$250/mm^3 and undetectable HIV viral load.

Participating Sites: Blantyre Malaria Project Research Clinic and Tisungane Clinic at Zomba Central Hospital

Study Design: This is a randomized controlled, open label, phase III trial of continued standard of care prophylaxis with daily trimethoprim sulfamethoxazole (TS) compared to discontinuation of standard of care TS prophylaxis and starting weekly chloroquine (CQ) prophylaxis or discontinuation of standard of care TS prophylaxis. Up to 1500 HIV-infected adults who have been receiving antiretroviral therapy (ART) for at least six months will be enrolled. They will be followed every 4-12 weeks (every 4 weeks for the first 24 weeks, then every 12 weeks thereafter) to determine if prophylaxis with TS or CQ is associated with improved morbidity and mortality. We will measure CD4 cell count and HIV viral load every 24 weeks to assure that ART failure is detected early and to compare rates of immunologic and virologic response to ART. Evaluation for causes of illness will be completed any time a participant is ill.

Study Duration: Follow up will conclude approximately 32 months after the last participant is enrolled (total 32-66 months for each participant); total planned study duration is five and a half years.

Study Regimen/Intervention: Daily prophylaxis with trimethoprim-sulfamethoxazole (TS) either two tablets daily (each containing 80 mg trimethoprim, 400 mg sulfamethoxazole) or one tablet daily (each containing 160 mg trimethoprim, 800 mg

sulfamethoxazole), weekly prophylaxis with chloroquine (CQ) one or two tablets (total 300 or 310 mg chloroquine base) or no prophylaxis

Primary Objective:

To determine if prophylaxis with TS or CQ, compared to no prophylaxis is associated with improved morbidity and mortality among adults receiving ART beyond 6 months

Primary Endpoint: Occurrence of severe events (deaths, WHO stage 3 or 4 event)

Secondary Objectives:

To assess the effect of prophylaxis with TS or CQ on the virologic, immunologic and clinical response to ART

To assess the efficacy of TS in preventing infection with bacteria or malaria

To assess the safety and tolerability of TS and CQ prophylaxis

Exploratory Objectives:

To evaluate the effect of TS and CQ prophylaxis on the incidence of drug-resistant organisms

To evaluate the efficacy of antimalarial treatment

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2 BACKGROUND INFORMATION AND SCIENTIFIC RATIONALE

2.1 Background Information

Several studies have demonstrated that daily trimethoprim-sulfamethoxazole (TS) prophylaxis reduces morbidity and mortality among people living with HIV (PLHIV) in sub-Saharan Africa.[1-4] As a result of these studies the World Health Organization (WHO) recommended administering TS prophylaxis to PLHIV. However, these studies were completed prior to the widespread availability of antiretroviral therapy (ART) and the applicability of the results to individuals on ART has not been definitively evaluated. A critical question remains about the need for and duration of TS prophylaxis and its public health impact: Is there a benefit to TS prophylaxis after patients have initiated and responded to ART?

In North America and Europe, TS prophylaxis is used to prevent *Pneumocystis jirovecii* pneumonia (PCP) and toxoplasmosis. Common practice has been to discontinue TS prophylaxis after the CD4 cell count reaches >200/mm^3. However, a recent multicenter study demonstrated that even with a CD4 cell count of 100-200/mm^3, there is minimal benefit of TS prophylaxis if the patient is on ART and the viral load is undetectable.[5] The risk of opportunistic infections, at least those infections common in Western countries, is very low once ART is successful, even with low CD4 cell counts.

In contrast, studies in Africa to determine the point when TS prophylaxis no longer confers an advantage with respect to survival or morbidity has not been examined directly. There is consistent evidence to support the use of CD4 cell count of >200 cells/mm^3 as a threshold above which TS prophylaxis survival benefit is absent among individuals receiving ART. In Uganda, there was no documented survival advantage conferred by TS prophylaxis in addition to ART once the CD4 count was above 200/mm^3.[3] A similar finding was recently published in a report of participants enrolled in the Development of Antiretroviral Therapy in Africa (DART) trial, in which the investigators compared the outcomes of individuals who received TS prophylaxis versus those who received no prophylaxis, based on the decision of the responsible clinician.[6] From the initiation of ART until 72 weeks on ART, TS provided a survival benefit if the CD4 cell count was <200/mm^3. Among those with a CD4 cell count >200/mm^3, the point estimate of the odds ratio favored TS prophylaxis of 0.77 but the difference between the two groups was not statistically significant (95% confidence interval 0.43 to 1.38). TS

prevented death due to illnesses attributed to infections that could be prevented by TS and also deaths due to illnesses that would not have been TS-preventable.

The benefit of prophylaxis is through the prevention of bacteremia, pneumonia, enteritis and, in some circumstances, malaria among adults and children who did not have access to ART. However, this broad benefit has not been consistently documented in adults on ART. In the DART study, the only clear disease-specific benefit of TS was in the prevention of malaria. TS did not prevent stage 4 illnesses.[6] Another study that was designed as a clinical trial to assess the benefit of TS prophylaxis in a population of adults on ART was stopped early because the group on TS prophylaxis had less malaria and diarrheal illnesses than the control group.[7] Four deaths occurred during the study—three in the TS treatment group and one in the control group. None of the deaths were related to malaria, diarrhea or TS toxicity. Because the follow up was limited, the effect of these mild intercurrent illnesses on ART response and survival could not be assessed.

The mechanism by which TS improves mortality is not known. In most settings, TS prevents both bacterial infection and malaria as disease-specific morbidities but is associated with decreased mortality due to illnesses from all causes. The relative benefit of the effect of the prevention of bacterial versus malaria infections has not been evaluated. It is possible that the prevention of malaria is the key to the apparent benefit of TS in settings where malaria is common. Malaria infection is associated with transient increases in HIV viral load.[8] However, it is not known if these increases are associated with loss of virologic suppression leading to HIV disease progression.

At the study sites, daily TS prophylaxis is currently used by all PLHIV. The Ministry of Health in Malawi currently recommends TS prophylaxis for all PLHIV as part of a standard package of preventive services.[9] TS prophylaxis is recommended to be continued for life in the absence of severe side effects as there are no randomized, controlled trials to determine when to stop TS in persons on ART. Long-term CQ use to prevent malaria in pregnancy as part of a separate NIH-sponsored clinical trial began in February 2012 (NCT 01443130). CQ is not currently used in the public sector outside of the research setting in Malawi for malaria prevention or treatment.

2.2 Study Hypothesis and Rationale

The role of TS prophylaxis in the context of the ART regimen is an important issue now facing ART programs in Africa. Currently, the updated WHO recommendation calls for TS prophylaxis in PLHIV

with a CD4 cell cut off of ≤350 cells/mm^3 in areas where bacterial infection and malaria are prevalent.[10] However this recommendation is based on expert opinion and not on results of appropriately conducted randomized, controlled trials. Some data from resource-limited settings suggest that it is safe to discontinue TS among those with immune recovery and CD4>200 cells/mm^3 in response to ART.[11;12] To date, no randomized clinical trials have assessed the safety and timing of discontinuation of TS prophylaxis following immune recovery in response to ART in resource-limited settings.[13]

Our study is designed to address the following two inter-related questions:

1. Is there a benefit to TS prophylaxis among adults who have viral suppression and good clinical response on ART?
2. If so, is the benefit due to antimalarial or antibacterial properties?

We hypothesize that there will be a long-term benefit on survival and disease control in the context of prophylaxis and that the benefit will be largely attributed to prevention of malaria. Specifically our main study hypotheses are as follows:

1. TS and CQ will decrease the rates of morbidity and mortality among adults after ≥6 months of ART.
2. CQ prophylaxis will be associated with more prolonged viral suppression and higher CD4 cell counts than TS prophylaxis or no prophylaxis.

We have therefore designed a study that will address these questions, taking advantage of the unique opportunity in Malawi, where malaria is uniformly susceptible to CQ.[14;15] In addition to a TS intervention arm and a control arm, we will include an arm that receives only CQ prophylaxis. Individuals who receive CQ will be receiving extremely effective malaria prevention, but will not be protected against bacterial infection. Thus, one arm represents a population that receives medication to prevent both bacterial and parasitic infection, one arm will receive medication to prevent just malaria and one arm will not receive active prophylaxis.

This study has the opportunity to inform HIV policy throughout Africa and it must be conducted in a rigorous manner so that the results can be generalized to the care administered to millions of people. The potential impact of the study is clear: TS prophylaxis at ~1 billion doses/year could potentially be removed from the ART regimen or, if it is beneficial, its use could be more broadly reinforced as a method to save lives and prolong the efficacy of the available ART. A control arm that discontinues TS

prophylaxis after good clinical and virologic response to ART is included in the study because there is clinical equipoise regarding the benefit of prophylaxis in this population. A randomized clinical trial is needed to definitively inform health policy in Malawi and other Sub-Saharan African countries who currently continue administering TS prophylaxis to PLHIV after successful therapy.[13]

Some studies suggest that daily CQ may reduce HIV viral load, prolonging the effects of antiretroviral therapy.[16;17] These studies use doses of 125-250mg of CQ twice daily, much higher than the standard prophylactic weekly dose of 500mg. The impact of weekly CQ prophylaxis on viral load is not known. In analyzing data from this study, we will be able to control for malaria as well as bacterial infections to determine if CQ prophylaxis is associated with HIV viral load suppression. If we find that CQ prophylaxis is associated with a prolonged duration of viral suppression, further studies to explore this association may be warranted.

Although ideally this study would be placebo controlled, the study team has concerns about the complexity of a placebo regimen. Participants would have to take one pill every week and a different one every day. Such an increase in the complexity of the medication regimen may endanger adherence to ART. In addition, this study design will allow us to assess the impact of the different regimens on overall drug adherence.

2.3 Potential Risks and Benefits

2.3.1 Potential Risks

Potential risks anticipated for the study participants include study drug-specific effects, risks associated with blood drawing, potential increased risk of opportunistic infections, and potential loss of confidentiality regarding HIV status.

The drugs used in this trial are not investigational new drugs. They have both been widely used for many decades throughout the world and particularly in Africa, at the doses and for the indications they will be used for in this trial. Their safety profiles are favorable and well known. However, as with any drug, risks exist. Though CQ is considered safe, adverse reactions to this drug may occur. Commonly reported symptoms include headache, malaise, dizziness, blurred vision, difficulty focusing, muscle weakness and mild gastrointestinal upset. Non-urticarial pruritis, without rash, is a problem that is more common among dark-skinned patients. The symptom usually begins within the first day after the

initial dose and may last up to seven days. Severe adverse reactions are extremely rare.[18] CQ is well known to be safe in pregnancy and is routinely recommended for both malaria treatment and malaria prophylaxis in pregnant women.

Common adverse reactions associated with TS use include rash, urticaria, loss of appetite, nausea and vomiting. Rare, serious side effects include agranulocytosis, aplastic anemia, disease of the hematopoietic system, fulminant hepatic necrosis, severe allergic reaction, Stevens-Johnson syndrome, and toxic epidermal necrolysis.[19] TS is considered to be relatively contraindicated in pregnancy (Class C) because of theoretical risks of neural tube defects in the first trimester and kernicterus in the third trimester. However, both the World Health Organization[13] and the Malawi Ministry of Health presently recommend TS prophylaxis for PLHIV, including pregnant women.

Finger pricks and venipuncture are associated with small risks of bleeding, hematoma and infection. To minimize this risk, the skin is cleaned with alcohol prior to puncture, sterile, unused needles and lancets will always be used and pressure will be held at the puncture site after removal of the needle or lancet. Although the quantity of blood drawn would not lead to any ill effects on the participants' health, some adults feel faint with phlebotomy. The risks will be minimized by having trained technicians perform the procedure. Clinicians will be available for evaluation if there is any untoward effect.

Study subjects who are assigned to the CQ or no treatment arms may be at increased risk for opportunistic infections. However, the fact that immune reconstitution will have already taken place in these individuals reduces this theoretical risk substantially.[20;21] Additionally, the risk of TS prophylaxis-associated adverse reactions must be weighed against the unknown, potential benefit of TS prophylaxis in this population. The potential risk of increased infection in those not taking TS will be minimized by close follow-up and monitoring that will lead to prompt diagnosis and treatment of bacterial infections and malaria should they occur.

As with any study involving HIV-positive individuals, there is a risk of loss of confidentiality regarding HIV status. Our study team is well-known in the community for conducting studies of malaria, so clinic attendance and home visits will not necessarily identify participants' HIV status. Efforts to reduce this risk will be a priority. Patient medical records will be kept in a locked cabinet in a locked room. The study protocol, documentation, data and all other information generated will be kept in strict confidence. No personal information will be released to any unauthorized third party without the

consent of the participant. Participant specimens and case report forms (CRFs) will be identified by a study code with the master key to be kept in a separate, locked cabinet.

2.3.2 Known Potential Benefits

Study participants will receive a higher standard of medical care than is typically available in Malawi. Close follow-up is likely to identify HIV-related illnesses sooner than they would otherwise be detected. We will pursue complete diagnostic evaluation of illness episodes and maintain a supply of medication to treat common diseases.

3 OBJECTIVES:

3.1 Primary objective and endpoints:

To determine if prophylaxis with TS or CQ, compared to no prophylaxis is associated with improved morbidity and mortality among adults receiving ART beyond 6 months.

The main endpoints for this objective are clinical endpoints:

- Primary endpoint: occurrence of a severe event (death, WHO stage 3 and 4 events)

Morbidity and mortality are high during the first months after initiation of ART, but the role for continued, life-long prophylaxis remains unknown. The addition of TS prophylaxis to life-long ART increases costs, pill burden and the risk of adverse drug reactions. It may also lead to increased rates of antifolate-resistant infections in communities that depend on TS as a first-line antimicrobial therapy and for malaria prevention, though this was not shown in a recent longitudinal cohort study in Uganda.[22] For these reasons, it is necessary to establish an evidence-based guideline to determine if TS prophylaxis can safely be stopped among those who have undergone immune reconstitution on ART with a CD4 count >250 cells/mm3 and undetectable viral load. A listing of WHO stage 3 and 4 events based on the WHO 2006 classification can be found in Appendix A. New and recurrent events will be included. The assessment of WHO stage 3 and 4 events will be reviewed independently by a committee blinded to treatment assignment.

3.2 Secondary objectives and endpoints:

1. To assess the effect of prophylaxis with TS or CQ on the virologic, immunologic and clinical response to ART
    - Endpoints (to be compared among those on prophylaxis with TS or CQ versus no prophylaxis):

        Laboratory:

        1) Virologic: Prevalence of undetectable (<400 copies/mL) HIV viral load assessed every 24 weeks

        2) Immunologic: CD4 cell count assessed every 24 weeks

        Clinical:

1) Occurrence of any WHO HIV stage 2, 3 or 4 illness or death

Because intercurrent infections are associated with increases in HIV viral load, we hypothesize that the prevention of malaria and bacterial infections will allow for sustained viral suppression. The evidence that one study arm is associated with improved durability of response to ART will be measured by viral load, CD4 count and evidence of clinical disease.

2. To assess the efficacy of TS in preventing infection with bacteria or malaria

    - Laboratory Endpoint: Occurrence of bacterial infections and malaria

TS prophylaxis has been recommended as an intervention to prevent infections in PLHIV infection even in the presence of high rates of antifolate resistant organisms. In this study, we have the opportunity to compare intervention and control arms in the same study to determine the ability of TS to prevent infections in the context of high rates of TS-resistant bacteria and SP-resistant malaria. Because some bacterial infections, such as pneumonia, are difficult to confirm through laboratory analysis, we will analyze laboratory-confirmed cases as well as suspected cases.

3. To assess the safety and tolerability of TS and CQ prophylaxis

    - Endpoint: Occurrence of adverse events that are ≥Grade 3
    - Endpoint: Discontinuation of TS or CQ prophylaxis

Adverse events that are ≥Grade 3 as well as the rate of discontinuation of TS or CQ prophylaxis will be compared among study groups to determine if a prophylactic regimen with TS or CQ is safe and well-tolerated. Significant drug interactions should be captured in this analysis. Adverse events will be graded according to the DAIDS toxicity table found at: http://rsc.tech-res.com/safetyandpharmacovigilance/

3.3 Exploratory objectives and endpoints

1. To evaluate the effect of TS and CQ prophylaxis on the incidence of drug resistant organisms

    - Laboratory Endpoint: Occurrence of a bacterial or malaria infection with CQ or TS resistant organism

CQ or TS prophylaxis may select for organisms resistant to antimicrobials. Antimicrobial susceptibility testing will be performed on bacterial pathogens isolated from blood and cerebrospinal fluid samples

based on accepted standards established by the British Antimicrobial Society guidelines and used as the standard for clinical management of patients in the Blantyre area. Antimalarial resistance testing will be performed on positive malaria specimens and analyzed by genotyping for molecular markers associated with antimalarial drug resistance as described on our website http://medschool.umaryland.edu/malaria/protocols.asp) and in the literature.[23]

2. To evaluate the efficacy of antimalarial treatment
    - Endpoint: Clinical and parasitological response to antimalarial therapy in cases of uncomplicated malaria.

While assessing the efficacy of antimalarial treatment is not a primary aim in this study, evaluation of treatment outcomes in this study population is important from a public health standpoint. Should antimalarial treatment efficacy among the HIV-positive population be low, then alternative antimalarial treatment regimens would need to be explored. Standard definitions of adequate parasitological and clinical response to antimalarial therapy will be used based on current WHO guidelines (http://whqlibdoc.who.int/publications/2009/9789241597531_eng.pdf).

4 STUDY DESIGN

This is a randomized, controlled, open-label, phase III trial of standard of care TS prophylaxis and CQ prophylaxis compared to no prophylaxis in adults receiving ART. Adults who have been receiving ART for at least six months with a good clinical response and provide informed consent and fulfill the eligibility criteria will be randomized to one of three arms: (1) to continue standard of care trimethoprim-sulfamethoxazole (TS) prophylaxis, (2) discontinue standard of care TS prophylaxis and begin weekly CQ prophylaxis or (3) discontinue standard of care TS prophylaxis. Participants will be asked to return to the research clinic every four weeks for the first 24 weeks then every 12 weeks thereafter, and any time they are ill to facilitate both active and passive follow-up of the study endpoints. Participation will last for approximately 32-66 months. Participants who develop a WHO clinical stage 3 or 4 illness (see Appendix A for a complete listing), experience a sustained decline in their CD4 count below 200 cells/mm^3, or who experience ART failure will be placed on standard of care TS prophylaxis. Those with confirmed ART failure will be evaluated for second-line therapy according to the Malawi Ministry of Health guidelines.

The study population will include up to 1500 Malawian adults aged 18 years or older living with HIV in or near Blantyre, Malawi, Central Africa who have been receiving antiretroviral therapy for at least 6 months with good clinical response to ART, have an undetectable HIV viral load and a CD4 count $\geq$250/mm^3. Participants must intend to stay in Blantyre for the entire study period. Major exclusion criteria include severe acute illness, chronic treatment or secondary prophylaxis with any drug with antimalarial or antibacterial activity, history of hypersensitivity to antifolate drugs or CQ, anemia, thrombocytopenia, neutropenia, liver or kidney failure, and pregnancy.

5 STUDY POPULATION

5.1 Site Description

Ndirande Health Centre. The study will be conducted at the Blantyre Malaria Project (BMP) Research Clinic at the Ndirande Health Centre on the outskirts of Blantyre, the commercial center of Malawi. Ndirande is a peri-urban township with a population of approximately 200,000 inhabitants. Other than private dispensaries and traditional medical practitioners, the Health Centre is the only source of medical care serving this large and relatively poor population. The Centre provides basic medical care free of charge for all members of the community including pre-natal clinics and facilities for deliveries. We have been working at this site since 1997. We initially began our collaboration with the district health center to conduct studies of antimalarial drug efficacy. In 2003, we began the observational study of PLHIV-infection in the district. To prepare for the study and for future trials, we established a voluntary counseling and testing (VCT) facility at the health center. This was the first VCT facility located at a district health center in Malawi. As a result of the program's success, the Ndirande District Health Centre was also one of the first district health centers to establish an ART clinic, where HIV care including TS prophylaxis and ART are provided without cost to the population. At present, adults receiving ART are always prescribed TS prophylaxis. Our studies in Ndirande have included active and passive follow-up for up to 27 months, with frequent home visits to residences of study participants throughout the Ndirande community.

Tisungane Clinic at Zomba Central Hospital. Tisungane Clinic, an outpatient ART clinic, located on the grounds of Zomba Central Hospital in Zomba, Malawi and is co-managed by Dignitas International and the Malawi Ministry of Health. Zomba is the administrative capital of Zomba District of Malawi (previously the capital city of Nyasaland and then Malawi before it moved to Lilongwe in 1974) and is one hour from Blantyre by car. It has a population of approximately 100,000 inhabitants of various Malawian ethnic backgrounds. The ART clinic is highly successful and productive, having initiated over 20,000 patients on ART since 2004. In addition to providing ART, the clinic provides HIV testing and counseling, services to prevent mother-to-child HIV transmission and TB services to HIV-TB co-infected patients.

Queen Elizabeth Central Hospital. Patients needing hospitalization as per standard of care in Malawi for diagnoses including but not limited to respiratory distress, sepsis, severe dehydration and severe malaria may be referred to the Queen Elizabeth Central Hospital (QECH), a large public tertiary-level hospital in Blantyre. Areas in the male medical and female medical wards are designated for study participants requiring hospitalization. The BMP and the Malawi-Liverpool-Wellcome Trust Clinical Research Programme (MLW) are both housed on the hospital campus and are affiliated with the University of Malawi College of Medicine. The MLW has provided a blood and CSF microbiology service for the Adult and Paediatric wards at QECH for over 10 years.

Zomba Central Hospital. Patients needing hospitalization as per standard of care in Malawi for diagnoses including but not limited to respiratory distress, sepsis, severe dehydration and severe malaria may also be referred to Zomba Central Hospital, a public hospital in Zomba. Areas in the male medical and female medical wards are available for study participants requiring hospitalization. Dignitas International's Tisungane Clinic is housed on the hospital campus. Dignitas staff will assist in supervision and coordination of care for participants admitted to Zomba Central Hospital, as is done with an ongoing study of inpatients with cryptococcal meningitis onsite.

5.2 Participant recruitment and retention

Individuals enrolled in ART clinics at Ndirande, Zomba and other government clinics if necessary will be eligible after six months on ART. The ART clinic staff will inform patients in the ART program about the possibility of enrolling in this study. Potential participants will be invited to meet with a study clinician who will discuss this study with them. Then, if the potential participant expresses interest, the clinician will proceed with the informed consent process, followed by laboratory specimen collection and evaluation, medical history and physical examination, and evaluation of inclusion and exclusion criteria.

5.3 Inclusion Criteria

- Age 18 years or older
- Documented HIV-1 infection
- Initiation of ART through a government-sponsored ART program at least six months prior
- Undetectable HIV viral load (<400 copies/mL)

- CD4 count ≥250/mm^3
- TS prophylaxis prescribed for at least the previous 2 months
- Intention to remain in the study area until the end of the study period
- Informed consent from participant
- Female study volunteers of reproductive potential must have a negative urine pregnancy test performed within 20 days before randomization.
- Female study volunteers of reproductive potential who participate in sexual activity that could lead to pregnancy must use contraception (male or female condoms, diaphragm or cervical cap with spermicide, intrauterine device, or hormone-based contraceptive) while receiving their assigned study drug and for one month after stopping the medications.

5.4 Rationale for inclusion criteria

Age 18 years or older. Studies of prophylaxis for PLHIV have been conducted separately for children and adults. Independent of HIV status, the epidemiology of infectious diseases differs markedly between adults and children. HIV-infected children over two years of age represent a small subpopulation in Malawi, since most children with congenital infection do not survive through infancy. They likely have unique virologic and immunologic factors that have contributed to their long-term non-progression. In addition, despite being open to enrollment to persons of all ages, few children were enrolled in the Ndirande Incidence Study, so it is unlikely that adequate numbers of children could be enrolled in a clinical trial to permit meaningful analysis of this subgroup. We have therefore chosen not to include children in this study.

Documented HIV-1 infection. To ensure data generated and study analyses are relevant to PLHIV, only persons with documented HIV-1 infection will be included. Documentation includes a rapid HIV test or any licensed ELISA test kit that is confirmed by a second rapid HIV test by a different method, a repeat ELISA, or a Western blot or plasma HIV-1 RNA.

Initiation of ART through a government-sponsored ART program at least six months prior
In order to maintain relative uniformity of risk of ART failure, we have chosen to limit the population to those initiating ART at least six months prior. Patients who begin ART are expected to demonstrate immunologic and virologic responses to therapy after 4-6 months.

Undetectable HIV viral load.

Those with a detectable HIV viral load (<400 copies/mL) may be failing antiretroviral therapy and will therefore be at higher risk for severe events due to continued immunosuppression. To reduce this risk, we will enroll participants with an undetectable HIV viral load at study start.

CD4 count ≥250/mm^3.

Although still limited, CD4 count measurement is being recommended for the evaluation for HIV staging and ART initiation. Due to the increased availability of this measure of immune function, it is feasible to use CD4 count as a criterion for stopping TS prophylaxis in addition to clinical response to ART. CD4 count of 200 to 350/mm^3 has been proposed as a possible threshold to discontinue TS prophylaxis by the WHO.[13] In addition, our studies have demonstrated that among adults who are not receiving ART, a CD4 count less than 200/mm^3 is associated with a much higher rate of morbidity and mortality than those above this level.[24] Because the CD4 count can fluctuate as much as 50 cells/mm^3 and we would like the minimum CD4 count to be at least 200 cells/mm^3, we have selected 250 cells/mm^3 as the minimum CD4 count requirement.

TS prophylaxis prescribed for at least the previous 2 months

To maintain uniformity of the study population, we have chosen to include those currently on daily TS prophylaxis at study start.

Intention to remain in the study area.

Participants will require long-term follow-up every 4-12 weeks (every 4 weeks for the first 24 weeks, then every 12 weeks thereafter) from enrollment until the close of the study. Follow up will likely be at least 32 months and the maximum follow up period is expected to be 66 months. In order to recruit participants who are likely to achieve adequate follow-up and whose bacterial and malarial infections are likely to be identified and treated by study personnel, we will recruit those who plan to remain in the area.

Female study volunteers of reproductive potential must have a negative urine pregnancy test performed within 20 days before initiating their assigned study drug.

Female study volunteers of reproductive potential (defined as girls who have reached menarche and women who have not been post-menopausal for at least 24 consecutive months, i.e. who have had menses within the preceding 24 months, and have not undergone surgical sterilization (e.g. hysterectomy, bilateral oophorectomy, tubal ligation or salpingectomy) must have a negative urine pregnancy test performed within 20 days before initiating randomization.

Female study volunteers of reproductive potential who participate in sexual activity that could lead to pregnancy must use contraception (male or female condoms, diaphragm or cervical cap with spermicide, intrauterine device, or hormone-based contraceptive) while receiving their assigned study drug and for one month after stopping the medications.

If participating in sexual activity that could lead to pregnancy, female study volunteers of reproductive potential (defined as girls who have reached menarche and women who have not been post-menopausal for at least 24 consecutive months, i.e. who have had menses within the preceding 24 months, and have not undergone surgical sterilization (e.g. hysterectomy, bilateral oophorectomy, tubal ligation or salpingectomy) must use at least one of the following forms of contraception while receiving their assigned study drug and for one month after stopping the medications: condoms (male or female), diaphragm or cervical cap with spermicide, intrauterine device, or hormone-based contraceptive. If the female volunteer is not of reproductive potential (girls who have not reached menarche, or women who have been post-menopausal for at least 24 consecutive months, or women who have undergone surgical sterilization, (e.g. hysterectomy, bilateral oophorectomy, tubal ligation or salpingectomy) she is eligible without requiring the use of a contraceptive method. Patient-reported history can serve as acceptable documentation of sterilization, other contraceptive methods, menopause, and a child's reproductive potential.

5.5 Exclusion Criteria

- Severe acute illness (defined as requiring hospitalization at the time of screening or other conditions such as laboratory abnormalities as determined by the investigators)

- Chronic treatment (requiring therapy for >14 days) or secondary prophylaxis (for toxoplasmosis, Pneumocystis pneumonia, or tuberculosis for example) with any drug with antimalarial or antibacterial activity
- History of hypersensitivity to antifolate drugs or CQ
- Laboratory exclusion criteria
    - Hemoglobin <8.0 gm/dL
    - Platelet count <50,000/mm^3
    - Absolute granulocyte count <500/mm^3
    - Serum alanine aminotransferase (ALT) concentration >210 U/L for men, >160 U/L for women
    - Serum creatinine concentration >3.3mg/dl (291.7µmol/L) for men, and >2.7mg/dl (238.7µmol/L) for women)
- History of visual field or retinal changes
- History of preexisting auditory damage
- History of porphyria
- History of psoriasis
- History of liver disease
- History of seizure disorder
- History of glucose-6-phosphate dehydrogenase (G6PD) deficiency
- History of ECG and cardiac conduction abnormality or cardiomyopathy
- History of myopathy

5.6 Rationale for exclusion criteria

Severe acute illness. To avoid the possibility of undue inducement to participate in the study in order to receive more urgent medical attention, persons with severe acute illness, defined as requiring hospitalization at the time of screening or other conditions as determined by the investigators will be invited to return for evaluation for study eligibility when they have recovered from their acute illness.

Chronic treatment (requiring therapy for >14 days) or secondary prophylaxis with any drug with antimalarial or antibacterial activity. Potential participants who are currently receiving chronic treatment or secondary prophylaxis (for toxoplasmosis or PCP, for example) with drugs that will

interfere with the endpoint assessment must be excluded. Participants who are completing a short course of antimicrobial therapy (less than or equal to 14 days) will not be excluded.

History of hypersensitivity to antifolate drugs or CQ. Individuals with allergies to any component of the study drugs will not be enrolled.

Hemoglobin <8.0 gm/dL; platelet count <50,000/mm^3; absolute granulocyte count <500/mm^3. These are the laboratory criteria for WHO clinical stage 3 disease. Individuals should not enroll in the study and undergo randomization until they have had hematological recovery.

Serum alanine aminotransferase (ALT) concentration >210 U/L for men, >160 U/L for women; serum creatinine concentration >3.3mg/dl (291.7µmol/L) for men, and >2.7mg/dl (238.7µmol/L) for women: There is a rare association between TS and fulminant hepatic failure. TS and CQ should be used with caution in individuals with hepatic or renal insufficiency. These values for ALT and creatinine are based on the normal values used at Queen Elizabeth Central Hospital, Blantyre, Malawi, and are five and three times the upper limit of normal, respectively.

History of visual field or retinal changes. Long-term daily use of CQ can cause irreversible retinal damage. It is unknown if those with preexisting visual field or retinal damage would be at higher risk for these effects, so they will be excluded to avoid possible additive toxic effects in this population.

History of preexisting auditory damage. CQ can cause reduced hearing in those with preexisting auditory damage, so these persons will be excluded to avoid possible toxic effects.

History of porphyria. CQ can cause or exacerbate preexisting porphyria, so these persons will be excluded to avoid possible adverse effects.

History of psoriasis. CQ can cause or exacerbate preexisting psoriasis, so these persons will be excluded to avoid possible adverse effects.

History of liver disease. CQ is known to concentrate in the liver and could potentially exacerbate underlying hepatic disease.

History of seizure disorder. Adverse effects of CQ include convulsive seizures, so persons with a history of seizure disorder will be excluded to avoid possible adverse effects.

History of glucose-6-phosphate dehydrogenase (G6PD) deficiency. CQ may potentiate hemolysis in patients with preexisting G6PD deficiency, so these persons will be excluded to avoid possible adverse effects.

History of ECG and cardiac conduction abnormality or cardiomyopathy. CQ rarely causes hypotension, electrocardiographic change and cardiomyopathy, so persons with a history of any of these conditions will be excluded.

History of myopathy. CQ may cause myopathy leading to progressive weakness and atrophy of proximal muscle groups, so persons with a history of myopathy will be excluded.

6 STUDY INTERVENTIONS

6.1 Regimen (dose, schedule, route)

Participants will be randomized to receive one of the following regimens:

1) Trimethoprim-sulfamethoxazole: either two tablets (each containing 80 mg trimethoprim, 400 mg sulfamethoxazole) or one tablet (each containing 160 mg trimethoprim, 800 mg sulfamethoxazole) to be taken every day by mouth
2) Chloroquine: one or two tablets (total 300 - 310 mg chloroquine base), to be taken every seven days by mouth
3) Control: no therapy

The participants will remain in the same treatment group throughout the study and will receive medications until the close of the study unless they meet the criteria for treatment discontinuation.

6.2 Study product formulation and preparation

Trimethoprim-sulfamethoxazole (TS) will be provided in tablet form containing 80 mg trimethoprim and 400 mg of sulfamethoxazole, or 160 mg trimethoprim, 800 mg sulfamethoxazole, manufactured by pharmaceutical companies approved by the U.S. Food and Drug Administration (FDA) or PEPFAR for TS manufacturing. A backup supply will be maintained at the study site.

Chloroquine (CQ) will be provided in either 500 mg tablet form containing 300 mg chloroquine base, 250 mg chloroquine tablet containing 155 mg chloroquine base, or 200 mg hydroxychloroquine tablets containing 155 mg chloroquine base, manufactured by pharmaceutical companies approved by the U.S. Food and Drug Administration (FDA), Global Fund or PEPFAR for CQ manufacturing.

Note: The Global Fund products are authorized for use by the regulatory authority in the country where they will be used, and must either be prequalified under the WHO Prequalification Programme, authorized for use by a stringent regulatory authority as defined by The Global Fund guidance, or recommended for use by The Global Fund Expert Review Panel, a group hosted by the WHO.

The study drugs will be stored according to the label. The CQ and TS tablets will be stored at 25°C, with excursions permitted. The medications will be kept in an air conditioned, locked storage closet on

the second floor of the BMP Ndirande Research Clinic. A working supply of the drugs will be kept in the study dispensary. Both the storage closet and the dispensary have temperature monitoring with minimum/maximum thermometers read daily by a member of the staff. CQ and TS tablets will be distributed to study participants no later than 35 days prior to the expiration date on the package.

A supply of TS and CQ will be dispensed to participants to be sure there is sufficient medication if the participant is delayed in returning for the next visit. Participants will also be instructed to keep the medication in a secure place in their home away from direct sunlight and other sources of heat. Refer to the study manual of procedures for details of drug dispensation and adherence counseling.

6.3 Study product supply and accountability

6.3.1 Acquisition

TS will be acquired from the Malawi Ministry of Health ART clinics supply. CQ and a backup TS supply will be purchased through a pharmaceutical supply company and delivered to the Blantyre Malaria Project or to Dignitas International, Zomba.

6.3.2 Accountability

The PI will ensure that personnel responsible for study drug acquisition, storage and dispensing are knowledgeable of the DAIDS policy for pharmacy facilities. Refer to the study manual of procedures for details of drug accountability.

6.4 Assessment of participant adherence with regard to study interventions

Participants will be instructed to return every 4-12 weeks (every 4 weeks for the first 24 weeks, then every 12 weeks thereafter) with the bottles of study drug and antiretroviral therapy. The study staff will interview participants about their overall adherence to both the ART regimen and, if appropriate, the study drug, and adherence in the past week in a non-threatening manner using the Special Programs of National Significance (SPNS) questionnaire (Appendix D).[25] Prior to inquiring about adherence, study staff will normalize non-adherence by stating that this is not uncommon, especially in a population on multiple daily therapies, and will ask about the reasons for non-adherence. The study staff will continue this dialogue with study participants by recognizing their reasons for non-adherence,

emphasizing the importance of adherence, and helping to overcome barriers to adherence. They will also note on the case record form (CRF) how many pills remain as a secondary measure of adherence to validate the self-report.

6.5 Concomitant Medications and Procedures

Participants will be permitted to take any necessary medication during the study, as deemed clinically appropriate by the study team. Treatment will be administered in accordance with the treatment guidelines. Participants will be strongly encouraged to seek all medical care and treatment first from the study team, and discouraged from independent use of antimicrobials, antimalarial drugs or other medications from non-study sources. The study team will routinely document the use of all medications including traditional remedies in the concomitant medication log.

Any concomitant medication will be permitted, if it is medically necessary. Drugs with antimalarial properties will be avoided, if possible, when not used to treat a malaria infection. Whenever a concomitant medication is initiated or the dose changed, investigators will review the concomitant medications' most recent package inserts, or other updated information to obtain the most current information on drug interactions, contraindications, and precautions.

6.5.1 Prohibited Medications and Procedures

There are no prohibited medications after enrollment in the study.

6.5.2 Required Medications and Procedures

- Study drug if assigned to daily TS or weekly CQ
- Standard antimalarial therapy if uncomplicated malaria is diagnosed (currently artemether-lumefantrine or amodiaquine-artesunate) unless there is a contraindication
- Antiretroviral therapy administered according to the Malawi Ministry of Health Guidelines (see Appendix C). Any antiretroviral therapy regimen provided by the local government clinics is acceptable.

7 STUDY PROCEDURES/EVALUATIONS

The following table summarizes events included in the study schedule:

	Screening	Enrollment	Every 4 weeks for 1st 24 weeks, then every 12 weeks	Additional evaluations every 24 weeks	Final study visit, time of termination	Premature discontinuation of study treatment	Unscheduled Visits
Informed consent	√						
Review of past medical history	√						
Review of current complaints		√	√		√	√	√
Medication history	√		√		√	√	
Bednet use		√	√		√	√	
WHO performance scale		√		√	√	√	
CBC, ALT, creatinine	√			√	√	√	
Urine pregnancy test	√	√[a]	√[a]				
CD4 count	√			√	√	√	
HIV viral load	√			√	√	√	
PBMC				√[b]			
Filter paper sample		√	√		√	√	
Physical examination	√	√ (limited)	√ (limited)	√	√	√	√
Visual acuity assessment		√		√	√	√	
Provision of medication		√	√				
Pill count and adherence interview			√		√	√	

[a] Urine pregnancy testing will be performed at all study visits only where pregnancy is suspected
[b] PBMC collections will occur only individuals who consent for sub-study 2 participation (appendix G)

For participants diagnosed with malaria, the following schedule will be followed in addition to the above schedule:

Days after malaria diagnosis →	0	1	2	3	7	14	21	28	Unscheduled visit (before d28)
Procedure ↓									
Clinical Assessment	C	C	C	C	C	C	C	C	C
Temperature	C	C	C	C	C	C	C	C	C
Questioning about antimalarial drug use	C	C	C	C	C	C	C	C	C
Finger stick blood sample for malaria smear	C	R	R	R	R	R	R	R	C
Finger stick blood sample for filter paper sample for PCR analysis	R	R	R	R	R	R	R	R	R
Administration of antimalarial drug (per Malawi Ministry of Health Protocol)	C	C	C						

C= procedures that are part of clinical care

R= research procedures

7.1 Clinical Evaluations and Procedures

Medical History-comprehensive (obtained by interview and patient's medical passport if available):

- Review of conditions that led to ART initiation
- Complete ART medication history
- Antibiotic and antimalarial use history
- Any other underlying medical conditions
- Review of current complaints
- Allergies, with special attention to antifolates and CQ
- For females, date of last menstrual period

History-interim (obtained by interview and patient's medical passport if available)

- Evaluation of current complaints
- Evaluate complaints of visual changes, hearing changes, and muscle weakness.
- Evaluation of ongoing symptoms
- Determination of concurrent medications (especially antibiotics and antimalarials)

Pill count and adherence interview (obtained by interview and counting pills brought in by participants)

WHO performance scale (obtained by interview)

Participants will be asked about their daily activity to determine their WHO performance scale:

- 1: Asymptomatic, normal activity
- 2: Symptomatic, normal activity
- 3: Bedridden <50% of the day during last month
- 4: Bedridden ≥50% of the day during last month

Bednet use (obtained by interview)

Participants will be asked about bednet use within the previous 4-12 weeks (every 4 weeks for the first 24 weeks, then every 12 weeks thereafter), whether they slept under a bednet last night and whether or not the bednet has been treated with insecticide.

Physical examination

The physical examination includes vital signs (temperature, heart rate, respiratory rate), weight, height (at study entry only) and a physical examination. At screening and every 24 weeks, a complete physical examination will be performed by a study clinician. At the enrollment visit, a limited physical examination will be routinely conducted by study nurses (or a study clinician) who document vital signs (temperature, heart rate, respiratory rate) and ask about current complaints; if the nurse or clinician elicits any health problem or finds in their professional judgment that further evaluation is indicated, the participant then has a targeted or complete physical examination by a study clinician. At other follow-up visits every 4-12 weeks (every 4 weeks for the

first 24 weeks, then every 12 weeks thereafter), the limited physical examination will be routinely conducted by study nurses (or a study clinician) who document vital signs (temperature, heart rate, respiratory rate) and ask about current complaints including concomitant medication use, vision or hearing changes, muscle weakness, or any other health problems since the last visit. The nurse or clinician also assesses the general health status of the participant. If the nurse or clinician elicits any health problem or finds in their professional judgment that further evaluation is indicated, they note on the participant record that a clinician exam is needed and the participant is then evaluated by a study clinician for an extended physical examination and documented treatment plan. If the nurse or clinician does not elicit any health problems and deems that further evaluation is not warranted at the time of the visit, they indicate on the participant record that an extended physical exam by a clinician is not needed for the current visit. Visual acuity will be checked at enrollment and every 24 weeks using the Snellen eye chart.

7.2 Laboratory Evaluations

7.2.1 Clinical Laboratory Evaluations

Complete blood count

Complete blood counts will be performed in a Coulter counter to determine hemoglobin, hematocrit, WBC with differential, and platelet count.

ALT and Creatinine

These laboratory results will be obtained using a Beckman Coulter AU480 biochemistry analyzer.

Urine pregnancy test

This evaluation will be performed on a fresh specimen collected at the time of the clinic visit.

Viral load

HIV-1 viral load will be measured by real time PCR using the Abbott RealTime HIV-1 Assay. The limit of detection is 40 copies/mL for 1.0 mL input.

CD4 count

CD4 counts will be assessed using Becton-Dickinson FacsCount.

Malaria smears

Both thick and thin smears will be obtained for patients with suspected malaria. Parasite density will be quantified each time malaria smears are obtained at the time of the visit if the participant is ill or if it is an antimalarial efficacy follow-up visit. Standard operating procedures are followed to assure uniform and high quality malaria smears and accurate results. Thick smears are read by counting the number of parasites seen per 200 white blood cells. Quantification will be based on an expected 8,000 white blood cells/mm^3 or the actual white blood cell count if one is available. Thin smears are read by counting the number of parasites seen per 500 red blood cells. Exact parasite density is calculated based on the red blood cell count. Thin smears will be used to identify the malaria species each time the diagnosis of malaria is made.

Malaria drug resistance analysis

Filter paper samples collected will undergo extraction and parasite DNA will be analyzed for polymorphisms associated with chloroquine as described on our website (http://medschool.umaryland.edu/malaria/protocols.asp) and sulfadoxine-pyrimethamine resistance in the literature.[23]

Blood culture

Blood culture is obtained from a venous or arterial blood draw to evaluate for the presence of bacteria. Specimens will be placed in an automated incubator. Pathogens may be stored for future analyses. Blood culture analysis is performed using an automated system (BacTalert®, Bio-Merieux) at MLW. Antimicrobial susceptibility testing will be performed on isolated pathogens based on accepted standards established by the Clinical Laboratory Standards Institute. Isolation of Diphtheroids, coagulase negative Staphylococci, Micrococci species or Bacillus species other than *anthracis* is recorded as contaminants. All other isolates are treated as significant. Bacterial organisms that are detected will be frozen and stored for potential re-evaluation of antimicrobial susceptibility testing at another laboratory.

Cerebrospinal fluid analysis culture

Cerebrospinal fluid is obtained from the lumbar puncture procedure performed when clinically indicated for diagnosing and treating meningitis or encephalitis in study participants. Specimens are incubated on blood agar plates and isolated pathogens will undergo drug susceptibility testing based on accepted standards established by the Clinical Laboratory Standards Institute. Bacterial organisms that are detected will be frozen and stored for potential re-evaluation of antimicrobial susceptibility testing at another laboratory.

7.2.2 Laboratory external quality assurance

External quality assessment will be maintained in accordance with the standard requirements of DAIDS. The investigators and laboratory personnel will work closely with the DAIDS laboratory team and its subcontractors (including pSMILE and IQA), to reach and sustain laboratory standards in accordance with GCLP.

7.2.3 Antiviral resistance testing

While antiviral pharmacokinetics and HIV resistance testing are outside the scope of the main clinical trial, these parameters could influence study outcomes and are therefore important to evaluate. We have designed a substudy to address HIV resistance testing in the context of this clinical trial (Appendix F).

7.2.4 EBV viral load testing

Chloroquine has been shown to have an impact on the lytic life cycle of EBV and therefore may have an impact on EBV-associated immune activation and EBV-related infections and malignancies. Selected serum samples will be tested for EBV viral loads from enrollment and follow up stored specimens.

7.3 Specimen Preparation, Handling and Shipping

Specimens will be handled according to the specifications for management of biohazards from the University of Maryland. For analyses performed at MLW, the Johns Hopkins Laboratory or the College of Medicine, specimens will be packaged in cooler boxes with ice and transported by study staff. When necessary, specimens may be shipped to the University of Maryland and other collaborating

laboratories in accordance with the COMREC materiel transfer agreement procedures. Dried filter paper specimens that are not known to carry infectious agents can be transported without any special precautions. Cryopreserved parasites, PBMCs and sera may be shipped to collaborating laboratories in liquid nitrogen dry shippers that meet all the relevant requirements and specifications.

7.4 Biohazard Containment

As the transmission of HIV and other blood-borne pathogens can occur through contact with contaminated needles, blood, and blood products, appropriate blood and secretion precautions will be employed by all personnel in the drawing of blood and shipping and handling of all specimens for this study, as currently recommended by the Centers for Disease Control and Prevention and the National Institutes of Health. All dangerous goods materials, including diagnostic specimens and infectious substances, will be transported according to instructions detailed in the International Air Transport Association (IATA) Dangerous Good Regulations.

7.5 Sequence of Procedures/Evaluations: Timing and Definitions

7.5.1 Screening

Attendees of government-sponsored ART clinics will be informed about the possibility of joining our study. Any patients who express interest will be invited to meet with a study clinician, who will briefly discuss the details of the study. No study-related evaluations will be undertaken prior to obtaining informed consent. The clinician will ask the participant about their plans to remain in the area and their willingness to attend the Ndirande Health Centre for all scheduled and unscheduled follow-up. Potentially eligible participants will then be asked to read the informed consent in either English or Chichewa. The informed consent will be read and explained verbally in Chichewa to those who are unable to read. This verbal explanation will be attended by a witness who will also sign the consent form. After reading or listening, the potential participant will be asked to sign the informed consent form. For those who cannot write, a thumbprint will be placed on the form, with the signature and date of a witness. The document also allows the participants to indicate how to handle specimens that remain after the study procedures have been completed. Any use of study samples that is outside the scope of the objectives of this protocol will be submitted for prior review and approval by the appropriate IRBs. After informed consent is obtained, participants will be assigned a screening identification number. Then, a case record form including demographic information, previous medication exposure, ART history, allergy history and physical examination will be completed. The

participant will be sent to the laboratory for venipuncture to obtain a complete blood count, ALT, creatinine, CD4 count, and HIV viral load. A total of 10-12 milliliters of blood will be drawn by venipuncture for this initial screening evaluation. Females will undergo urine pregnancy testing by providing a urine sample alone in a closed toilet area. The potential participant will then be given a follow-up appointment to return to the clinic in order to find out if the lab results are within the eligible range for participation in the clinical trial. Screening evaluations to determine eligibility must be completed within 20 days prior to study entry unless otherwise specified. If a results are delayed at the laboratory or results cannot be obtained from the collected specimen (clotting, mechanical error, etc.), the screening specimens may be re-drawn.

7.5.2 Enrollment

If the participant meets all of the inclusion criteria and none of the exclusion criteria, a study number (PID) will be assigned. After assignment of a PID, baseline measurements of visual acuity and WHO performance score will be assessed and recorded. A filter paper specimen will be obtained. The participant will then be escorted to the study pharmacy. Drug assignment will be made as described in the randomization section. For those randomized to receive TS or CQ, a supply of study drug will be given to each participant with instructions for how to take the medication. The first dose of the drug will be administered on the dispensary if the participant is assigned to an intervention arm. Participants will be asked to provide detailed directions to their home to facilitate active follow-up.

7.5.3 Follow-up

Follow-up visits may be one of three types: routine visit, unscheduled visit, or antimalarial efficacy follow-up.

Routine visits occur every 4-12 weeks (every 4 weeks for the first 24 weeks, then every 12 weeks thereafter). Initially, to ensure participant compliance with the study schedule and study drug regimen (if assigned to TS or CQ), participants will be followed every 4 weeks for the first 24 weeks. Thereafter, participants will be followed every 12 weeks, as is the standard of care for ART clinics in Malawi. Filter paper samples will be obtained. The encounter will include an interval history, regimen adherence questionnaire and pill counts, questions about off-study drug use and bednet use and a physical examination. In addition, study clinicians will record any adverse events that were not previously noted since the last visit. Females whose last menstrual period was more than four weeks prior or whose clinical evaluation suggests pregnancy will undergo urine pregnancy testing. At routine

visits every 24 weeks, and the final study visit, approximately 10-12 milliliters of blood will be drawn via venipuncture for measurement of CD4 count and HIV viral load as well as to measure CBC, ALT and creatinine; also during these visits visual acuity will be assessed and the WHO performance score will be recorded. If blood specimens are not able to be analyzed due to specimen clotting or equipment malfunction, a repeat blood draw will be done for the test that is needed. When participants are followed-up every 12 weeks, the study team will attempt to contact them via cell phone (if number is provided) every 4-6 weeks to ask about their health, and request that they come to the clinic for any current issues.

Unscheduled visits due to interval illnesses may occur at any time during the follow-up period. During unscheduled visits, the participant will be evaluated according to standardized procedures for the evaluation and treatment of illness as defined by the Ministry of Health. If bacteremia is suspected, participants will have blood collected for blood culture. If meningitis is suspected, participants will have cerebrospinal fluid collected for culture. Isolated pathogens will undergo drug susceptibility testing. Any signs or symptoms of malaria will prompt the collection of a malaria smear. If the participant is diagnosed with malaria, defined as any level of parasitemia with symptoms associated with malaria (fever documented or reported during the previous 48 hours, myalgia, weakness, pallor or headache), he or she will receive therapy for malaria according to Malawi national policy.

For patients diagnosed with uncomplicated clinical malaria, they will be enrolled in a 28-day efficacy study to include antimalarial efficacy follow up. Follow up will occur on days 1, 2, 3, 7, 14, 21 and 28. Finger prick specimens for malaria smear and filter paper collection will be obtained on every day of follow up. Participants who have clinical treatment failure will be administered rescue therapy according to the Malawi national guidelines. Cases of severe malaria will be referred to the hospital. Further evaluation and management will be undertaken by the study team according to national guidelines.

7.5.4 Discontinuation of Study Treatment Assignment

If a participant is diagnosed with a WHO clinical stage 3 or 4 illness during the course of the study, or if their CD4 count is <200 cells/mm^3 or they experience ART failure, they will be given daily TS prophylaxis and followed according to the study protocol. If they had been assigned to CQ prophylaxis, the CQ will be stopped. They will not be terminated from the study for this reason.

If a participant becomes pregnant during the study, she will be given TS prophylaxis and remain in the study. She will be treated according to Malawi national guidelines detailing the treatment of HIV in pregnancy, which currently includes daily TS prophylaxis. If she was previously randomized to daily TS, she will continue taking daily TS. If she was previously randomized to take weekly CQ, she will discontinue weekly CQ. If she was previously randomized to no prophylaxis, she will begin taking daily TS. Pregnancy outcome information will be recorded on a pregnancy outcome CRF.

7.5.5 Final Study Visit

The final study visit will occur from approximately 32-66 months after enrollment . A WHO performance score will be measured and approximately 10-12 milliliters of blood will be drawn by venipuncture for measurement of CBC, ALT, creatinine, CD4 count and HIV viral load. Additional assessments will be based on clinical findings.

At the final study visit, participants will discontinue their study treatment assignment and will receive prophylaxis according to Malawi Ministry of Health Guidelines. Management of their HIV care will be transferred to the Ndirande health center ART clinic or other government-sponsored ART clinic.

7.5.6 Early Termination Visit

Early termination may occur if participants choose to withdraw from the study. If early termination occurs, the procedures associated with a routine visit will be performed. In addition, CD4 count, HIV viral load, CBC, ALT and creatinine will be obtained and the WHO performance scale will be assessed if they were not done in the past four weeks. If study participants are terminated due to missed visits or loss to follow-up, every effort will be made to ascertain their clinical status for the duration of the expected follow-up after premature termination from the study.

7.6 Clinical management

All participant outpatient clinical management of HIV and other health issues will be housed at the Ndirande Health Center Research Clinic for the duration of the study. Clinical, diagnostic and therapeutic services will be provided for study participants at the Ndirande Health Centre Research Clinic during routine working hours. Study nurses and clinicians will provide outpatient clinical care under the supervision of study physicians and study coordinators. The study physicians and study coordinators will provide primary oversight of clinical aspects of the trial.

Specific detailed case definitions and standard treatment regimens are described in the manual of standard operating procedures. Diagnoses of illnesses experienced by participants will be documented based on updated criteria for diagnoses established by the ACTG group (Appendix E) when feasible in this setting. The treatment guidelines will be followed by study clinicians for outpatient treatments conducted at the Ndirande Health Centre Research Clinic, subject to modification according to the clinical judgment of the supervising physician.

If they require hospitalization, study participants will be admitted to the Queen Elizabeth Central Hospital or to Zomba Central Hospital. Criteria for hospitalization will be the same for all study participants, regardless of treatment assignment, and will be outlined in the study Manual of Procedures. Essential medications and basic diagnostic tests are provided at no charge by each hospital. In the event that specific essential medications routinely used at the hospital are temporarily unavailable due to periodic shortages that have been known to occur, the study will provide these essential medications for study participants from a stock that will be maintained for this purpose.

Participants who are diagnosed with malaria during the course of the study will be treated for malaria according to national guidelines, and they will continue their assigned study treatment during their treatment for malaria. TS will continue to prevent bacterial infections during antimalarial treatment and CQ should be continued to maintain the steady state of the drug.

7.7 Participant tracing and follow up

At enrollment, participants are requested to give the location of their home including a description of how to find it, as well as permission to come to the home to find them in the case of a missed visit or follow up as needed. Participants who do not return for scheduled visits, either for routine visits every 4-12 weeks (every 4 weeks for the first 24 weeks, then every 12 weeks thereafter) or follow up of a disease episode, will be sought at their home if they have previously given study staff permission to visit them at home. Study team members who seek participants at their home will identify themselves as staff from the Blantyre Malaria Project (BMP) or from Dignitas International and inquire about the participant. The study team will encourage a participant to return to the study clinic for procedures associated with the visit. If the participant is not found within one week of the missed visit, the tracing

team will search for them again at the time of the next routine visit. We will attempt to ascertain at minimum vital status for all participants who are lost to follow up.

8 ASSESSMENT OF SAFETY

8.1 Methods and Timing for Assessing, Recording, and Analyzing Safety Parameters

8.1.1 Adverse Events definitions

An Adverse Event (AE) is any untoward medical occurrence in a patient or clinical investigation participant administered a study product/intervention(s) and that does not necessarily have a causal relationship with this treatment. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) study product/intervention(s), whether or not related to the medicinal (investigational) study product/intervention(s).

An Unanticipated Problem (UP) is defined as an event (including onsite and offsite adverse event reports, injuries, side effects, breaches of confidentiality, deaths or other problems) that occurs any time during or after the research study, which in the opinion of the PI:

- Involves harm to one or more participants or others, or placed one or more participants or others at increased risk of harm AND
- Is unexpected AND
- Is related to the research procedures

Complications related to ART or HIV infection will not be considered UPs.

8.1.2 Adverse Event Procedures and Reporting Requirements

The occurrence of an AE may come to the attention of study personnel during study visits and interviews or by a study participant presenting for medical care, or upon review by a study monitor. Any medical condition that is present at the time of the participant is enrolled will be considered a baseline condition, and not reported as an AE. However, if it deteriorates at any time during the study it will be recorded as an AE.

AEs will be captured on the appropriate CRF. AEs will be recorded in study source documents, and an assessment of whether they are associated with the study drug, the ART regimen, an intercurrent illness or another cause will be made. Information to be collected includes event description, date of

onset, clinician's assessment of seriousness and severity, relationship to study product (assessed only by those with the training and authority to make a diagnosis, which would include MD, PA, Nurse Practitioner, Clinical Officer, DO, or DDS), expectedness and time of resolution/stabilization of the event. AEs will be followed to adequate resolution or stabilization. If the AE has not stabilized or resolved at the end of the study period, it will be followed for a maximum of one year after study completion.

Seriousness of Event: AEs will be assessed by study clinicians to determine the seriousness of the outcome of the event. The April 1996 International Conference on Harmonisation (ICH) guidance, "Good Clinical Practice: Consolidated Guidance," (ICH E6) defined a serious adverse event (SAE) as "any untoward medical occurrence that at any dose:

- Results in death**,**
- Is life-threatening,
- Requires inpatient hospitalization or prolongation of existing hospitalization,
- Results in persistent or significant disability/incapacity, or
- Is a congenital anomaly/birth defect."

"Important medical events that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the patient or may require intervention to prevent one of the outcomes listed in the definition above" may also be considered to be serious. (October 1994 ICH guidance (E2A), "Clinical Safety Data Management: Definitions and Standards for Expedited Reporting.")

Severity of Event: AEs will be assessed by the investigator using a protocol defined grading system in Appendix B and the DAIDS Adult Toxicity Tables. (http://rsc.tech-res.com/safetyandpharmacovigilance/) or locally derived normal ranges (for creatinine and ALT). For events not included in the DAIDS Adult Toxicity Tables or protocol-defined grading system, the following guidelines will be used to quantify intensity for clinical adverse events:

GRADE 1	**Mild**	Symptoms causing no or minimal interference with usual social & functional activities
GRADE 2	**Moderate**	Symptoms causing greater than minimal interference with usual social & functional activities
GRADE 3	**Severe**	Symptoms causing inability to perform usual social & functional activities

Grade 4	**Potentially Life-threatening**	Symptoms causing inability to perform basic self-care functions OR Medical or operative intervention indicated to prevent permanent impairment, persistent disability
Grade 5	**Death**	

When intensity changes occur more frequently than once a day, the maximum severity for the event should be listed.

Relationship to study products: AEs will have their relationship to study product assessed using the following terms:

- **Definitely related**. The adverse event and administration of study agent are related in time, and a direct association can be demonstrated.
- **Probably related**. The adverse event and administration of study agent are reasonably associated in time, and the adverse event is more likely explained by study agent than other causes.
- **Possibly related**. The adverse event and administration of study agent are reasonably related in time, and the adverse event can be explained equally well by causes other than study agent.
- **Probably not related**. A potential relationship between study agent and the adverse event could exist (i.e. the possibility cannot be excluded), but the adverse event is most likely explained by causes other than the study agent.
- **Not related**. The adverse event is clearly explained by another cause not related to the study agent.
- **Pending**. Pending may be used as a temporary relationship assessment only for death and only if data necessary to determine relationship to study agent are being collected. The site is required to submit a final assessment within 3 business days after reporting the death. If no final assessment is made within 3 business days after the date of submission, the event will be assessed as possibly related to study agent. Any additional information received at a later time, including an autopsy report, should be submitted as a Follow-up Report.

A suspected adverse drug reaction (SADR) is an adverse event that could potentially have a causal relationship to the study agent (definitely, probably, possibly, probably not related, or for deaths, pending).

Expectedness (Expected versus Unexpected)

Expected refers to the perspective of events previously observed, not on the basis of what might be anticipated from the pharmacological properties of the study agent. (ICH E2A) Expected adverse events related to study drugs are found in the following table:

Expected Adverse Events Related to Study Drugs

Study Drug	Expected Adverse Events
Trimethoprim-sulfamethoxazole	Rash, urticaria, loss of appetite, nausea, vomiting, agranulocytosis, aplastic anemia, disease of the hematopoietic system, fulminant hepatic necrosis, severe allergic reaction, Stevens-Johnson syndrome, toxic epidermal necrolysis
Chloroquine	Headache, malaise, dizziness, blurred vision, difficulty focusing, muscle weakness, electrocardiogram changes, gastrointestinal upset, mouth ulcers, diarrhea, vomiting, non-urticarial pruritis, leukopenia, methemoglobinemia, retinopathy

Unexpected refers to events whose nature or severity (intensity) is not consistent with those included in the package insert/summary. (ICH E2A) If an unexpected adverse drug experience is observed that is definitely or probably related to TS or CQ, it will be reported to the U.S. Food and Drug Administration using the MedWatch safety information and adverse reporting system via the online system at www.fda.gov/MedWatch/report.htm. An unexpected adverse drug experience is defined as any adverse drug experience that is not listed in the current labeling for the drug product. This includes events that may be symptomatically and pathophysiologically related to an event listed in the labeling, but differ from the event because of greater severity or specificity. "Unexpected," as used in this definition, refers to an adverse drug experience that has not been previously observed (i.e., included in the labeling) rather than from the perspective of such experience not being anticipated from the pharmacological properties of the pharmaceutical product.

8.2 Expedited Adverse Events

8.2.1 Expedited Adverse Event Reporting to DAIDS

Because this study will be conducted with licensed drugs used for approved indications and not under investigational new drug application, expedited adverse event reporting to DAIDS is not required.

8.2.2 Requirements for Adverse Event Reporting

Only serious, unexpected, related AEs and all deaths will be reported in this expedited manner.

Regulatory requirements for reporting to the University of Malawi, the University of Maryland and the DAIDS Clinical Representative will be observed. Reporting procedures will follow ICH 4.11, 5.17 and Clinical Safety Data Management: Definitions and standards for expedited reporting. AEs will be reported as described in the table below:

	DAIDS Clinical Representative	**University of Malawi**	**University of Maryland**
Contact information	e-mail to DAIDS clinical representative	Hand delivery or e-mail to College of Medicine Research and Ethics Committee (COMREC; comrec@medcol.mw)	Via CICERO (Collaborative Institutional Comprehensive Evaluation of Research Online) system
Related, unexpected SAEs	Within 48 hours	Within 48 hours	Within 5 days

9 CLINICAL MANAGEMENT

Study clinicians will receive advanced training in ART management and will continue professional education activities related to care of PLHIV throughout the study period.

9.1 Toxicity Management

Toxicity experienced by study participants will be managed according to the adverse event, clinical or laboratory finding associated with the study drug.

For TS, the WHO has established a grading scale for toxicity in adults and adolescents and a recommended treatment guideline at (http://www.who.int/hiv/pub/guidelines/ctxguidelines.pdf). Adverse events (AE) related to TS will be managed according to these guidelines. For Grade 3, TS will be discontinued until the event has completely resolved (usually 2 weeks) at which point reintroduction or desensitization can be considered, as described in the WHO guidelines. Other AEs associated with the study drug will be managed by the clinical coordinators and other investigators with the oversight of the independent safety monitor.

For CQ, participants who experience a worsening in visual acuity or other visual complaints, muscle weakness, or hearing defects while taking CQ will discontinue CQ prophylaxis and will be referred to the ophthalmology or appropriate clinic for further evaluation and treatment. They will be placed on TS prophylaxis after CQ is discontinued.

9.2 Other Disease Events

Study participants will be monitored closely for the development of clinical stage 3 and 4 HIV/AIDS-defining illnesses listed in Appendix A. If this should occur, then the participant will be given daily TS prophylaxis and followed according to the study protocol. These illnesses and others will be diagnosed based on updated published ACTG diagnostic criteria (Appendix E) when feasible in this setting.

For all other illnesses, study participants will be treated according to standard guidelines developed by the investigators and study staff.

9.3 Pregnancy

At each routine follow-up visit, women will be asked the date of their last menstrual period. If more than four weeks has elapsed since the last period and/or if physical examination or other clinical indicators suggest pregnancy, a urine pregnancy test will be done. Females who become pregnant during the study will discontinue their study medication and be given daily TS prophylaxis per Malawi Ministry of Health Guidelines through routine care and will continue in the follow-up schedule to contribute data for study endpoints included in the intention-to-treat analysis. At the end of pregnancy, these participants may have the study treatment assignment reintroduced if agreed upon by the participant and deemed appropriate by the study coordinator.

Female study volunteers of reproductive potential (women who have not been post-menopausal for at least 24 consecutive months, i.e. who have had menses within the preceding 24 months), or have not undergone surgical sterilization (e.g. hysterectomy, bilateral oophorectomy, tubal ligation or salpingectomy) must have a negative serum or urine pregnancy test performed prior to entry.

If participating in sexual activity that could lead to pregnancy, the female study volunteer must use a form of contraception: condoms (male or female) with or without a spermicidal agent, diaphragm or cervical cap with spermicide, intrauterine device or hormonal-based contraception during study duration.

If the female volunteer is not of reproductive potential (women who have been post-menopausal for at least 24 consecutive months, or women who have undergone surgical sterilization, e.g. hysterectomy, bilateral oophorectomy, or salpingectomy) she is eligible without requiring the use of contraception.

9.4 Breastfeeding

Participants in the study will be permitted to breastfeed while taking study medications. The current standard of care in Malawi for all PLHIV including pregnant and breastfeeding females calls for daily TS prophylaxis.[9]

The use of weekly CQ prophylaxis in breastfeeding mothers is expected to result in a maximum daily dose of 0.7% of the maternal start dose via breastfeeding.[26] Use of CQ is considered compatible with breastfeeding according to the American Academy of Pediatrics[27] and is considered safe for use in pregnancy. CQ is currently used for presumptive treatment and prophylaxis of pregnant women in

many tropical countries including Papua New Guinea.[28] Previous studies of 300mg weekly chloroquine prophylaxis in pregnancy demonstrate improved newborn birth weight and improved hematologic status in mothers at delivery.[29-31] No adverse effects to breastfeeding infants have been documented, but the effects of CQ on breastfed infants have not yet been systematically evaluated in a large patient population.

9.5 Criteria to Discontinue Treatment Assignment and Receive TS

- Confirmed CD4 count <200 cells/mm^3
- Development of antiretroviral treatment failure (confirmed viral load >1000 copies/ml[32])
- Development of a WHO stage 3 or stage 4 opportunistic infection

9.6 Criteria for Permanent Treatment Discontinuation

- Require chronic treatment with an antimalarial drug
- Study drug-related toxicity when reintroduction or desensitization cannot be considered per WHO guidelines
- Request by subject to terminate treatment
- Clinical reasons believed life threatening by the investigators such as severe allergic reaction, even if not addressed in the toxicity section of the protocol

9.7 Criteria for Premature Study Discontinuation

- Request by the subject to withdraw
- If the investigators find that the study is no longer in the best interest of the subject
- Volunteer judged by the site investigator to be at significant risk of failing to comply with the provisions of the protocol as to cause harm to self or seriously interfere with the validity of the study results
- At the discretion of the Study team/investigator, IRB, Office for Human Research Protections (OHRP), or NIAID

Study participants who discontinue their study drug assignment will remain in the study; study results will be analyzed on an intention-to-treat basis. Severe events that occur during a period of loss to follow-up and that are confirmed will be included in study analyses. Whenever possible, participants who withdraw or who are withdrawn from the study will be followed for intention-to-treat analysis.

10 STATISTICAL CONSIDERATIONS

10.1 Overview and General Design Issues

Several studies have demonstrated that daily trimethoprim-sulfamethoxazole (TS) prophylaxis reduces morbidity and mortality among PLHIV in sub-Saharan Africa.[1-4] The benefits appear to be due to prevention of opportunistic infections such as bacteremia, pneumonia and enteritis and in some circumstances, prevention of malaria. In this clinical trial we will assess whether it is safe to withdraw TS prophylaxis after immune reconstitution (≥6 months on ART), or TS prophylaxis continues to show a substantial protective effect. We also hope to determine if sustained viral suppression is related to preventing malaria, bacterial infections or both. Participants will include up to 1500 Malawian adults aged 18 years or older living with HIV in or near Blantyre, Malawi, Central Africa who have been receiving antiretroviral therapy for ≥6 months and have an undetectable HIV viral load and a CD4 count ≥250/mm^3. They will be randomized to one of three study arms: TS daily prophylaxis, CQ weekly prophylaxis and a control arm with no prophylaxis. TS prophylaxis is expected to prevent both malaria and bacterial infections. It is an effective prophylactic agent even with underlying high rates of bacterial and malarial resistance.[3;4;31] In this setting CQ is expected to be highly efficacious at preventing malaria, but not opportunistic infections. The study design is a randomized, controlled, clinical trial.

10.2 Study Endpoints

Primary Endpoint:

1. Occurrence of a severe event (death, WHO stage 3 and 4 events). The primary analysis will compare the distributions of time to first occurrence of a severe event in volunteers randomized to continuation of TS prophylaxis and to withdrawal of prophylaxis.

Secondary Endpoints:

1. Undetectable (<400 copies/mL) HIV viral load (yes or no) assessed every 24 weeks
2. Median CD4 cell count assessed every 24 weeks
3. Occurrence of any WHO HIV stage 2, 3 or 4 illness or death

4. Occurrence of infection with bacteria or malaria

5. Occurrence of ≥Grade 3 adverse events that require discontinuation of TS or CQ prophylaxis

Exploratory Endpoints:

1. Occurrence of infection with TS or CQ resistant organism

2. Clinical and parasitological response to antimalarial therapy in cases of uncomplicated malaria

10.3 Study Hypotheses

This study is designed as a non-inferiority trial. Our hypothesis is that it is safe to discontinue TS prophylaxis in PLHIV with no detectable viral load and CD4 count ≥ 250/mm3. – i.e., that in this population TS prophylaxis will provide no benefit in reducing the incidence of severe events, or else the benefit is too small to justify continued prophylactic use of TS. The statistical null hypothesis for the primary analysis is that the rate of severe events is at least 35% lower with TS prophylaxis than with no prophylaxis. Rejecting the null hypothesis will be considered evidence for non-inferiority of discontinuing TS to continuing TS, with a non-inferiority margin of a 35% preventive effect of continuing TS.

A secondary null hypothesis to be tested is that the rate of severe events is at least 35% lower with CQ prophylaxis than with no prophylaxis.

Additional hypotheses we plan to test are that the virologic response to ART is better among those on prophylaxis with TS or CQ, compared to individuals not on prophylaxis.

10.4 Primary and Major Secondary Analyses, Sample Size and Power

10.4.1 Analysis of severe events

We plan to randomize approximately 467 participants each to the continuation of TS and discontinuation of TS arms and approximately 467 participants to the discontinuation/CQ arm

(total=1401) during a 36-month enrollment period, and to follow participants for 32-66 months (average 49 months, if there is equal enrollment throughout the enrollment period). We assume that loss to follow-up will account for approximately 15% of the potential follow-up time, evenly distributed throughout the study, so that the potential follow-up time available (not accounting for the occurrence of severe events) will average 41.6 months per study participant, for a total of approximately 1619 person-years in each study arm. As of July 2014, our loss to follow-up rate is 3%, so our allowance for losses to follow-up seems reasonable or possibly conservative, given the duration of the study. If there are small differences in event rates or accumulation of person-time, we may enroll up to 1500 participants (500 per arm).

The primary analysis will compare continuation of TS and discontinuation of TS. It will consist of a 95% confidence interval (CI) for the effect of TS, relative to no prophylaxis, in preventing the occurrence of a severe event, based on proportional hazards (Cox) regression modeling. The TS effect is defined as 1-HR, where HR is the hazard ratio from the Cox regression model. The analysis will be done according to the intention-to-treat principle; that is, each individual's data will be analyzed according to the study arm to which the individual was randomized, regardless of the treatment and amount of treatment actually received. Study participants who develop a WHO clinical stage 3 or 4 illness, experience a sustained decline in their CD4 count below 200 cells/mm^3, or who experience ART failure will be given TS prophylaxis and will continue to be followed in the study.

The analytical method for comparing CQ prophylaxis and no prophylaxis (i.e., discontinuation of TS) will be the same as for comparing continuation and discontinuation of TS.

Secondary analyses will compare TS prophylaxis to no prophylaxis, and CQ prophylaxis to no prophylaxis, with respect to total number of severe events experienced (i.e., including multiple events in individual study participants), using Poisson regression modeling. TS and CQ prophylaxis will also be compared to each other with respect to first occurrence and all occurrences of severe events. We do not plan an adjustment for multiple comparisons.

For estimating the power of the study, the statistical null hypothesis is that the effect of TS prophylaxis in preventing the first occurrence of a severe event (WHO stage 3 or 4 event or death), relative to no prophylaxis, is at least a 35% reduction in the hazard rate for first occurrences over the study period. Our assumption (alternative hypothesis) for power calculation is that there is no preventive effect of TS, i.e., that HR=1. The comparison will be based on the upper limit of a two-sided 95% CI for the TS effect. An upper limit < 35% -- i.e., a reduction significantly less than 35% at the one-sided 2.5%

significance level – will be considered sufficient evidence for discontinuing TS prophylaxis in this population. Using a formula of Fleming[34], which is a generalization of a formula of Schoenfeld[35], for 80% power to reject the null hypothesis, approximately 170 subjects with a primary endpoint event would be needed in two groups. For equal hazard rates in all groups, the required total number of subjects with ≥ 1 event in the three study arms combined would be approximately 255. For 90% power, the numbers of subjects with events are 228 in two groups and 342 in the entire study.

Results of the study up to an interim database lock of July 2014 show an overall primary endpoint event rate of 6.8 severe events (WHO stage 3 and 4 events and deaths) per 100 person-years in all participants. If we assume that this rate is constant through the end of the study, then we would expect about 330 primary endpoint events will be documented (1619 person-years/arm x 3 arms x 6.8 events/100 person-years = 330). Assuming the number of severe events follows a Poisson distribution, the mean of the distribution for a participant with average follow-up time (41.6 months) will be 0.236 events, and the probability that the participant will experience at least one severe event will be 0.210. Assuming an equal incidence rate without prophylaxis (i.e., no effect of TS), we then expect for each comparison about 196 study participants in both study arms combined to experience at least one severe event, which gives us an expectation of approximately 85% power that the upper limit of the 95% CI for the effect of TS will be < 35%. If event rates are equal for all three study arms, the total expected number of subjects with ≥ 1 severe event is about 294.

For the secondary comparison of CQ prophylaxis to no prophylaxis, using the same hypotheses, methodology, and assumptions as above, the number of cases required for 80% power (approximately 170) and number of expected cases in the two study arms combined (approximately 196) are the same as for the comparison of continuation and discontinuation of TS.

10.4.2 Analysis of loss of viral suppression

Besides severe events, we are interested in assessing the value of TS and CQ prophylaxis in preventing loss of viral suppression. As for first occurrence of a severe event, separate comparisons of 1) TS continuation and discontinuation, 2) TS continuation and CQ prophylaxis, and 3) CQ prophylaxis and TS discontinuation will be done using Cox regression modeling to estimate two-sided 95% CIs for effect of prophylaxis. Since severe events are expected to occur mainly in participants who do not maintain their viral suppression (i.e., who develop a detectable viral load), we define minimally acceptable prophylaxis, as for occurrence of a severe event, as at least a 35% effect in preventing loss of viral suppression. Then the same numbers of losses of viral suppression are

needed for 80% power to obtain a CI for effect of prophylaxis with an upper bound < 35%, assuming there is no effect, as for first occurrence of a severe event. We expect the probability of maintaining viral suppression to be approximately 75% for participants on TS prophylaxis who have the average amount of follow-up (49 months).[39] Then about 75 participants in each of the study arms will be expected to lose their viral suppression during the study. The power to obtain an upper confidence limit < 35% for effect of prophylaxis is then about 75% for each of the comparisons.

10.5 Enrollment/Stratification/Randomization/Blinding Procedures

10.5.1 Enrollment

Individuals enrolled in the ART clinic in government sponsored clinics in Blantyre will be eligible after remaining on ART for six months. Screening will occur at the ART clinic where the participant is currently receiving therapy. Enrollment will occur at the Ndirande Research Clinic.

10.5.2 Stratification

There will be no stratification of enrollment.

10.5.3 Randomization Procedures

After a potential participant has given informed consent and screening procedures show that they are eligible as evaluated by a study staff member, they will be randomized to one of the three study arms in a 1:1:1 ratio. A potential participant's study file will be taken to the data management office, where randomization will occur online in real time via the 21 CFR part 11-compliant internet data entry system that facilitates both online and offline data entry with frequent uploads to a central database. A backup hard copy randomization scheme will be devised by the study statistician prior to study start in case of technology failure. The study statistician has no contact with participants or the study staff that cares for the participants. The study data entry staff onsite will enroll the participant by recording the study arm assignment and study identification number for the participant and return the form to study staff. After enrollment, the participant will meet with the study pharmacist or a study nurse assigned to the dispensary. This person will administer the first dose of the drug, if appropriate, and instruct the participant about the medication.

10.5.4 Blinding Procedures

Treatment arms will not be masked as each of the intervention arms has a different regimen (TS is once daily while CQ is once weekly). We have chosen not to conduct a blinded study with placebos because of the concern that the complicated placebo regimen may interfere with adherence to ART. Also, we would like the opportunity to assess the effect of each prophylaxis regimen on treatment adherence. Laboratory technicians will be blinded to the treatment assignment of the participants, although the clinical staff will have access to the treatment assignment. The assessment of WHO stage 3 and 4 events will be reviewed independently by a committee blinded to treatment assignment.

10.6 Participant Enrollment Follow-Up

A maximum of 1500 participants will be enrolled over approximately a three year period. Study participants will be followed for approximately 32 months after the end of enrollment (32-66 months total follow-up).

10.7 Time under Surveillance

Time under surveillance will be defined as the time from randomization to the date of the last visit. If the participant has no contact with the study team for up to 12 weeks during the first 24 weeks of follow-up or up to 16 weeks of follow-up after 24 weeks but resumes contact with the study team, that period of lost follow-up will still be considered under surveillance as data regarding the primary outcome will be collected and contribute to the final analysis. If a volunteer misses 17 or more consecutive weeks, then the volunteer can return to the study follow-up schedule, and we will attempt to capture the severe events that occurred. However, the period during which the participant was absent will be removed from the time under surveillance, and events identified as having occurred during that period will not be included in the per protocol analysis.

10.8 Data Safety and Monitoring

In addition to the regular safety monitoring conducted by the investigators, this trial will be reviewed at least annually by the NIH NIAID Data and Safety Monitoring Board (DSMB). An independent biostatistician and/or database management group will prepare the DSMB report for the scheduled DSMB meetings. Follow-up intervals of reporting will be at the discretion of the DSMB based on the

trends of the data, but are planned to occur approximately every six months for safety and once per year for efficacy. The NIH NIAID DSMB can meet more frequently as needed. The DSMB will be independent from the investigators and will have full unblinded access to all accumulating data. The biostatistician and/or database management group will prepare reports that include:

- Study accrual by month and by study site
- Eligibility violations
- Baseline characteristics
- Protocol adherence report
- Data completeness report
- Periodic summary adverse event report
- Primary and secondary endpoints summaries, overall and for key subgroups.

The initial DSMB review is planned specifically to assess safety data to assure there are not dramatic differences in survival between treatment groups, and to determine the appropriate frequency of DSMB reporting. If the trial executive committee or DAIDS Clinical Representative has efficacy or safety concerns, an unscheduled DSMB review will be requested.

10.9 Site Monitoring Plan

Site monitors under contract to the National Institute of Allergy and Infectious Diseases (NIAID) Division of AIDS (DAIDS) will visit participating clinical research sites to review the individual subject records, including consent forms, CRFs, supporting data, laboratory specimen records, and medical records (physicians' progress notes, nurses' notes, individuals' hospital charts), to ensure protection of study subjects, compliance with the protocol, and accuracy and completeness of records. The monitors also will inspect sites' regulatory files to ensure that regulatory requirements are being followed and sites' pharmacies to review product storage and management.

10.10 DSMB Analyses of Study Progress and Safety

The DSMB will review safety and enrollment data approximately every six months after the first study participant is enrolled and at other intervals if deemed necessary by the sponsor or study investigators with input from the DSMB. Data sets for these analyses will be created in a joint effort between the data management team and the independent statistician.

The major purpose of these analyses will be to review study progress and safety data. Safety outcome measures to be monitored include severe adverse events (SAEs) that are deemed probably or definitively associated with the study interventions and the total of SAEs in each group. The number of primary endpoint events (deaths, WHO stage 3 and 4 events) as well as ≥Grade 3 adverse events and the rates of discontinuation of TS or CQ prophylaxis will thus necessarily be included in the safety review.

Based on their assessment of study enrollment and the safety and tolerability of the prophylaxis, the DSMB will make a recommendation that the study continue with no modification; continue with modification (including the possibility of terminating one of the prophylaxis arms); or be discontinued, either permanently or temporarily pending further review.

TS prophylaxis has been studied in adults living with HIV in Africa, and the drug has been very well tolerated. If, however, an increased rate of drug-associated SAEs is found in either prophylaxis arm, we will reconsider the continuation of this study.

10.11 Analysis Plan

An extensive statistical analysis plan will be developed by the Statistical and Data Coordinating Center for the study. The statistical analysis plan will be reviewed and finalized with the study biostatistician, investigators and study sponsor prior to database lock and analysis. The following are the main analyses planned for the primary and secondary objectives.

10.11.1 Primary

Objective: To determine if prophylaxis with TS or CQ, compared to no prophylaxis is associated with improved morbidity and mortality among adults receiving ART beyond 6 months

Endpoint: Occurrence of a severe event (WHO stage 3 or 4 event or death)

Analysis: The primary analysis will be estimation of a two-sided 95% CI for the preventive effect of TS prophylaxis. TS discontinuation will be considered non-inferior if the preventive effect for severe events is <35%.The preventive effect is defined as 1-HR, where HR is the hazard rate (TS relative to no TS) estimated form a proportional hazards (Cox) regression model with a single 0-1 covariate for treatment arm (TS or no TS). Models with additional covariates (bednet use, adherence and CD4 cell count, and interactions with treatment arm) will be fit in secondary analyses.

All severe events, including multiple events within an individual, will be analyzed using Poisson regression modeling with adjustment for differential probabilities of a second or third event within an individual and for over-dispersion as necessary. Evaluation of all severe events will permit comparison to similar studies and facilitate evaluation of these important public health outcomes. The analyses will be done according to the intention-to-treat principle – i.e., including all available follow-up time from randomized participants, with participants allocated to treatment arms according to the randomization, regardless of treatment or amount of treatment actually received. In addition, one or more per protocol analyses may be done if there is substantial lack of adherence or administration of a treatment other than the one to which an individual was randomized.

Severe events that occur during a period of loss to follow-up and that are confirmed will be included in study analyses. We will also conduct an analysis that censors participants at the time they develop an indication to begin TS prophylaxis (e.g. low CD4 count, ART failure, etc), regardless of treatment assignment (i.e., participants on TS prophylaxis will also be censored at this point to avoid bias).

The same type of analysis as described above for TS continuation will be done to measure the effect of CQ prophylaxis, relative to no prophylaxis and also relative to TS prophylaxis, in preventing severe events.

10.11.2 Secondary

Objective: To assess the effect of prophylaxis with TS or CQ on the virologic, immunologic and clinical response to ART.

Endpoints:

Virologic*:* Undetectable (<400 copies/mL) HIV viral load (yes or no) assessed every 24 weeks.

Immunologic: CD4 cell count assessed every 24 weeks

Clinical: Incidence of WHO HIV stage 2, 3 or 4 defining illnesses or death

Analysis: To measure the effects of prophylaxis with TS or CQ on the virologic response to ART, we will compare loss of viral suppression in the TS continuation and TS discontinuation arms, and in the CQ prophylaxis and TS discontinuation arms, using Cox proportional hazards regression modeling as in the analysis described in Section 10.12.1 above for first occurrence of a severe event. We will also compare percentages of volunteers with undetectable HIV viral load (plasma HIV copy number of <400 copies per mL) in intervention and no-intervention arms using longitudinal logistic regression modeling with GEE methodology to allow for within-subject correlation of observations at different time

periods. The measured viral load will also be compared among the treatment groups, using longitudinal linear regression with GEE methodology. All these regression models (proportional hazards, logistic and linear) will include a categorical variable to indicate treatment group. The modeling will be done without covariates other than treatment group identification and also with presence or absence of malaria and interaction between treatment group and presence/absence of malaria included.

Similar comparisons as above will be done for CD4 counts. For proportional hazards and logistic regression analyses of CD4 count, a dichotomous variable will be defined indicating whether or not CD4 count is below a specified threshold value. The main threshold value of interest is 250 cells/mm^3, but various other threshold values will also be used: 150, 200, 300, 350, and 400 cells/mm^3.

Since follow-up times will vary among study volunteers, proportional hazards regression modeling will be used to compare the treatment groups with respect to incidence of an illness defined as WHO HIV stage 2, 3 or 4 or death.

Objective: To assess the efficacy of TS in preventing infection with bacteria or malaria.

Endpoint: Occurrence of all suspected and also laboratory-confirmed infection with bacteria or malaria.

Analysis: Incidence of overall infections and resistant infections will be compared between TS and no prophylaxis using Poisson regression analysis, as for the primary analysis. The analysis will incorporate variables indicating treatment group and bednet use as well as interaction between treatment group and bednet use.

The methods described above for primary and secondary analyses will also be used to compare the TS and CQ arms. The latter analyses will be considered exploratory.

Objective: To assess the safety and tolerability of TS and CQ prophylaxis.

Endpoint: Occurrence of ≥Grade 3 adverse events and rate of discontinuation of TS or CQ prophylaxis.

Analysis: Incidence of drug-related Grade 3, 4 and 5 events and incidence of discontinuation of TS or CQ prophylaxis will be calculated with 95% confidence intervals. The incidence rates will be

compared between the three treatment arms for ≥Grade 3 events and between the TS and CQ arms for discontinuation of prophylaxis using Poisson regression analysis.

10.11.3 Exploratory

Objective: To evaluate the effect of TS and CQ prophylaxis on the incidence of drug resistant organisms.

Endpoint: Occurrence of all bacterial infections with antibiotic resistant organisms

Analysis: Incidence of drug resistant organisms in the TS (bacteria and malaria) and CQ (malaria) and no prophylaxis arms will be compared using Poisson regression analysis.

Objective: To evaluate the efficacy of antimalarial treatment.

Endpoint: Clinical and parasitological response to antimalarial therapy in cases of uncomplicated malaria.

Analysis: To analyze the efficacy of antimalarial treatment, we will employ Kaplan-Meier 28-day survival estimated and compare using Cox proportional hazards, as recommended by the WHO (http://whqlibdoc.who.int/publications/2009/9789241597531_eng.pdf).

Intention-to-treat analysis will be used for all primary comparisons. This analysis will include all participants who were randomized, regardless of attendance of follow up visits and the duration of study drug administration. For participants who are no longer actively followed at the end of the study, every effort will be made to obtain the vital status. Per protocol analysis will be performed as a secondary analysis for those who complied with follow up visits at least every 8 weeks and study and ART medication. Analyses will be done in which data on study volunteers who switch from one study intervention to another are censored at the time they develop an indication for TS prophylaxis, regardless of treatment assignment.

11 DATA HANDLING AND RECORD KEEPING

The principal investigator is responsible to ensure the accuracy, completeness, legibility and timeliness of the data reported. All source documents will be completed in a neat, legible manner to ensure accurate interpretation of data. Black ink is required to ensure clarity of reproduced copies. When making changes or correction, the original entry will be crossed out with a single line and the change will be dated and initialed. The original should not contain erasures, overwrites, correction fluid or tape. Records will be kept in locked files.

Copies of the electronic CRF (eCRF) will be provided for use as source documents and maintained for recording data for each subject enrolled in the study. Data reported in the eCRF derived from source documents should be consistent with the source documents or the discrepancies should be explained.

DAIDS and/or it designee will provide guidance to investigators on making corrections to the source documents and eCRF.

11.1 Data Management Responsibilities

All source documents and laboratory reports will be reviewed by the study team and data entry staff to ensure that they are accurate and complete. Adverse events (AEs) must be graded, assessed for severity and causality, and reviewed by the PI, an investigator, or a clinical coordinator. Data collection is the responsibility of the clinical trial staff at the Blantyre Malaria Project Research Clinic and at Dignitas International under the supervision of a clinical coordinator and the investigators. During the study, the investigator must maintain complete and accurate documentation for the study. The Statistical and Data Coordinating Center for this study will be responsible for the data management, quality review, analysis, and reporting of the study data.

11.2 Source Documents and Access to Source Data/Documents

Appropriate medical and research records will be maintained for this trial, in compliance with ICH E6 and any applicable DAIDS policies. Study investigators and the clinical coordinator will have access to all study records. Study nurses and clinicians will have access to laboratory source documents and CRFs of participants who are being actively enrolled and followed. Laboratory staff will have access to

laboratory source documents. Data entry clerks and the Data Manager will have access to case record forms (CRF). Authorized representatives of DAIDS will be permitted to examine (and when required by applicable law, to copy) clinical records for the purposes of quality assurance reviews, audits and evaluation of the study safety and progress.

Standard case record forms (CRF) will be provided for each participant. Participants will be identified by the Participant Identification Number (PID). All data on the CRF will be recorded in black ink legibly. A correction will be made by striking through the incorrect entry with a single line and entering the correct information adjacent to it. The correction will be initialed and dated by the investigator or a designated, qualified member of the study team. Any requested information that is not obtained as specified in the protocol should have an explanation noted on the CRF as to why the required information was not obtained.

The central source document will be the CRF, which includes history, physical examination, laboratory data, and diagnosis as determined by a study physician. This document will be completed for all participants. Termination forms will be recorded for all participants who leave the study, indicating the reason for exit. Laboratory tests will be labeled with PIDs. Results from laboratory testing as noted in the protocol analyte list will be recorded in laboratory source documentation. The results will also be given to the study staff for review and to record in the CRF.

11.3 Quality Control and Quality Assurance

The site developed a protocol-specific quality management plan in conjunction with the University of Maryland Center for Vaccine Development Office of Regulatory Affairs and Quality Management. This plan will be in place for quality management including how the data will be evaluated for compliance with protocol, which documents will be reviewed and methods of training staff.

The study will be conducted at the Blantyre Malaria Project Research Clinic at Ndirande Health Centre and at Tisungane Clinic at Zomba Central Hospital in Zomba. Recruitment activities will occur at outpatient ART clinics at these sites. Laboratory testing will also be performed at certified laboratories for testing according to the protocol analyte list.

Site monitoring will be conducted by a DAIDS contractor to assure protocol compliance, ethical standards, regulatory compliance and data quality.

Following written SOPs, the monitors will verify that the clinical trial is conducted and data are generated, documented (recorded), and reported in compliance with the protocol, GCP, and the applicable regulatory requirements. Reports will be submitted to DAIDS on monitoring activities.

We will provide direct access to source data/documents, and reports for the purpose of monitoring and auditing by the sponsor, and inspection by local and regulatory authorities.

The Data Manager will implement quality control procedures beginning with the data entry system and generate data quality control checks that will be run on the database. Any missing data or data anomalies will be communicated to the site for clarification/resolution.

12 CLINICAL SITE MONITORING

Study monitoring will be conducted to ensure the safety and conduct of the study complies with 45 CFR 46, GCP and ICH Guidelines, and DAIDS guidelines. A monitoring plan will be developed by DAIDS and implemented by DAIDS Clinical Site and Study Monitoring Contractor. This monitoring plan will be described in a separate document.

The site monitors will visit participating clinical research sites to review the individual subject records, including consent forms, CRFs, supporting data, laboratory specimen records, and medical records (physicians' progress notes, nurses' notes, individuals' hospital charts), to ensure protection of study subjects, compliance with the protocol and accuracy and completeness of records. The monitors also will inspect sites' regulatory files to ensure that regulatory requirements are being followed and sites' pharmacies to review product storage and management.

13 HUMAN SUBJECTS PROTECTIONS

The investigator will ensure that this study is conducted in full conformity with the principles set forth in The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research of the US National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research (April 18, 1979) and codified in 45 CFR Part 46 and/or the ICH E6; 62 Federal Regulations 25691 (1997). Key study personnel will maintain training in human subjects protection and good clinical practice.

13.1 Institutional Board/Ethics Committee

This protocol, the informed consent document and any subsequent modifications will be reviewed and approved by the Institutional Review Board or Ethics Committee responsible for oversight of the study including the College of Medicine of the University of Malawi and the University of Maryland. The University of Malawi College of Medicine Research Ethics Committee (COMREC) will be considered the local IRB and the University of Maryland IRB will be considered a remote IRB (Michigan State University IRB will defer to University of Maryland IRB) for the purpose of reporting SAEs.[40]

13.2 Protocol Registration

Prior to implementation of this protocol, and any subsequent full version amendments, each site must have the protocol and the protocol consent form(s) approved, as appropriate, by their local institutional review board (IRB)/ethics committee (EC) and any other applicable regulatory entity (RE). Upon receiving final approval, sites will submit all required protocol registration documents to the DAIDS Protocol Registration Office (DAIDS PRO) at the Regulatory Support Center (RSC). The DAIDS PRO will review the submitted protocol registration packet to ensure that all of the required documents have been received.

Site-specific informed consent forms (ICFs) will be reviewed or approved by the DAIDS PRO, and sites will receive an Initial Registration Notification when the DAIDS PRO receives a complete registration packet. Receipt of an Initial Registration Notification indicates successful completion of the protocol registration process. Sites will not receive any additional notifications from the DAIDS

PRO for the initial protocol registration. A copy of the Initial Registration Notification should be retained in the site's regulatory files.

Upon receiving final IRB/EC and any other applicable RE approval(s) for an amendment, sites should implement the amendment immediately. Sites are required to submit an amendment registration packet to the DAIDS PRO at the RSC. The DAIDS PRO will review the submitted protocol registration packet to ensure that all the required documents have been received. Site-specific ICF(s) WILL NOT be reviewed and approved by the DAIDS PRO and sites will receive an Amendment Registration Notification when the DAIDS PRO receives a complete registration packet. A copy of the Amendment Registration Notification should be retained in the site's regulatory files.

For additional information on the protocol registration process and specific documents required for initial and amendment registrations, refer to the current version of the DAIDS Protocol Registration Manual.

13.3 Informed Consent Process

Informed consent is a process that is initiated prior to the individual's agreeing to participate in the study and continuing throughout the individual's study participation. Extensive discussion of risks and possible benefits of this therapy will be provided to the subjects and their families. Consent forms describing in detail the study interventions/products, study procedures, and risks are given to the subject and written documentation of informed consent is required prior to starting intervention/administering study product. Consent forms will be IRB-approved and the subject will be asked to read and review the document, or else verbally explained to the subject (verified by a witness). Upon reviewing the document, the investigator will explain the research study to the subject and answer any questions that may arise. The subjects will sign the informed consent document prior to any procedures being done specifically for the study. The subjects should have the opportunity to discuss the study with their surrogates or think about it prior to agreeing to participate. The subjects may withdraw consent at any time throughout the course of the trial. A copy of the informed consent document will be given to the subjects for their records. The rights and welfare of the subjects will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

13.4 Participant Confidentiality

Participant confidentiality is held in strict trust by the investigators, the staff and the study sponsor. This confidentiality is extended to cover testing of biological samples and genetic tests in addition to the clinical information relating to participants. The study protocol, documentation, data and all other information generated will be held in strict confidence. Only codes, not names, will be used on specimens and CRFs.

Documentation, data and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party, without prior written approval of the participant except as necessary for monitoring by the University of Maryland IRB or University of Malawi College of Medicine Research and Ethics Committee, the study sponsor, the National Institutes of Health or the United States Office for Human Research Protections.

The study monitors, University of Malawi College of Medicine Research and Ethics Committee (COMREC), IRBs or other authorized representatives of the sponsor may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records and pharmacy records. The clinical study staff will permit access to such records.

13.5 Study Discontinuation

The study may be discontinued at any time by the University of Maryland IRB, the University of Malawi College of Medicine Research and Ethics Committee (COMREC), NIAID, or other U.S. government agencies as part of their duties to ensure that research subjects are protected. If the study is discontinued, participants will be referred to the Ndirande Health Centre, Tisungane Clinic, Zomba Central Hospital or Queen Elizabeth Central Hospital for any further medical concerns.

13.6 Compensation

Participants will be compensated the cost of transport to and from the clinic for each visit (approximately US$2, subject to change based on inflation).

13.7 Exclusion of Children

Studies of prophylaxis for PLHIV have been conducted separately for children and adults. Independent of HIV status, the epidemiology of infectious diseases differs markedly between adults and children. HIV-infected children who are over two years of age represent a small subpopulation in Malawi, since most children with congenital infection do not survive through infancy. They likely have unique virologic and immunologic factors that have contributed to their long-term non-progression. In addition, despite being open to enrollment to persons of all ages, few children were enrolled in the Ndirande Incidence Study, so it is unlikely that adequate numbers of children could be enrolled in a clinical trial to permit meaningful analysis of this subgroup. We have therefore chosen not to include children in this study.

13.8 Future Use of Stored Specimens

Filter paper specimens, cryopreserved parasites, PBMCs and serum will be maintained after the study is complete if the participant has agreed to this during the informed consent process and indicated permission on the written document. Any analysis of these specimens that is outside the scope of the objectives of this protocol will be submitted for prior review and approval by the appropriate IRBs. Parasites, viruses and their genetic material may be unlinked for further analysis, without any identifying clinical information. Should a participant change their mind at any time and revoke authorization for specimen storage with identifying information, their remaining samples will be unlinked from identifying information prior to analysis or else destroyed at the participant's request.

14 PUBLICATION POLICY

Following completion of the study, the investigator may publish the results of this research in a scientific journal. The International Committee of Medical Journal Editors (ICMJE) member journals have adopted a trials-registration policy as a condition for publication. This policy requires that all clinical trials be registered in a public trials registry such as ClinicalTrials.gov, which is sponsored by the National Library of Medicine. Other biomedical journals are considering adopting similar policies. It is the responsibility of the study investigators to register this trial in an acceptable registry. Any clinical trial starting enrollment after 01 July 2005 must be registered either on or before the onset of subject enrollment.

15 SUBSTUDIES

Two substudies have been added to this protocol as of August 19, 2013.

15.1 Substudy 1: Effect of malaria infection on ART-resistant HIV virus subpopulation replication

Please see Appendix F for summary, specific aims and research strategy. This substudy will not seek additional informed consent from participants because the procedures require only laboratory analysis. Therefore, only samples from participants who agreed in their original consent to have their left over specimens used in the future and who meet the criteria of the substudy will be included.

15.2 Substudy 2: Mass cytometry analysis of T and B cell immune exhaustion in response to chronic malaria infection in HIV co-infected Malawian adults

Please see Appendix G for the summary, specific aims, and research strategy. This substudy will ask participants to sign a separate informed consent.

16 Plans For Distribution Of Research Findings To Study Community

In our twelve years of conducting research in Malawi, we have maintained close relationship with the local medical community. The PI and co-investigators regularly participate in local and national meetings in Malawi to discuss malaria and HIV issues as well as local research meetings at the University of Malawi College of Medicine. The findings will be presented at the annual College of Medicine Research Dissemination Day and at other appropriate conference venues. Study results will also be shared expeditiously with Malawian HIV and malaria control officials and the Malawi Ministry of Health and its local representatives. Results will be submitted for publication to local and international journals.

17 LITERATURE REFERENCES

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APPENDIX A: REVISED WHO CLINICAL STAGING FOR HIV/AIDS FOR ADULTS AND ADOLESCENTS WITH CONFIRMED HIV INFECTION

Based on WHO 2010 criteria found at:

http://whqlibdoc.who.int/publications/2010/9789241599764_eng.pdf

Clinical Stage 3

- Unexplained severe weight loss (>10% of presumed or measured body weight)
- Unexplained chronic diarrhoea for longer than one month
- Unexplained persistent fever (above 37.5°C intermittent or constant, for longer than one month)
- Persistent oral candidiasis
- Oral hairy leukoplakia
- Pulmonary tuberculosis
- Severe bacterial infections (e.g. pneumonia, empyema, pyomyositis, bone or joint infection, bacteraemia, severe pelvic inflammatory disease)
- Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis
- Unexplained anaemia (<8g/dl), neutropenia (<500/mm^3) and/or chronic thrombocytopenia (<50,000/mm^3)

Clinical Stage 4

- HIV wasting syndrome
- *Pneumocystis jiroveci* pneumonia
- Recurrent severe bacterial pneumonia
- Chronic herpes simplex infection (orolabial, genital or anorectal of more than one month's duration or visceral at any site)

APPENDIX A: REVISED WHO CLINICAL STAGING FOR HIV/AIDS FOR ADULTS AND ADOLESCENTS WITH CONFIRMED HIV INFECTION

- Oesophageal candidiasis (or candidiasis of the trachea, bronchi or lungs)
- Extrapulmonary tuberculosis
- Kaposi's sarcoma
- Cytomegalovirus infection (retinitis or infection of other organs, excluding liver, spleen or lymph nodes)
- Central nervous system toxoplasmosis
- HIV encephalopathy
- Extrapulmonary cryptococcosis including meningitis
- Disseminated non-tuberculous mycobacterial infection
- Progressive multifocal leukoencephalopathy (PML)
- Candida of trachea, bronchi or lungs
- Chronic cryptosporidiosis
- Chronic Isosporiasis
- Disseminated mycosis (histoplasmosis, coccidiomycosis)
- Recurrent septicaemia (including nontyphoidal *Salmonella*)
- Lymphoma (cerebral or B-cell non-Hodgkin)
- Invasive cervical carcinoma
- Atypical disseminated leishmaniasis
- Symptomatic HIV-associated nephropathy or symptomatic HIV-associated cardiomyopathy

APPENDIX B: SEVERITY OF GRADING AND USE OF NORMAL AND ABNORMAL LAB VALUES

	Grade 1	Grade 2	Grade 3	Grade 4
Hemoglobin	**8.5-10.0 g/dL**	**7.5-8.4 g/dL**	**6.5-7.4 g/dL**	**<6.5 g/dL**
Absolute Neutrophil Count	**1000-1300/mm3**	**750-999/mm3**	**500-749/mm3**	**<500/mm3**
Platelets	100,000 – 124,999/mm3	50,000 – 99,999/mm3	25,000 – 49,999/mm3	< 25,000/mm3
Creatinine	1.1 – 1.3 x ULN	1.4 – 1.8 x ULN	1.9 – 3.4 x ULN	≥ 3.5 x ULN
ALT	1.25 – 2.5 x ULN	2.6 – 5.0 x ULN	5.1 – 10.0 x ULN	> 10.0 x ULN

ULN = upper limit of normal

APPENDIX C: ANTIRETROVIRAL THERAPY IN MALAWI

The following table lists the current antiretroviral therapy regimens for adults in Malawi as of 2016:

Drug Regimen	**Drugs in the regimen**
First Line Regimens:	Tenofovir/Lamivudine/Efavirenz TDF/3TC/EFV Zidovudine/Lamivudine/Nevirapine AZT/3TC/NVP
Alternative First Line Regimens in case of side effects:	Abacavir/Lamivudine+Nevirapine ABC/3TC+NVP Abacavir/Lamivudine+Efavirenz ABC/3TC+EFV Zidovudine/Lamivudine+Efavirenz AZT/3TC+EFV Tenofovir/Lamivudine+Nevirapine TDF/3TC+NVP
Second Line Regimens:	Tenofovir/Lamivudine+Atazanavir/ritonavir TDF/3TC+ATZ/r Zidovudine/Lamivudine+Atazanavir/ritonavir AZT/3TC+ATZ/r
Third Line Regimen	Tenofovir/Lamivudine+Duranavir/ritonavir TDF/3TC+DRV/r Raltegravir+Lamivudine+ Duranavir/ritonavir RAL+3TC+DRV/r

This protocol will be amended to reflect changes and any other modifications in the ART regimen that are adopted in Malawi.

APPENDIX D: MEDICATION ADHERENCE QUESTIONNAIRE

National Significance (SPNS) Adherence Initiative (http://hab.hrsa.gov/abouthab/special/adherencemedication.html). An index based on the scoring of three simple questions is reportedly as good as or better than a longer self-report.

The SPNS questions are as follows:

1. How often do you feel that you have difficulty taking your HIV medications on time? By “on time” we mean no more than 2 hours before or 2 hours after the time your doctor told you to take it.
    1. All of the time
    2. Most of the time
    3. Rarely
    4. Never

2. On average, how many days PER WEEK would you say that you missed at least one dose of your HIV medications?
    1. Every day
    2. 4 to 6 days/week
    3. 2 or 3 days/week
    4. Once a week
    5. Less than once a week
    6. Never

3. When was the last time you missed at least one dose of your HIV medications?
    1. Within the past week
    2. 1 to 2 weeks ago
    3. 3 to 4 weeks ago
    4. Between 1 and 3 months ago
    5. More than 3 months ago
    6. Never

Scoring
>10 equals good adherence
<10 = poor adherence

APPENDIX F: SUBSTUDY 1 - Effect of malaria infection on ART-resistant HIV virus subpopulation replication

Title: Effect of malaria infection on ART-resistant HIV virus subpopulation replication

Persons living with HIV who have achieved viral suppression on ART may experience transient increases in viral load during malaria illness. Viral replication that takes place in a host on ART with previously undetectable HIV viral load may represent ART-resistant virus.

1. Introduction

HIV and malaria share a common distribution in that both are most prevalent in the poorest countries of sub-Saharan Africa. These two diseases exact a massive toll on life in Malawi and other countries in the region. The degree to which malaria and HIV interact is still unclear. A study by Kublin et al found that malaria infection is associated with transient increases in HIV viral load.[1] It is possible that the repeated episodes of malaria that people living in endemic regions like Malawi experience lead to increased viral replication causing more rapid disease progression or delayed immune recovery on ART. In people receiving ART, increased viral replication due to malaria may prevent viral suppression. Persistent viral replication, even at a low level, is critical for two reasons: it indicates that immunosuppression is likely on-going and it promotes the emergence of viral strains that are resistant to the therapy. While resistant viral strains are undesirable in any context, it is especially problematic in settings like Malawi where second-line therapy is not widely available due to the high cost.

2. Specific Aims

a. **Aim 1: To evaluate the prevalence of ART-resistance mutations before and after malaria infection in adults with durable control of virus replication on ART**
b. **Aim 2: To characterize sequence evolution in plasma virus among those with malaria infection compared to those without malaria infection**

3. Research Strategy

Significance

The role of antimalarial prophylaxis in the context of the ART regimen is an important issue now facing ART programs in Africa. Currently, the updated WHO recommendation calls for trimethoprim-sulfamethoxazole (TS) prophylaxis in persons living with HIV (PLHIV) with a CD4 cell cut off of $\leq$350 cells/mm^3 in areas where bacterial infection and malaria are prevalent.[2] However this recommendation is based on expert opinion and not on results of appropriately conducted

APPENDIX F: SUBSTUDY 1 - Effect of malaria infection on ART-resistant HIV virus subpopulation replication

randomized, controlled trials that have carefully evaluated the effects of malaria on HIV virus replication *in vivo*. The proposed work will provide an understanding of the longitudinal effects of malaria on ART resistance development. This knowledge is needed to inform policymakers of the consequences of malaria infection in persons on ART and the need for prophylaxis in areas where malaria is endemic. If results show that malaria infection promotes the development of ART-resistant HIV, this would advocate for targeted antimalarial prophylaxis in HIV-infected persons living in malaria-endemic areas. If findings indicate that malaria infection is not associated with the development of ART-resistant HIV, then this would indicate that other aspects of HIV preventive care be prioritized such as drug adherence counseling and nutrition.

Innovation

This study will be the first to evaluate the longitudinal impact of malaria infection on the development of ART-resistant HIV in a population of adults on ART living in a malaria-endemic area. It will provide evidence for or against genetic change and HIV resistance evolution following malaria infection in adults on ART, and will also illuminate the effect of malaria on HIV viral replication below levels commonly tested. This model could also potentially set a new standard for evaluation of other diseases that affect PLHIV to investigate the interplay of disease processes and to characterize the importance of preventive efforts.

Approach

Participants will be selected from among the current TSCQ Malawi study, a prospective, open-label, randomized study of daily trimethoprim-sulfamethoxazole or weekly chloroquine among adults on ART in Malawi. Study participants have non-detectable HIV viral load (<400 copies/mL), CD4 cell count of at least 250 cells/mm^3, and have been on ART for at least six months at study start. Participant dried blood spot (DBS) samples will be selected from the TSCQ Malawi study from among those who are not infected with malaria at baseline (n=100). DBS samples at baseline and after the malaria season begins will be compared among participants who acquire malaria infection (n=50) or who remain malaria negative (n=50) over the course of a malaria season. Plasma samples will be evaluated for the prevalence of non-nucleoside reverse transcriptase inhibitor (NNRTI) mutations using published methods.[3] Samples will be evaluated to document sequence evolution to characterize NNRTI resistance profiles before and after malaria infection, and among adults who

APPENDIX F: SUBSTUDY 1 - Effect of malaria infection on ART-resistant HIV virus subpopulation replication

acquire malaria infection compared to those who do not. Potential problems that may be encountered include a low prevalence of NNRTI resistance detection in this cohort, but this can be overcome by increased sampling of plasma from an expanded number of participants as a large cohort is currently being enrolled in the study. This will also allow us to establish a baseline rate of NNRTI resistance that is detectable and the expected sequence evolution to fuel sample size calculations for larger studies of ART resistance evolution as a result of malaria-HIV interactions.

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APPENDIX G: SUBSTUDY 2 - Mass cytometry analysis of T and B cell immune exhaustion in response to chronic malaria infection in HIV co-infected Malawian adults

Title: Mass cytometry analysis of T and B cell immune exhaustion in response to chronic malaria infection in HIV co-infected Malawian adults

1. Introduction

B and T cell exhaustion is a state of cellular dysfunction that occurs in conditions of chronic infection and results in inefficient effector function, expression of cellular inhibitory receptors, and, ultimately, ineffective disease control.[1,2] T cell exhaustion (described initially in $CD8^+$ followed by $CD4^+$ T cells) has been defined in mouse and human models; in particular as a response to chronic viral infections such as HCV and HIV.[1,3-5] Subsets of B cells and memory B cells (MBCs) have subsequently been found to express inhibitory and homing markers as well as exhibit a general hyporesponsiveness with reduced proliferation and a paucity of antibody production.[4,5] Recently, phenotypic B and T cell exhaustion have also been described in children with chronic *P. falciparum* (Pf) exposure.[2] HIV and malaria represent two of the most deadly infectious threats to humans worldwide and coexist in much of sub-Saharan Africa. While interactive effects of these two infections might vary by region, transmission intensity and age, it appears that HIV-associated immunosuppression does lead to increased clinical malaria episodes.[6-8] Repetitive and ongoing exposure to malaria is required to achieve and maintain acquired immunity.[9] The immunosuppression associated with HIV coupled with this inefficient acquisition of immunity to malaria may lower the threshold for symptomatic malarial disease in co-infected individuals.[8,10] We propose to examine the impact of malaria infection on immunologic exhaustion in asymptomatic HIV infected Malawian adults this pilot study designed to address the following hypotheses:

2. Specific Aims

a. **To measure the effect of malaria infection on T and B cell exhaustion among adults with HIV infection who are successfully treated with anti-retroviral therapy.**
   Hypothesis: Chronic P. falciparum infection in HIV-infected adults is associated with heightened immunologic T and B cell exhaustion compared to HIV-infected adults without Pf infection. These studies will involve longitudinal measurement at two time points, six

APPENDIX G: SUBSTUDY 2 - Mass cytometry analysis of T and B cell immune exhaustion in response to chronic malaria infection in HIV co-infected Malawian adults

months apart, of phenotypic markers of T and B cell exhaustion in a cohort of persons living with HIV (PLHIV) on ART and stratified to those with documented asymptomatic *Pf* parasitemia (n = 10) or aparasitemic individuals (n = 10). To reach 20 cases that meet these criteria, we may enroll up to 200 participants for this substudy. Results will also be compared to a cohort of HIV-negative, malaria-naïve adults (n = 5).

b. To measure the effect of malaria infection on B cell subset distribution among adults with HIV infection

Hypothesis: Chronic P. falciparum infection in HIV infected adults is associated with altered B cell subsets compared to HIV-infected adults without Pf infection. These studies will assess the distribution of B cell subsets at two time points, six months apart, to include naïve B cells, classic and atypical MBCs, as well as activated MBCs between adults with HIV infection on ART with or without documented *Pf* infection, and compared to healthy, HIV-negative, malaria-naïve adults.

3. Research Strategy:

Significance

Heightened awareness to the magnitude of the ongoing malaria and HIV crisis has taken on greater significance in the face of economic and political instability related to these poverty-promoting diseases.[11] The overlap of these two deadly infections in sub-Saharan Africa affects millions. To our knowledge, no systemic immunologic assessment of T and B cell immune exhaustion has been performed in Malawian PLHIV and very little is known about immunologic exhaustion in individuals with malaria.

Innovation

The proposed immunological investigations will elevate our understanding of the human immune response to Pf, HIV and to the interactive effect of dual infection. Conventional flow cytometry (CFCM) has been critical to the ability to describe complex immunologic processes; however, limitations of light spectrum and spectral overlap limit the practical analysis. Recently, the use of stable metal isotopes coupled to antibodies to bind single epitopes of interest, which are then detected via mass spectrometry (MFCM) allows for the simultaneous examination of vastly more biological parameters

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compared to CFCM. This increased dimensionality facilitates simultaneous examination of both the B and T cell arms of the immune system and allows for interactive interpretation on a scale not previously possible with CFCM. The current proposal aims to fill the wide gaps of knowledge in malaria and HIV immunity using the invaluable PBMC samples and malaria infection data collected longitudinally in a cohort of well characterized adult PLHIV in Malawi who have detailed clinical information gathered through intensive active and passive surveillance.

Approach

Rationale: Cellular exhaustion is a hierarchical process that involves stages in which effector cell function is lost, intrinsic inhibitory factors are expressed, and cell function ceases, limiting memory responses and resulting in impaired disease control.[1] T cells experience a stepwise loss of effector function (initially IL-2 followed by TNF-alpha and lastly IFN-gamma).[1] Sustained surface expression of cellular inhibitors such as programmed cell death-1 (PD-1), lymphocyte activation factor-3 (LAG-3), and CTLA-4 are associated with T cell exhaustion, leading ultimately to cellular apoptosis, as has been reported in HIV and malaria.[1,2,12] In B cells, expression of Fc receptor-like-4 (FcRL4) and the loss of CD21 in a subpopulation of MBCs has been described in conditions of poorly controlled HIV.[4] CD27 is a classic marker of MBC. Elevation of FcRL4 surface expression on $CD27^-$ "atypical" MBC,[13,14] and high surface expression of PD-1/LAG-3 in $CD4^+$ T cells has recently been described in conditions of chronic Pf exposure.[2] The transcription factor, T-bet, has been shown to drive $CD8^+$ T cell-specific HIV responses and suppress PD-1 expression, but its role is unknown in malaria.[15] ART may reverse some of these findings but alterations in MBC persist.[5] The loss of $CD4^+$ T cells in HIV may limit B cell costimulatory pathways resulting in diminution of antibody production and altered MBC response to co-circulating antigens such as malaria. Comprehensive studies are needed to address these hypotheses and illuminate immunologic interaction in dual infections.

Design and Methodology: PBMC will be drawn at baseline and after six months from subset of a cohort of Malawian adults living with HIV on ART (CD4 > 250 cells/mm^3) stratified to receive Bactrim (TS) prophylaxis, chloroquine (CQ) prophylaxis or no prophylaxis. Volunteers with asymptomatic malaria (likely from the 'no prophylaxis' group) will contribute PBMC. This cohort will be composed of 10 volunteers with two or more quantitative polymerase chain reaction (qPCR) assays prior to PBMC acquisition. A separate group of 10 volunteers without malaria infection (preferably from the 'TS

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group' due to known CQ immunomodulatory properties) will also contribute PBMC. A third group of 5 malaria-naïve, HIV negative volunteers will contribute PBMC as control under an existing protocol for immunological studies at the University of Maryland. Cells will be stained with a panel of 27 stable metal isotope-labeled monoclonal antibodies (mAbs), including 19 surface markers and 8 intracellular molecules. These metals are absent in cells, resulting in negligible background above instrument noise. Cell subsets to be studied include a T cell panel include surface staining for Th1, Th2, and T memory subsets (CD3, CD4, CD8, CD45RA, CD197 (CCR7)); a B cell panel (CD10, CD19, CD20, CD21, CD27, CD38, IgM, IgD, and IgG to enable the discrimination between naïve, switched and unswitched memory and antibody-secreting cells/plasmablast B cell subsets.[16-19]); Macrophages (CD14); and markers of immune exhaustion (PD-1, LAG-3, CTLA4, and FcRH4); as well as activation molecules (CD69, CD107); and 6 other intracellular epitopes reflecting intracellular activation states (IFN-gamma, TNF-alpha, IL-2, IL-6, IL-10, and the master Th1 regulator T-bet). In addition, a viability dye, Cisplatin,[20] as well as the DNA metalointercalator $^{191/193}$Ir to identify cells in MFCM, will be used to complete an initial 30-parameter panel. Conjugated mAbs will be purchased when commercially available (DVS Sciences) or will be conjugated in house using MaxPAR mAb conjugation kits. The Center for Vaccine Development (CVD) Immunology Section at the University of Maryland has a CyTOF® Mass Cytometer. List-mode FCS 3.0 data files will be analyzed using DVS.Cytobank (www.cytobank.org). Spanning-tree Progression Analysis of Density-normalized Events (SPADE); a method of detecting cell populations utilizing flow data files projected into "trees", will enable stratification of data into a cellular hierarchy. This technique minimizes the subjectivity of traditional biaxial gating strategies and allows for visualization of minor cell populations coupled with a color scheme denoting intensity using an agglomerative hierarchical clustering algorithm.

Aim 1: Identification and quantification of T and B cell markers of exhaustion: We hypothesize that markers of immune exhaustion will be seen in Malawian adults with HIV and will be further accentuated by infection with Pf as compared to healthy control volunteers. PBMC that have been thawed and stained with a single MFCM panel (described in *Design and Methodology*) will be analyzed as separate T cell ($CD3^+CD4^+$ and $CD8^+$), and B cell ($CD19^+CD20^+$) analyses. Markers of immune exhaustion (PD-1, LAG-3, CTLA4, and FcRH4) will be examined in cellular subsets. In the case of T cells, characteristics of exhaustion such as diminution of cytokine expression and increased expression of T cell inhibitory receptors will be examined in memory sub-populations, using

APPENDIX G: SUBSTUDY 2 - Mass cytometry analysis of T and B cell immune exhaustion in response to chronic malaria infection in HIV co-infected Malawian adults

hierarchical gating techniques. Similarly, B cell subsets will be examined, and markers of immune exhaustion documented. Results will be compiled by cohorts and three-way comparisons will be made between PLHIV versus Pf co-infected individuals, PLHIV vs. health controls, and co-infected individuals vs. healthy controls at two time points.

Aim 2: Identification and quantification of B cell subsets: In addition to surface marker expression of immunologic exhaustion, Pf has been associated with shifts in populations of MBCs including a distinct population characterized by a $CD10^{-}CD21^{-/lo}CD27^{-}$ phenotype, theorized to represent "exhausted" or atypical MBCs. Naïve mature B cells have the capability to expand to resting MBC, activated B cells or exhausted MBC. The loss of CD21 is a distinct characteristic of HIV disease progression.[4] Similarly, $CD21^{-}CD27^{-}$ atypical MBCs (a.k.a., exhausted MBC) have been described in asymptomatic Kenyan children with Pf,[2] and malaria-endemic Malian adults.[13] We hypothesize that adults infected with HIV will have evidence of enhanced activated B cells and atypical MBCs as compared to healthy controls. The additional role of co-infection with Pf in volunteers with HIV may accentuate the presence of these findings. In this aim, we will perform B cell gating strategies and examine mature ($CD19^{+}CD20^{+}CD10^{-}$) B cells with activated ($CD21^{-}CD27^{+}$), naïve ($CD21^{+}CD27^{-}$), atypical/exhausted ($CD21^{-}CD27^{-}$), or classical MBC ($CD21^{+}CD27^{+}$) phenotypes. We will compare results by study cohort, time point and *Pf* malaria status to examine immunologic differences in B cell subsets.

Statistical analysis of immunologic responses. The MFCM data will be normalized, subtracted from background and imported as FCS 3.0 files into DVS Cytobank or WinList for analysis. Three-way, phenotypic comparisons will be made between groups as described above. Cytobank allows for the generation of "heatmaps" enabling comparisons between fluorescence and MFCM measurements using log-converted ratios of the mean fluorescent intensities (MFI) of populations of interest versus the uninfected controls. MFCM data is converted to arcsinh values enabling one to deduce the mean replicates of a population of interest subtracted from the median scaled arcsinh value of a stimulated (or control) condition. Independent one-sample *t*-tests will be performed and p-values can be adjusted for multiple comparisons. Demographic and immunologic data will be stratified and evaluated by study cohort, time point and Pf malaria status. Mann-Whitney rank sum analysis will be used for continuous data not normally distributed and two-sided Student's *t*-test for timepoint data. Chi^2 analysis (Fisher's

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exact test (two-tailed) as appropriate) will be performed for categorical data. Multivariable Cox regression analysis will be utilized for time to event analysis. Significance is set at $P \leq 0.05$.

Potential Pitfalls and Alternative Approaches: The proposed single panel is comprehensive and economical. Accordingly, some important cellular inhibitors such as TIM-3, CD160, and T regulatory immunomodulatory factors are not included. Fortunately, this platform permits reanalysis of the comprehensive data generated to answer many additional questions. For instance, multifunctionality, a term referring to the ability of T cells to simultaneously produce multiple cytokines has been associated with increased protective responses against some infections, and is therefore generally accepted as an indicator of the 'quality' of T cell response.[21] Cytokines are being measured as part of the documentation of exhausted T cells but can be reanalyzed in the context of multifunctionality using Boolean gating and WinList FCOM applications. Malaria antigen-specific T cells may also be evaluated by stimulating PBMC with malaria antigen (i.e., apical membrane antigen 1 or circumsporozoite protein) available at the CVD. The key investigators are skilled with all presented applications and software and have generated preliminary data for a similar project in a cohort of Malian children. The CVD also has an LSR II SORP 4 and sorters on site so that CFCM can be performed for validation purposes.

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