EFFECTS OF COTRIMOXAZOLE WITHDRAWAL ON MALARIA PARASITEMIA PREVALENCE, PARASITE DENSITY AND MULTIPLICITY OF INFECTION IN HIV-INFECTED INDIVIDUALS WITH IMMUNE RECOVERY FOLLOWING ANTIRETROVIRAL TREATMENT IN HOMABAY COUNTY

BY

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DECLARATION

I declare that what has been presented here is my own original work and has not been presented in any other institution or university for the award of degree. This thesis, or any part of it, may not be reproduced without written permission from the author, my supervisors and Maseno University.

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We (the supervisors) confirm that the research findings in this thesis were carried out by the said candidate under our supervision.

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To my loving family, Anthony Ottichilo and Mable Ottichilo, Brother Edwin and Sister Stellah Ottichilo. In appreciation for their encouragement and overwhelming support.

ABSTRACT

Strategies to reduce HIV-related morbidity and mortality include scale up of Antiretroviral Therapy (ART) and provision of broad spectrum antibiotics. Cotrimoxazole (CTX) is a widely available low cost antibiotic recommended by WHO in settings with high infectious disease prevalence for treatment and prevention of opportunistic infections and malaria in all HIV-infected individuals. With immune reconstitution following ART, the risk of opportunistic infections greatly diminishes. Continuing CTX indefinitely raises concern about persistent antibiotic exposure, resistance and pill burden. The effect of CTX discontinuation on malaria remains undefined. This blinded Randomized Controlled Trial (RCT) investigated the effect of CTX discontinuation on malaria parasite prevalence, incidence, parasite density and Multiplicity of Infection (MOI) on HIV-infected adults with evidence of immune reconstitution. Five hundred participants were enrolled from Homabay County hospital and randomized into two study arms: discontinue CTX vs continue CTX. They were followed quarterly for 12 months and whenever they reported sick between February 2012 and September 2013. Blood was drawn from study participants at each visit and spotted on paper blots with subsequent DNA extraction. Malaria parasites were detected by qRT-PCR and MOI evaluated by nested PCR targeting MSP-1 (K1, MAD20 and RO33) and MSP-2 (FC27 and IC3D7) alleles. Chi-square was used to test differences in parasitemia prevalence over time between study arms. Where parasites were detected, parasite density values were log-transformed, and the difference between arms tested using generalized estimating equations. The frequency of mixed infections (MOI >1) was compared between the two study arms. Rates of parasitemia in the two study cohorts were calculated on the basis of person-time at risk. Among the 500 participants in the RCT, median $CD4^+$ count was 595 cells/mm^3 and the median ART duration was 4.5 years. Parasite prevalence at enrolment was: 4% for discontinue CTX arm and 6% for continue CTX arm. Within 3 months of CTX discontinuation, parasitemia prevalence increased steadily in the discontinue CTX arm during the year to >5-fold: 4% (11/248) at M3, 8% (21/249) at M6, 14% (33/244) at M9 and 22% (54/245) at M12. In comparison, the continue CTX arm had parasitemia prevalence of <1% (1/248) at M3, 2% (5/247) at M6, 2% (4/245) at M9 and 4% (10/245) at M12 ($P < 0.0034$). Post enrollment, discontinue CTX arm had 90 new infections compared to 23 in the continue CTX arm. Parasitemia incidence was 42.0 in discontinue CTX arm versus 9.9 per 100 person years in continue CTX arm; with an incident rate ratio of 4.3 (95% CI: 0.14-0.37; $p<0.001$). Among follow-up visits where parasites were detected, the discontinue CTX arm had a significantly higher mean parasite density (log10) than the continue CTX arm (4.42 parasites/mL vs. 3.13 parasites/mL, $p < 0.001$). Post enrolment, mixed infections (MOI >1) were only present in the STOP-CTX arm. Results from this study indict despite immune reconstitution following ART provision and use of ITNs. Discontinuation of CTX prophylaxis in individuals with HIV results in increased parasite prevalence, incidence, parasite density and MOI overtime, in malaria endemic regions. The increased malaria incidence seen in those who discontinue prophylaxis, is not due to a short-lived rebound effect following withdrawal of CTX but continues for a longer period. Therefore stopping CTX prophylaxis may not be recommended in the context of malaria in resource-limited settings.

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LIST OF ABBREVIATIONS AND ACRONYMS

Acronym	Definition
ART	Antiretroviral Therapy
AIDS	Acquired Immunodeficiency Syndrome
ACT	Artemisinin-based Combination Therapy
DHFR	Dihydrofolate Reductase
DHPS	Dihydropteroate Synthase
CD4	Cluster of differentiation 4
CTX	Co-trimoxazole
CE	Capillary Electrophoresis
CSP	Circumsporozoite Protein
DNA	De-oxyribo Nucleic Acid
GEE	Generalized Estimating Equations
GLURP	Glutamate Rich Protein
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immuno-deficiency Virus
KEMRI	Kenya Medical Research Institute
MOI	Multiplicity of Infections
MSP-1	Merozoite Surface Protein-1
MSP-2	Merozoite Surface Protein-2
POP-7	Performance optimized polymer
q-RTPCR	Quantitative Real Time Polymerase Chain Reaction
RBCs	Red Blood Cells
RFU	Relative Fluorescent Unit
C_T	Threshold cycle
USA	United States of America
USAMRD	United States Army Medical Research Directorate
USAMRMC ORP HRPO	United States Army Materials and Research Medical Command, Office of Research Protections, Human Research Protection Office
WHO	World Health Organization
WRAIR	Walter Reed Army Institute of Research

OPERATION OF TERMS

Term	Definition
Immune reconstitution	Rapid and sustained rises in absolute CD4 counts following effective ART.
Multiplicity of Infections	The number of genetically distinct plasmodium parasite types simultaneously infecting an individual
Parasitemia	Parasitemia in this study was defined as any parasites detected in both study arms

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CHAPTER ONE

INTRODUCTION

1.1 Background information

HIV remains one of the most common infections in sub Saharan Africa. Approximately 22 million people are infected and 72% of AIDS-related deaths worldwide occur in sub-Sahara Africa (WHO, 2006). Immunosuppression resulting from HIV infection predisposes HIV infected individuals to community acquired opportunistic infections such as Malaria, *Pneumocystis jirovecii pneumonia*, *Toxoplasma gondii*, and *Isospora belli* (AIDSinfo, 2013; Korenromp *et al.,* 2005). Strategies to reduce morbidity and mortality in HIV infected individuals due to opportunistic infections have involved the scale up of antiretroviral therapy as well as provision of broad spectrum antibiotics for prophylactic interventions.

Co-trimoxazole, a fixed-dose combination of sulfamethoxazole and trimethoprim (TMP/SMZ), is a widely available, off-patent, low-cost antibiotic that is used in resource-limited settings (WHO, 2006). The use of CTX either as primary or secondary prophylaxis for prevention of opportunistic infections has been part of the standard care in the management of HIV-infected individuals since the early 1990s. This is supported by several studies that showed significant beneficial effects of CTX on morbidity and mortality amongst people with early as well as advanced HIV (Anglaret *et al.,* 1999; Grimwade *et al.,* 2003; Watera *et al.,* 2006; Wiktor *et al.,* 1999).

Recommendations for CTX use in HIV infected adults vary by setting. In the United States and Europe, CTX is recommended for HIV infected adults with severe immunosuppression ($CD4^+$ <200)(Benson *et al.,* 2004; McNaghten *et al.,* 1999). Following Antiretroviral Therapy (ART), CTX can be discontinued once immune reconstitution is documented (Benson *et al.,* 2004; Furrer *et al.,* 1999; Weverling *et al.,* 1999). In Africa, infections such as

malaria, bacterial pneumonia and diarrhea remain common in both the general and HIV-infected population (WHO, 2006). Therefore, CTX is recommended at higher $CD4^+$ counts. For countries with high prevalence of HIV and limited health infrastructure, the WHO endorses universal CTX for all HIV-infected individuals (WHO, 2006). Notably, these guidelines were created prior to the scale-up of ARTs. Presently in sub-Saharan Africa, there are marked differences in national policy regarding CTX prophylaxis in the context of ARVs, with some countries such as Kenya and Uganda continuing prophylaxis indefinitely and others recommending discontinuation when patients meet certain clinical thresholds (ART use for 12 months and a $CD4^+ > 350$ cells/mm^3) (WHO, 2006). Continuing CTX prophylaxis indefinitely however, raises concerns such as increases in cost of care (Lara *et al.*, 2012), risk of haematological toxicity (Moh *et al.,* 2005), hypersensitivity skin reactions (Mermin *et al.,* 2004), pill burden and unnecessary antibiotic exposure. Once started on ART, patients' immune function improves and the risk of opportunistic infections reduces (Kasirye *et al.,* 2015). The threshold for CTX discontinuation following ART remains undefined in limited resource settings.

Sub-Saharan Africa carries a high burden of both HIV and malaria, thus co-infection is common in many areas (Alemu *et al.,* 2013). HIV infection, through immune suppression, affects the acquisition and persistence of immune response to malaria (Van *et al.,* 2008). Cotrimoxazole, is well known as an antibacterial drug but less as an antimalarial, however its antimalarial activity which is similar to pyrimethamine-sulfadoxine (SP) cannot be ignored. Several clinical trials have reported CTX efficacy against malaria both in children and adults (Thera *et al.,* 2005; Walker *et al.,* 2010). In the 70 s and 80 s, CTX was reported to be as effective as chloroquine for treatment of malaria. Parasite clearance rates were similar but fever clearance rates were higher in the chloroquine group due to its antipyretic properties. (Wilkinson *et al.,*1973). The antimalarial effects of CTX are dependent on its ability to

inhibit parasite dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), thereby blocking parasite folate synthesis (Walker *et al.,* 2010). The fact HIV individuals have to be on CTX prophylaxis indefinitely points to some form of protection against malaria parasitemia attacks due to its antimalarial properties. Studies show high parasite density contributes to the pathogenesis of severe malaria (Bejon *et al.,* 2007; Gonçalves *et al.,* 2014). The mean parasite density is substantially higher during episodes of severe malaria than during mild or asymptomatic infections (Gonçalves *et al.,* 2014). Therefore in the context of malaria, the effect of discontinuing CTX prophylaxis on malaria prevalence and parasite burden in patients on ART and with evidence of immune reconstitution has needs to be determined. A prior CTX discontinuation study on HIV infected individuals in Uganda was stopped only four months after commencement, due to observed high rates of malaria and diarrhoea (Campbell *et al.,* 2012). Additionally, rates and quantity of parasitemia were not determined; nor was timing of increased parasitemia evaluated. Therefore, there is a need for a study with longer follow-up period, to determine whether the increased malaria incidence seen in those who discontinue prophylaxis, is due to short-lived rebound or would continue for a longer period.

Natural infections with *Plasmodium falciparum* are often composed of multiple concurrent genetically distinct parasite clones (Pinkevych *et al.,* 2015). Such multiclonal infections are more common in areas of high transmission. This scenario of multiclonal infections is often referred to as Multiplicity of infection (MOI). Multiplicity of infection is simply defined as the number of genetically distinct clones in an infection. Multiplicity of infection may be predictive of the individual's subsequent risk of clinical malaria (Pinkevych *et al.,* 2015; Vafa *et al.,* 2008). Multiplicity of infection poses numerous challenges to malaria control as recombination between genetically distinct parasite clones (out-crossing) is a major risk factor for generating novel parasite variants with clinically important phenotypes such as

virulence, drug resistance or immune evasion (Auburn *et al.*, 2012). It is therefore necessary to determine the effect of CTX discontinuation on *P.falciparum* clonal diversity, it is possible withdrawal of prophylaxis will render HIV infected individuals susceptible to clones they were previously protected against and this may have an effect on the individual's subsequent risk of clinical malaria.

1.2 Statement of the problem

The use of CTX is now part of the standard care and management of people living with HIV. WHO recommends use of CTX as prophylaxis against opportunistic infections, for all HIV-infected individuals regardless of clinical stage, in areas high HIV prevalence and limited health infrastructure. Notably, these guidelines were created prior to the scale-up of Antiretroviral Therapy (ART). With immune reconstitution following ART, the risk of opportunistic infections diminishes greatly. Opinions differ on merits and demerits of continuous use of CTX in immune reconstituted individuals. Advantages cited by proponents of stopping CTX prophylaxis include prevention of drug toxicities, decrease of the pill burdens and decrease to the risk of microorganism’s resistance. Malaria and HIV infection are each responsible for staggeringly high morbidity and mortality in sub-Saharan Africa. In regions where both diseases are endemic, HIV infection may increase the burden of malaria by increasing the susceptibility to infection. Cotrimoxazole although not recommended for malaria prophylaxis, similar to pyrimethamine-sulfadoxine, it has anti-malarial activity. The effect of discontinuing CTX prophylaxis on malaria incidence in patients on ART is not well defined. A prior CTX discontinuation study on HIV infected individuals in Uganda was stopped only four months after commencement, due to observed high rates of malaria and diarrhoea in individuals who stopped CTX prophylaxis. Rates and quantity of parasitemia were not determined; nor was timing of increased parasitemia evaluated in this study.

1.3. Objectives

1.3.1 General objective

To determine the effect CTX withdrawal has on malaria parasitemia prevalence, parasite density and multiplicity of infection in HIV-infected individuals with immune recovery following ART.

1.3.2 Specific objectives

1. To determine the effect of CTX withdrawal on malaria parasitaemia prevalence and incidence, in HIV-infected individuals with immune recovery following ART.
2. To determine the effect of CTX withdrawal on malaria parasite density, in HIV-infected individuals with immune recovery following ART.
3. To determine the effect of CTX withdrawal on Multiplicity of malarial Infection, following CTX withdrawal, in HIV-infected individuals with immune recovery following ART.

1.3.3 Null hypothesis (H_0)

1. There are no differences in malaria parasitaemia prevalence and incidence in HIV infected individuals, with immune recovery randomized to continue or discontinue CTX.
2. There are no differences in parasite density in HIV infected individuals, with immune recovery randomized to continue or discontinue CTX.
3. There are no differences in Multiplicity of Infection in HIV infected individuals, with immune recovery randomized to continue or discontinue CTX.

1.4 Significance of the study

The control and management of opportunistic infections in HIV infected individuals on antiretroviral treatment entail use of CTX prophylaxis. Existing evidence suggests that

individuals with HIV are more likely to become infected with malaria, and additionally likely to suffer high parasite burden (Alemu *et al.,* 2013). By all measurements (parasitemia prevalence, parasite density, parasitemia incidence and multiplicity of infection) over the 12 months observation period, the study participants who discontinued CTX prophylaxis had higher malaria burden compared to the subjects on CTX prophylaxis. This study demonstrates clearly, the increased incidence of malaria infections in HIV individuals who discontinue CTX is not due to a transient rebound after withdrawal of the long term conferred protection, but continues for a longer period. This suggests that despite immune reconstitution malaria endemicity may be the most relevant factor to consider in the decision to stop CTX.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria and HIV co-infections

Human Immunodeficiency Virus (HIV) and malaria infections often coexist in patients in many parts of the world due to wide geographic overlap. This is particularly true in sub-Saharan Africa, where an estimated 40 million people are living with HIV and more than 350 million episodes of malaria occur yearly (Hewitt *et al.,* 2006). The interaction between malaria and HIV infections is of global health importance. On one hand, HIV-related immunodeficiency results in infected individuals being more vulnerable to malaria, likely to suffer high parasite burden and exhibit reduced efficacy to antimalarial drugs (Ezeamama *et al.,* 2012) On the other hand, malaria is a powerful stimulator of the immune system and individuals exposed frequently to malaria have enhanced serum levels of immunoglobulins, an accelerated rate of IgG turnover and high T-cell turnover (Cohen *et al.,* 2005) . Therefore, malaria has an adverse effect on HIV infection both by stimulating the T-cell turnover and by impairing T-cell cytotoxic function, resulting in increased viral replication and thus HIV disease progression (Ryan-Payseur *et al.,* 2011). This interaction needs to be further investigated in a context where access to CTX prophylaxis and antiretroviral therapy could have a protective effect against malaria in HIV infected individuals. Although not defined as an opportunistic disease (Kyeyune *et al.,* 2014), a malarial episode may become more serious with HIV influence as reported by many studies (Sandison *et al.,* 2011; Walker *et al.,* 2007; Laufer *et al.,* 2006).

2.1.1 Cotrimoxazole use in HIV infected individuals

Cotrimoxazole (CTX), fixed-dose trimethoprim-sulfamethoxazole, is a low-cost and widely utilized broad spectrum antibiotic used to prevent opportunistic infections in patients with HIV. Prophylaxis with CTX has been shown to decrease mortality, morbidity, and hospitalizations among HIV-infected adults (Anglaret *et al.,* 1999; Lowrance *et al.,* 2007) and children (Bwakura-dangarembizi *et al.,* 2014), primarily by decreasing rates of malaria, pneumonia, and diarrhea, as well as severe bacterial infections, even in settings with high prevalence of CTX resistance(Campbell *et al.,* 2012; Walker *et al.,* 2010).

Recommendations for CTX use in adults with HIV vary by setting. In the United States and Europe, CTX is recommended for HIV infected adults with severe immunosuppression (CD4 count < 200 cells/mm3) to prevent Pneumocystis jiroveci pneumonia and toxoplasmosis (McNaghten *et al.,* 1999). Following antiretroviral therapy, CTX can be discontinued once immune reconstitution is documented (CD4 count > 200 cells/mm3) (Aberg *et al.,* 2014; Furrer *et al.,* 1999). In resource-limited countries, the threshold for CTX discontinuation following ART remains undefined. The 2006 WHO guidelines recommend CTX prophylaxis for HIV infected patients with a CD4 cell count of <350 cells/mm^3 (WHO, 2006). For settings with a high prevalence of HIV and limited health infrastructure, WHO guidelines recommend that all HIV infected adults take CTX prophylaxis (WHO, 2006). In Africa, infections such as malaria, bacterial pneumonia remain common in both the general and HIV-infected population. Therefore, CTX is recommended at higher CD4 counts to decrease mortality and morbidity in HIV-infected individuals (Walker *et al.,* 2010; WHO 2006). However, these guidelines were developed prior to the scale-up of ART. The increased use of ART in sub-Saharan Africa warrants re-examination of this policy. Potential benefits of CTX discontinuation include lower risk of drug toxicity or drug–drug interactions, decreased risk of antimicrobial resistance, decreased costs of care, and lower pill burdens for patients. Only

one other randomized control trial to date has examined CTX discontinuation after ART-initiation in adults. However, the study was halted by the investigators at the recommendation of the Data Safety Monitoring Board after just 4 months, due to observed high rates of malaria and diarrhoea in individuals who stopped CTX prophylaxis (Campbell et al., 2012). Rates and quantity of parasitemia were not determined; nor was timing of increased parasitemia evaluated in that study, thus the need for a study with longer follow-up period. It is also not clear whether the incidence of malaria after CTX discontinuation occurs at similar levels as the HIV-uninfected local population or if while on CTX, the loss of natural immunity leads to a 'rebound' increase in malaria when CTX is stopped. Moreover, it is also uncertain if these findings from Uganda would generalize to other African settings with varied malaria endemicity.

2.2. Cotrimoxazole prophylaxis and malaria prevalence

Cotrimoxazole is a drug combination made up of Trimethoprim and Sulfamethoxazole. Trimethoprim (2, 4-diamino-5-(3, 4, 5 trimethoxybenzyl pyrimidine) fall under a group of compounds with antibacterial and antimalarial activity (Manyando *et al.*, 2013). It is an inhibitor of dihydrofolate reductase and has been shown to act as a sulfonamide potentiator (Bushby *et al.,* 1968). Similar to Sulfadoxine-pyrimethamine (SP) Trimethoprim and Pyrimethamine bind and inhibit dihydrofolate reductase (DHFR), whereas Sulfamethoxazole and Sulfadoxine target dihydropteroate synthase (DHPS) leading to impaired thymidine biosynthesis. A 1:5 combination of trimethoprim (8 mg/kg bodyweight) and sulfamethoxazole (40 mg/kg bodyweight) is documented to effectively treat chloroquine-resistant *Plasmodium Falciparum* infections (Fasan *et al.,* 1971). The efficacy of CTX in lowering the prevalence of malaria cases has been well-documented in clinical trials in children (Kamya *et al.,* 2007), pregnant women (Kapito-Tembo *et al.,* 2011) and adults (Thera *et al.,* 2005). Cotrimoxazole has been shown to be 99.5% effective in averting malaria

versus 95% effectiveness with Sulfadoxine/Pyrimethamine, and both have about 80% therapeutic efficacy for the treatment of malaria (Thera *et al.,* 2005). When combined with insecticide-treated nets, CTX prophylaxis reduced the prevalence of clinical malaria in HIV infected children by 97%, whereas these treated nets alone reduced this prevalence by 43% (Kamya *et al.,* 2007). Co-trimoxazole in patients with $CD4^+$ cell counts of less than 200 cells per μL, in combination with insecticide-treated nets, has also been considered as a part of preventive strategies against malaria. The high efficacy of co-trimoxazole in the prevention of clinical malaria raises the question of the discontinuation of this combination in HIV-infected patients with restored immunity under antiretroviral treatment: although they may not need this prophylaxis anymore for the prevention of opportunistic infections, they might still benefit from its antimalarial effect.

2.3 Cotrimoxazole prophylaxis and malaria parasite density

Frequent and recurrent infections with *Plasmodium falciparum* in areas with stable malaria transmission result in a semi-immune state in adults which allows for an increasing proportion of infections to stabilize at variable parasite densities without severe disease. These parasitemias can last for months and may either be asymptomatic or mildly symptomatic and tolerated without treatment but likely contribute to transmission (Schneider *et al.,* 2007). HIV-infected adults with decreased CD4 counts (especially those with CD4 counts less than 200) living in areas of stable malaria transmission have an increased incidence of symptomatic malaria infections compared to HIV-uninfected adults and there is evidence of an inverse relationship between level of parasitemia and $CD4^+$ count (Hewitt *et al.,* 2006; Laufer *et al.,* 2006; Patnaik *et al.,* 2005). Given the antimalarial activity of CTX, continuous use points out to a prolonged conferred protection against malaria attacks that may be lost if prophylaxis is stopped.

2.4 Multiplicity of Infection (MOI) in Malaria

The concept of Multiplicity of Infection (MOI) arises from the genetic diversity exhibited by plasmodium parasite at all levels of endemicity (Bendixen *et al.,* 2001). Multiplicity of Infection is simply defined as the number of genetically distinct *plasmodium* parasite types simultaneously infecting an individual (Vafa *et al.,* 2008). Multiplicity of Infection is an important parameter to measure for two reasons: 1) its potential bearing on clinical disease outcome and, 2) the information it can provide about disease transmission (Mwingira *et al.,* 2011). The relevance of MOI to disease manifestation and immune response is well established, but mechanisms are not yet understood and correlations are sometimes contradictory (Vafa *et al.,* 2008). While some studies have documented a positive association between MOI and symptomatic malaria others have found an inverse association, suggesting that the clinical and epidemiological consequences of MOI are important but not yet fully understood (Galinsky *et al.,* 2015).

Genetic diversity within *P. falciparum* is a major characteristic and a factor by which the parasites survive the hosts' immune responses. It results from allelic polymorphism, recombination, chromosome rearrangements, and antigenic variation (Agyeman-Budu *et al.,* 2013). In high transmission settings, individuals infected with *P. falciparum* harbor multiple parasite genotypes as compared to low transmission areas where majority of the infections are monoclonal (Lopez *et al.,* 2012). Different genetic markers such as Circumsporozoite Protein (CSP), Merozoite Surface Antigens (MSP) 1 and 2 and Glutamate Rich Protein (GLURP) have been used to determine the diversity of malaria parasites. This current study therefore sought to evaluate the impact of CTX withdrawal on the MOI in HIV infected adults with evidence of immune recovery following ART.

2.4.1 Merozoite Surface Antigens

The Merozoite Surface Antigens; MSP-1, MSP-2 and GLURP genes, code for highly diverse surface antigens found in malaria parasites and are commonly used as markers for genotyping *P. falciparum*. These three genes are unlinked, single copy with extensive polymorphism. Therefore, the diversity of these markers indicates parasite clonal diversity. Since the parasite is haploid during the human asexual part of the parasite life cycle, these markers are stable in human host and can be used to define parasite populations within the host (Hughes *et al.*, 1995; Hughes *et al.*, 1992; Anderson *et al.*, 2000).

The *Plasmodium Falciparum* MSP-1 gene is approximately 5,000 base pairs (bp) long, located on chromosome 9 (Pan *et al.*, 1999). Sequence comparison has shown that MSP-1 gene is divided into 17 blocks. The gene has 7 variable blocks interspersed by five conserved and five semi-conserved blocks (Kiwanuka *et al.*, 2009). In blocks 1, 3, 5, 12, and 17, the sequences are conserved whereas blocks 2, 4, 6, 8, 10, 14 and 16, the sequence show extensive diversity (Figure 2.1). The remaining blocks 7, 9, 11, 13, and 15 have semi conserved sequences (Raj *et al.*, 2004). Each of the variable blocks is dimorphic with two alleles K1 and MAD20. However Block 2 near the N-terminal of MSP-1 gene is the most dimorphic with respect to size and sequence and has three distinct allelic families; MAD20, K1 and RO33 (Heidari *et al.*, 2007).

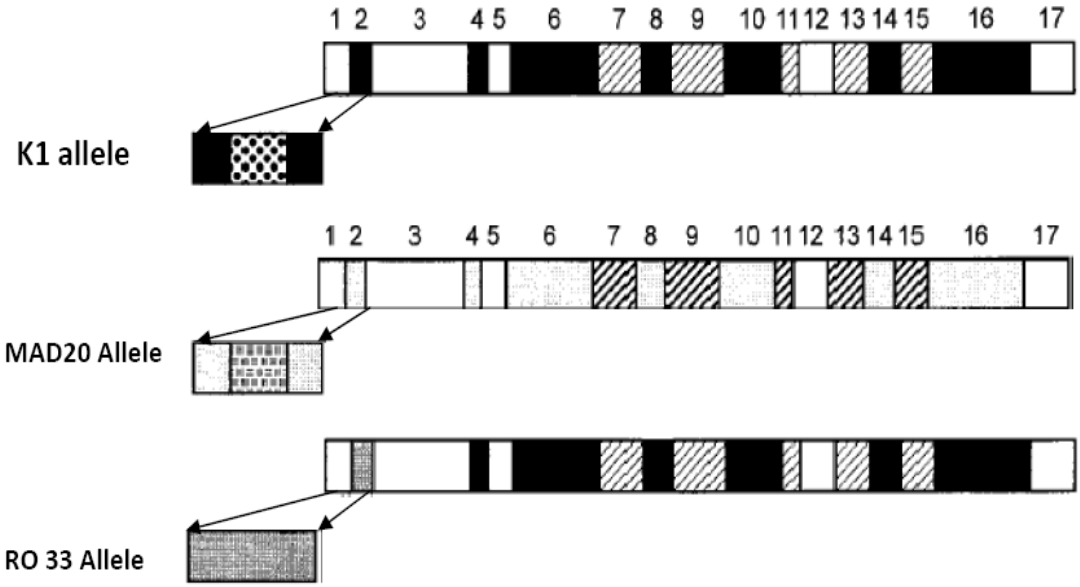


Figure 2.1: Schematic presentation P. falciparum msp-1 gene

The conserved blocks are shown as, open boxes, semi conserved blocks as hatched boxes and the polymorphic blocks as closed boxes. Allele families, K1 and MAD20 are present in all the polymorphic blocks. Block 2 has an additional allele family RO33. Adapted from (Da Silveira *et al.*, 1999).

The MSP-2 has also been used as a candidate antigen for malaria vaccine development. The MSP-2 gene located on chromosome 2 comprises of conserved amino (N) and carboxyl (C) terminal domains (Block1 and Block 5), highly polymorphic central repeats (Block 3) that are flanked by dimorphic domains (Block 2 and 4). The polymorphic central region is composed of repeats flanked by non-repetitive sequences (Figure 2.2). The repetitive sequences form the two allelic families: FC27 and IC3D7 (Ghanchi *et al.*, 2010). These polymorphic regions are used as genetic markers and provide a means to assess the composition of parasite population. Of these three loci *msp-2* has been found to be the most polymorphic (Heidari *et al.*, 2007).

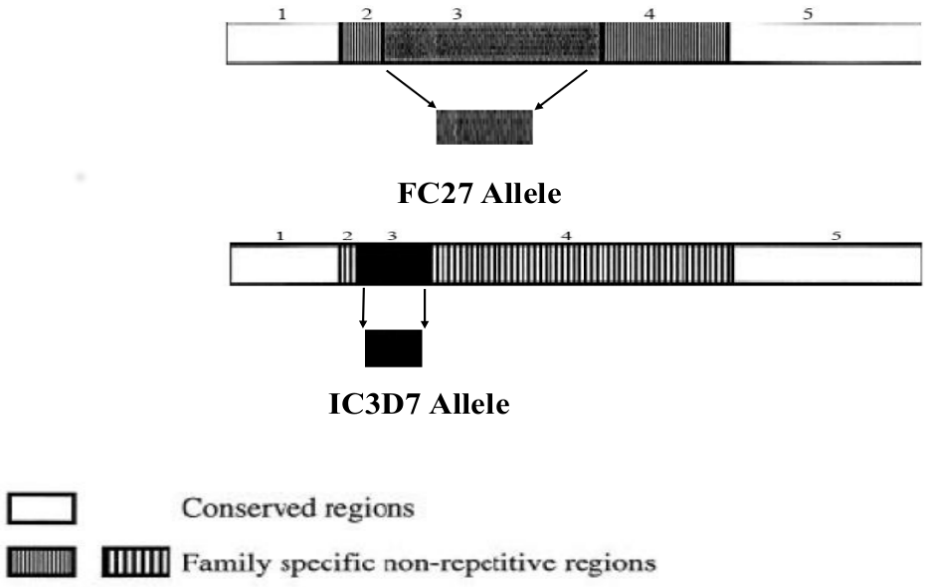


Figure 2.2: Schematic diagram of P. falciparum msp-2 gene Adapted from (Ekala *et al.,* 2002).

GLURP is a 220 kDa polypeptide that is expressed in all asexual stages of the parasite. The GLURP gene is located on chromosome 10 and consists of two tandem repeats (RI and RII) and appears only as one allelic family. The RII is most diverse and therefore normally targeted for genotyping (Høgh *et al.,* 1993). In the current study, only MSP-1 and MSP-2 were used to genotype the plasmodium parasite. This is due to the fact that, although GLURP has a high number of tandem repeats, it is less polymorphic than MSP-1 and MSP-2 (Greenhouse *et al.,* 2006).

2.5 Plasmodium falciparum genotyping.

In order to determine genetic diversity, several techniques exist that have been employed in clinical trials of anti-malarial drug efficacy against *Plasmodium Falciparum*. These genotyping techniques include;

2.5.1 Reverse Transcriptase PCR (RT-PCR)

RT-PCR is used for specific detection and genetic characterization of *P. falciparum* gametocytes in the blood of malaria infected individuals and uses highly polymorphic pfg377 gene as target (Menegon *et al.,* 2000). This technique, other than being highly sensitive, it also useful for detecting low or sub-patent levels of these forms in natural infections. This method however is expensive and has not been successful in distinguishing alleles of genes which differ by just a single nucleotide (Hunt *et al.,* 2005).

2.5.2 Proportional sequencing

This method is rapid, and in principle has been applied to any single nucleotide polymorphism (SNP) at any locus, with only minimal requirements for optimisation and assay development (Hunt *et al.,* 2005). This method estimates allele frequencies in mixed infections by measuring the peak heights in direct sequencing electropherograms. This contrasts with RT-PCR, in which rigorous development of each individual assay is needed. Proportional sequencing can accurately estimate the proportions of alleles at a given locus within a mixture of parasites (Hunt *et al.,* 2005).

2.5.3 Pyrosequencing

Pyrosequencing has been used reliably to quantify alleles in mixed malaria infections. It is a real time sequencing method that detects release of pyrophosphate during nucleotide incorporation by an enzyme cascade that generates light proportional to the amount of nucleotide incorporated (Takala *et al.,* 2006). This technique allows sequencing of short stretches of nucleotides (10-20 bp) surrounding known polymorphisms without sequencing the rest of the conserved sequence (Takala *et al.,* 2006). This method has been shown to provide accurate and precise measurements of allele frequencies and of the degree of DNA methylation (Tost *et al.,* 2003; Wasson *et al.,* 2002).

All of these methods, including Pyrosequencing, have advantages and disadvantages. RT-PCR is a more sensitive method than Pyrosequencing at detecting very low frequency alleles (<5%); however, it has a lower throughput and requires more optimization than Pyrosequencing (Cheesman *et al.,* 2003). While Proportional sequencing has similar applications and accuracy as Pyrosequencing, it is more expensive and has a lower throughput (Hunt *et al.,* 2005). Because Pyrosequencing sequences short stretches of nucleotides (10–20 bp), for certain very polymorphic loci (e.g. domain I of *P. falciparum* apical membrane antigen-1, another vaccine candidate antigen), it is not possible to set down a sequencing primer every 20 bp. In this instance, proportional sequencing may be more appropriate (Hunt *et al.,* 2005).

2.5.4 Microsatellite Marker Analysis

Multilocus genotyping has been used to study the population structure of *P. falciparum* (Snounou *et al.,* 1998; Anderson *et al.,* 2000), and in vaccine trials to assess the molecular impact of the vaccine on parasite MOI (Waitumbi *et al.,* 2009). This technique involves a nested PCR amplification of the loci of interest. The primary PCR uses primer pairs corresponding to the conserved sequences, spanning the polymorphic regions of block 2 of MSP-1 gene and block 3 of MSP-2 gene. Amplicons from the primary PCR are used as templates for the nested reaction. In this study allelic discrimination was achieved by capillary electrophoresis (CE) and not the traditional method of running the PCR amplicons in agarose gel electrophoresis and resolving based on fragment size. Adequate discrimination of alleles especially in high transmission settings where the MOI is high has been shown to be poor when gel electrophoresis due to limited resolving power of gel electrophoresis (Gupta *et al.,* 2010). When compared to conventional gel electrophoresis, capillary electrophoresis (CE) has been shown to provide better resolution of *P. falciparum* alleles (Gupta *et al.,* 2010). In addition to the high resolution, CE has been recommended by the

WHO for allele discrimination and sizing due to its high throughput, ability to multiplex by using different fluorescent dyes for different alleles, thereby reducing cost and time (WHO, 2007).

CHAPTER THREE

METHODOLOGY

3.1. Study area

Study participants were recruited from HIV treatment and care clinic known as the Patient Support Center (PSC) of Homa Bay District Hospital in Homabay County (0.6833° S, 34.4500° E). Homabay county, has the highest regional prevalence of both HIV at 25.7% and malaria 29.5% in Kenya (National AIDS and STI Control Programme & National AIDS Control Council, 2014). Therefore a region with the potentially highest benefit from CTX.

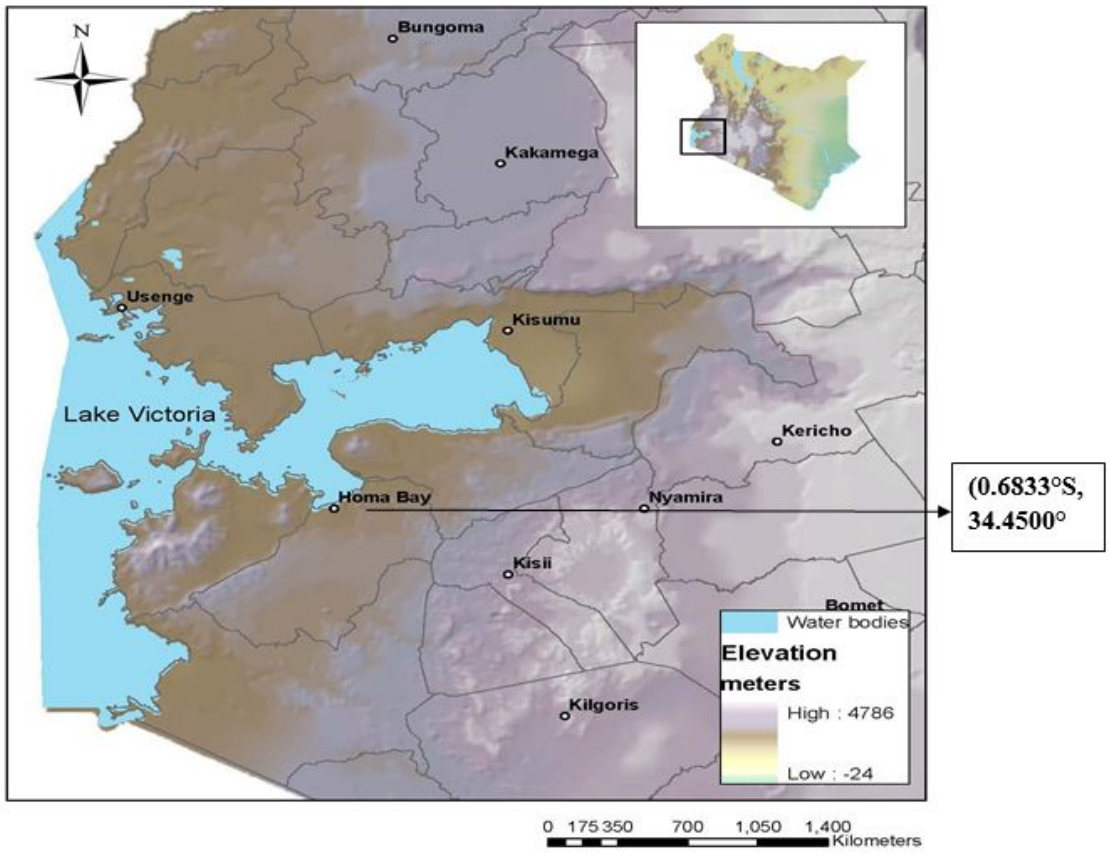


Figure 3.1: A map of Kenya showing sites from which blood samples were collected (Source: www.usamrukenya.org)

3.2 Study population

Individuals who were 18 years or older were enrolled between February 1, 2012, and August 27, 2012 after obtaining a written informed consent. Study participants were eligible if they met the following inclusion criteria: HIV seropositive, and taking first-line antiretroviral (ARVs) with evidence of immune recovery (ARVs for ≥ 18 months and CD4 count ≥ 350 cells/mm3). Currently taking CTX for HIV and willing to return to the clinic every 3 months for the 12-months study follow-up period. Exclusion criteria included individuals who were pregnant at enrolment (by urine HCG testing) or breastfeeding, taking second-line ARVs, or had a documented allergy to CTX.

3.2.1 Study Design

This study was an blinded, two-arm randomized clinical trial (RTC), that was part of an on-going parent study with the primary aim of determining whether CTX prophylaxis can be discontinued in ART-treated, immune-reconstituted adults without significant harm. Adults who met the study inclusion criteria were consented, enrolled and randomized into either the CTX continuation or CTX discontinuation arm. A computer-generated blocked randomization sequence was used to assign participants (1:1) to either the CTX continuation or CTX discontinuation arm. CTX continuation arm received CTX prophylaxis, while CTX discontinuation arm stopped prophylaxis at time of enrollment. Each participant's arm allocation was concealed from study investigators, staff, and participants in a randomization envelope until all assays were conducted and completed. All the participants in the RTC following enrollment were followed up every 3 months at the clinic and were encouraged to return to the clinic at any time they were sick.

Study retention was similar in both arms. A total of 490 participants (98%) were retained to the end of scheduled follow-up (the Month 12 study visit). A total of 5 participants were lost to follow up, 3 in the continue CTX and 2 in the discontinue CTX arm. There were 4

withdrawals, 1 in the continue CTX and 3 in the discontinue CTX arm. There was 1 death in the continue CTX arm (Figure 3.1).

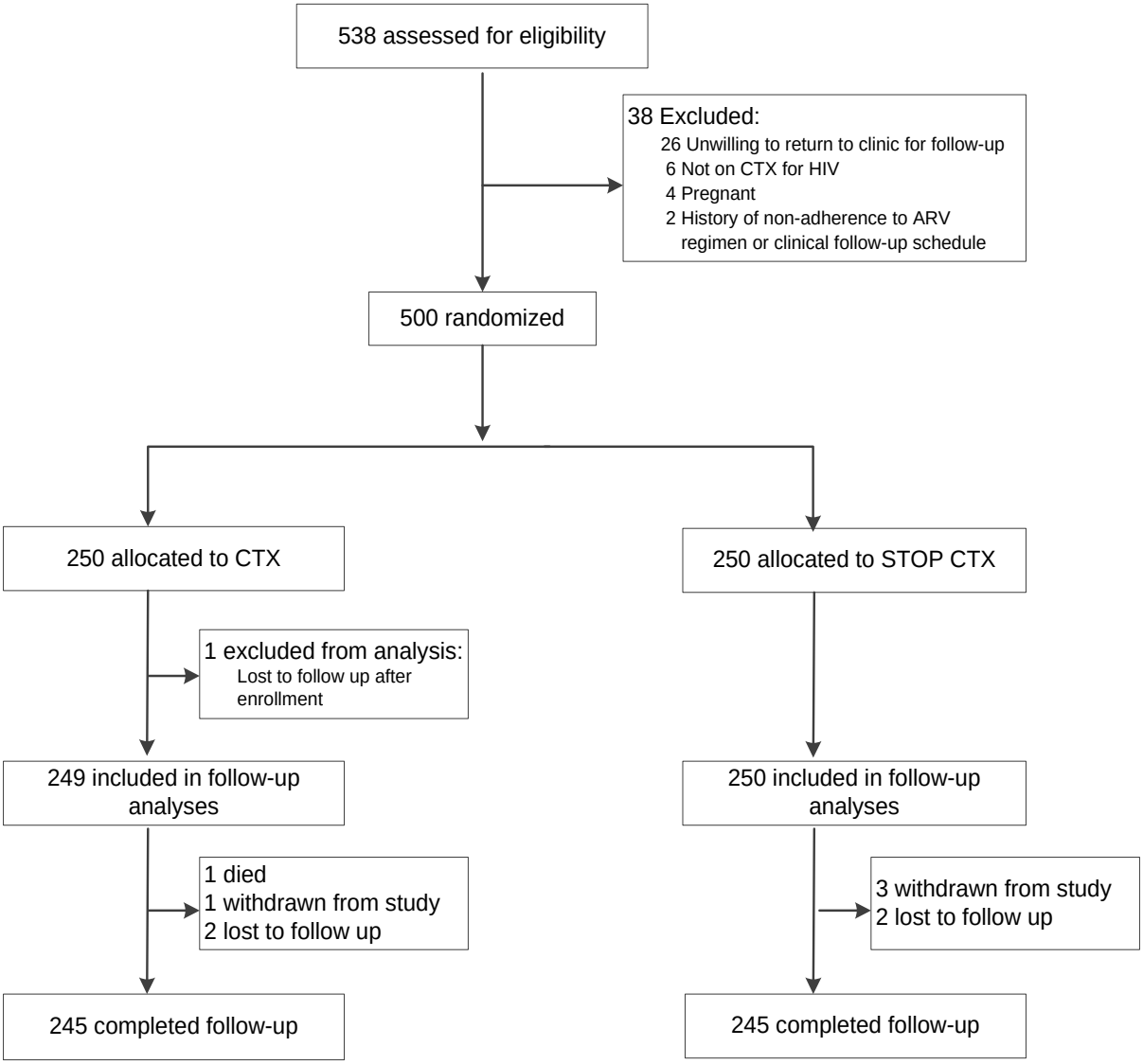


Figure 3.2: Figure showing the study profile of individuals enrolled and randomized into the two study arms (CTX continuation vs discontinuation arm)

3.3 Sample size determination

Sample size determination was based on the following formula: (Noordzij *et al.*, 2010).

$$n = \frac{[(a+b)^2(p_1q_1 + p_2q_2)]}{x^2}$$

n = the sample size in each of the groups
p1 = proportion of individuals with malaria in treatment group 1
q1 = proportion of individuals without malaria in treatment group 1 (= 1− p1)
p2 = proportion of individuals with malaria in treatment Group 2
q2 = proportion of individuals without malaria in treatment group 2 (= 1− p2)
x = minimal suspected difference between those who stopped CTX and those who continued CTX (minimal clinically relevant difference)
a = conventional multiplier for alpha = 0.05
b = conventional multiplier for power = 0.80 (Noordzij *et al.*, 2010).

Morbidity estimates were based on prior morbidity incidence data from an RCT Ugandan study (Campbell *et al.*, 2012). A difference in event rate of 10% (0.10) was chosen as clinically relevant (Noordzij *et al.*, 2010). Based on recently published findings from studies with a similar design, the proportion of subjects with malaria in the continue CTX arm was 14% (p1 =0.14) and in the discontinue CTX arm, 25% (p2 = 0.25) (Campbell *et al.*, 2012). Therefore q1 and q2 become 0.86 and 0.75, respectively. Assuming a power of 80% (0.80) and an alpha of 0.05.

$$\frac{(1.96+0.842)^2(0.14\times 0.86)+(0.25\times 0.75)}{0.10} = 241$$

This means that a minimum sample size of 241 subjects per group was needed to answer the research question. This study had a sample size of 250 individuals per arm in the final analysis.

3.4. Sample collection

At every visit, 1mL of blood was drawn in EDTA tubes. This blood was blotted on Whatman FTA-classic cards (Sigma-Aldrich, Inc. USA) and stored in zip lock bags with silica gel at Homabay county referral hospital before being transported to WRP/KEMRI research laboratory and stored in a dry place for use in q-PCR malaria diagnosis and parasite genotyping.

3.5 Nucleic acid extraction

Total DNA was isolated using QIAamp DNA mini kit (QIAGEN sciences, Maryland, USA), accordance to manufacturer's instructions. In brief, three punched-out circles from the Whatman FTA-classic cards (Sigma-Aldrich, Inc. USA) were placed into a 1.5 ml micro centrifuge tube and 180 µl of tissue lysis buffer ALT added to the content and incubated at 85°C for 10 minutes. This was followed by addition of 20 µl QIAGEN proteinase K in order to digest the proteins. The mixture was vortexed and incubated on a hotplate at 56°C for 1 hr. Tissue lysis buffer AL was then added to the sample, mixed thoroughly by vortexing and incubated at 70°C for 10 min, after which 200 µl of absolute ethanol (96-100%) was added to the sample and mixed thoroughly by vortexing.

The de-proteinized DNA preparation was then purified on a QIAamp mini spin column via centrifugation for 1 minute at 6000 × g and the tube containing the filtrate discarded. The spin column was then transferred to a clean collection tube and 500 µl of buffer AW1 added and centrifuged for 1 min at 6000 × g after which the filtrate was discarded and the spin column placed in another clean collection tube. Five hundred µl of buffer AW2 was then be added to the spin column and the preparation centrifuged for 3 minutes at 20,000 × g. Finally the purified DNA was eluted from the spin column by addition of 110 µl of elution buffer AE and stored at -20^{o}C for use in determining Plasmodium parasitemia and parasite densities.

3.6. Determination of Plasmodium parasites and parasite densities

Quantitative Real time PCR (qRT-PCR) targeting the Plasmodium *genus* 18S ribosomal gene was performed with the following forward and reverse primers (PLU3F 5'-GCT-CTT-TCT-TGA-TTT-CTT-GGA-TG-3', PLU3R 5'-AGC-AGG-TTA-AGA-TCT-CGT-TCG-3') and probe (PLU3P 5'-ATGGCCGTTTTTAGTTCGTG-3') described previously (Kamau *et al.,* 2011). To obtain maximum sensitivity, we followed the qRT-PCR approach described by Waitumbi *et al*, was followed (Waitumbi *et al.,* 2011). Briefly, the PCR was performed in a final volume of 10 µL with 2 µL of the template, 5 µL of 2X QIAGEN Quatitec probe RT-PCR master mix (QIAGEN Inc., CA), 0.4 µL of 0.4 µM probe and primers mix, 0.1 µL of reverse transcriptase enzyme mix, 1.6 µL of 4 µM magnesium chloride and 1.1 µL of PCR grade RNAse/DNAse free water. Reactions were carried out in the 7300 analytical PCR system thermo cycler (Applied Biosystems, CA, USA). Since the total DNA extraction process did not have an RNAse step, to increase the assay sensitivity, the amplification process started with a 30 minutes reverse transcription step at 50° C to convert RNA to cDNA (Waitumbi *et al.,* 2011). This was followed by heating at 94° C for 10 min to inactivate the reverse transcriptase, then 45 cycles of 95° C for 15 s and 60° C for 1 minute to amplify the target cDNA and genomic DNA.

A sample was considered positive if the cycle threshold (C_T) exceeded the C_T values of the negative control; in this case the negative control included non-infected human DNA and non-template control (Figure 3 panel A). The positive control used was blood with an estimated parasite count of 469,920 parasites/µL from a donor infected with *P. falciparum* was obtained from the National Institute for Biological Standards and Control (NIBSC; Hertfordshire, UK). A standard curve from C_T values obtained from 10-fold serial dilution of the positive control was constructed for each run. A plot of the log of initial target copy number for a set of standards versus C_T should give a straight line (Higuchi, Fockler,

Dollinger, & Watson, 1993). Figure 3 panel B shows a typical example of a standard curve. It is from this standard curve that copy numbers of the known sample were calculated by interpolation of the experimentally determined CT. The 7300 instrument software (7300 system sequence detection software version 1.3, Applied Biosystems, Foster City, CA, USA) performed the whole process of calculating the C_T values preparing the standard curve, and determining the copy numbers of the sample.

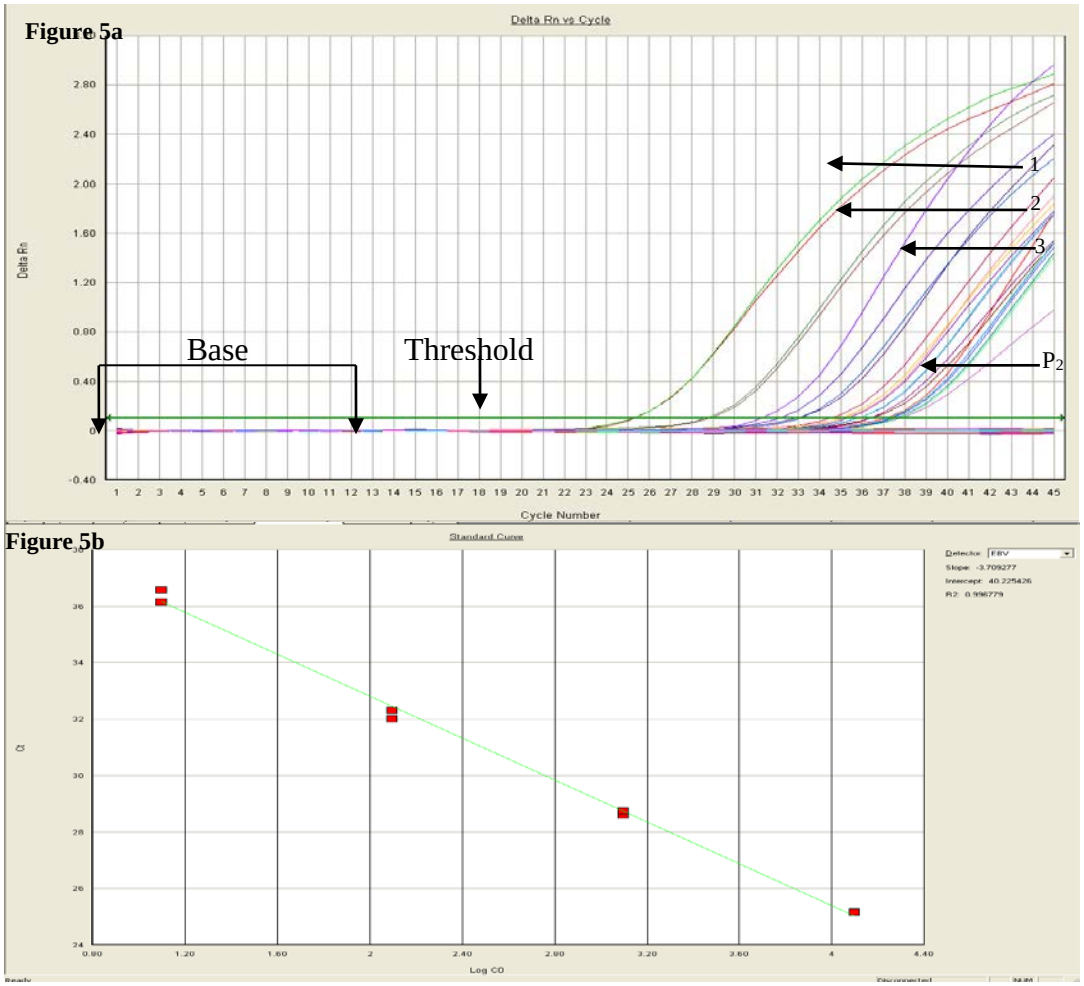


Figure 3.3: (a) Standard curves generated from sample of known concentration. Duplicate curves 1, 2, and 3 represent 10-fold serial dilutions of the standard positive control DNA. P1 and P2 represent individual curves for patients' DNA samples. The curves shift to the left with increasing copy number (b) Standard curves generated from blood with a known concentration. The logarithm of the change in fluorescence, Rn, is plotted against the cycle number for the 18S ribosomal gene. The unknown patient samples are extrapolated from this curve.

3.7. *Plasmodium falciparum* MSP-1 and MSP-2 genes genotyping

Polymorphic regions of the MSP-1 and MSP–2 was amplified by primary round of PCR using DNA extracted from each sample with primers targeting the entire block 2 of MSP-1 and block 3 of MSP-2 followed by nested PCR using products from primary PCR for each sample with allele specific primers to amplify the K1, MAD-20 and the RO33 alleles for MSP-1 and FC27 and IC3D7 for MSP-2 (see appendix 2). For every batch of PCR reaction, a positive and a negative control was included for all the alleles. DNA known and verified to contain each of the alleles was used as a positive control, whereas PCR grade double distilled water was used as the negative control.

Each of the reaction tube contained 3µL of DNA, 15.9 µL ultra-pure grade RNAse/DNAse free water (Applied Biosystems, CA, USA), 2.5 µL of 1X MyTaq ™ buffer (Bio-line, UK), 0.625 µL of 0.25 pm/µL of forward and reverse primers (Applied Biosystems, CA, USA), 2 µL of 2 mM Mgcl2, 0.3 µL of 0.125 mM dNTPs and 0.1 µL of 0.02 U of MyTaq™ DNA polymerase (Bio-line, UK). The reactions were carried out in the HID Veriti® 96-Well Thermal Cycler (Applied Biosystems, CA, USA). The cycle conditions for both MSP-1 and MSP-2 genes included: initial denaturation at 95° C for 1 min, followed by 35 cycles of denaturation at 95° C for 15 sec, annealing at 58° C for 15 sec and extension at 72° C for 10 sec.

3.7.1 Nested PCR

In the secondary reaction, allele specific primers were used to amplify the K1, MAD-20 and the RO33 alleles of MSP-1 and FC27 and IC3D7 alleles of MSP-2 (Appendix 2). For these, the reverse primers were labeled with different fluorescent dyes at the 5´ end; K1 with NED (yellow), MAD20 with PET (red), RO33 with VIC (green), FC27 with 6-FAM (blue) and ICD37 with VIC (green). All primer sets were obtained from Applied Biosystems, Foster City, CA. The reaction volume for both msp-1 and msp-2 allelic amplification was 25 µL

containing 1 µL of the primary amplicons, 19.5 µL PCR grade RNAse/DNAse free water, 2.5 µL of 1X MyTaq ^{TM} buffer (Bio-line, UK), 0.3125 µL of 125 nm of each allelic type primers, 1 µL of 1mM Mgcl2, 0.315 µL of 125 µM dNTPs and 0.1 µL of 0.02 U of MyTaq™ DNA polymerase (Bio-line, UK). The cycle conditions for the MSP-1 alleles were as follows: initial denaturation at 95° C for 1 min, followed by 35 cycles of 95° C for 15 sec, annealing at 61° C for 15 sec, extension at 72° C for 10 sec. For the MSP-2 alleles, the cycle conditions consisted of an initial denaturation step at 95 °C for 1 min, followed by 35 cycles of 95 °C for 15 sec, annealing at 58 °C for 15 sec, extension at 72 °C for 10 sec. All amplifications were performed in HID Veriti® 96-Well Thermal Cycler (HID Veriti, Applied Biosystems, CA, USA). The PCR products were wrapped in aluminium foil to avoid photo bleaching of the fluorescent dyes and stored at -20^{o} C until required.

3.7.2 Capillary Electrophoresis and Fragment Analysis of MSP-1 and MSP-2

Capillary Electrophoresis was performed in a 3130 Genetic Analyzer (Applied Biosystem) as recommended by the manufacturer. All reagents including the polymer were from Applied Biosystem. Capillary Electrophoresis generates a size estimate for DNA fragments relative to a size standard. Both the sample and the size standard DNA are co-loaded into the capillary electrophoresis system. After run completion, DNA fragments are displayed as peaks, with fragment size calculated in base pair (bp), a height calculated in relative fluorescence units (rfu) and a calculated area underneath the associated peak (Figure 4). Stutter peaks were eliminated by setting a background cut-off 300 relative fluorescent unit (rfu) as previously described (Liljander *et al.*, 2009).

A reaction mix (950 µL), enough for 96 samples in a 96 well plate was prepared by adding 50 µL of LIZ standard (GeneScan-500LIZ® was used for smaller fragments less than 500 bp such as K1, MAD20, RO33 and FC27 whereas GeneScan-1200LIZ® between 500 - 1200 bp fragments such as IC3D7) in 900 µL of Hi-di (Highly deionized) formamide. The mix was

pulse vortexed and centrifuged at 8000 rpm (Microfuge®22R Centrifuge, Beckman Coulter). Nine μL of the mix was then dispensed into the 96 well plates (MicroAmpTM Applied Biosystems). One μL of amplified product from the nested reaction was then added to the 96-well plate, vortexed and then briefly centrifuged at 3000 rpm (Sorvall RT6000B centrifuge) to ensure uniform mixing, eliminate bubbles and sediment particles. Samples were denatured at 95° C for 5 min and then chilled in ice to maintain the single strands prior to loading into the Genetic Analyzer. The samples were then loaded in the 36 cm capillary unit containing performance-optimized polymer, (POP-7TM) for resolution and fragment sizing. Capillary Electrophoresis output was exported into GeneMapper® Software version 4.0 (Applied Biosystems) for fragment analysis.

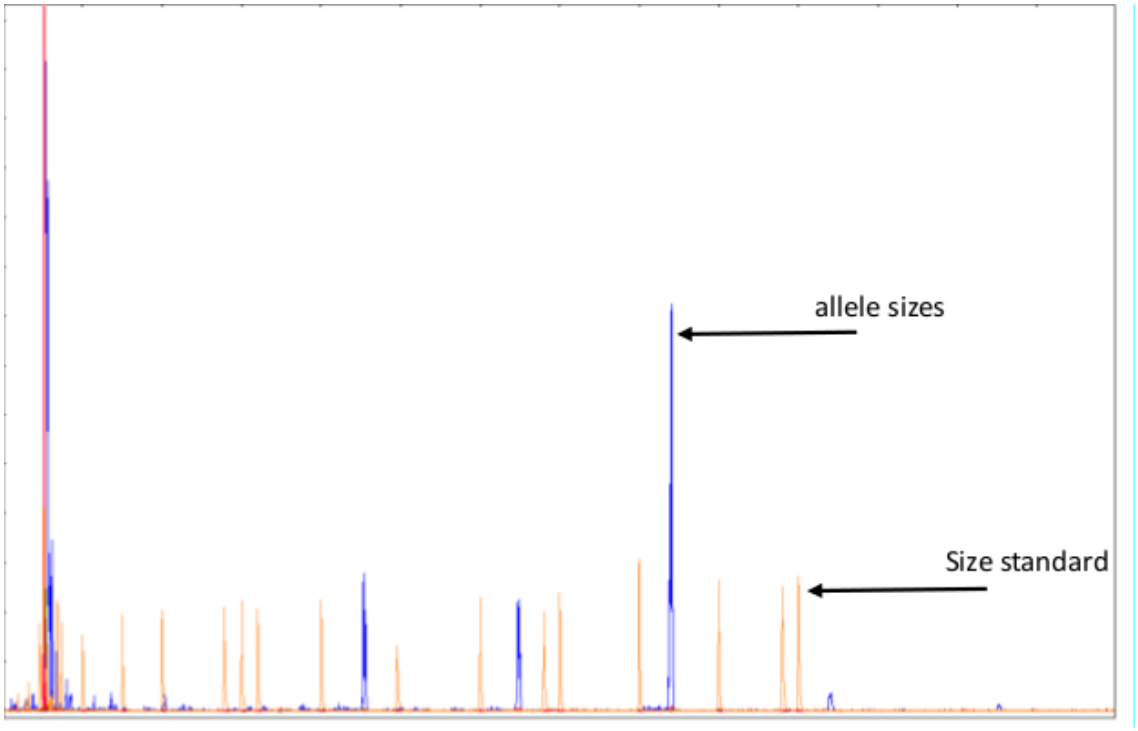


Figure 3.4: PCR amplicons and molecular size markers following CE.

Raw data output prior to analysis by the genemapper software. In this case, the blue colored tracings shows three amplicons of different allele sizes whereas the orange peaks show the size standard (LIZ 500) used to calibrate the allele sizes.

3.8. Statistical Analysis

Data were analyzed for discontinue and continue CTX arms at enrolment, and all scheduled and sick follow-up visits. Scheduled visits where the participants were also sick were included in both the scheduled visit analyses and sick visit analyses. Descriptive statistics of malaria parasitemia, parasite density and the number of genotypes in each allelic family of MSP-1 and MSP-2 (MOI) were computed. Parasitemia in this study was defined as any parasites detected in both study arms. Chi-square tests were used to test for differences between study arms in parasitemia prevalence over time. In the subset of visits where

parasites were detected, parasite density values were log-transformed, and the difference between arms over the follow-up period was tested using a generalized estimating equations (GEE) model with identity link, independence working correlation structure, and robust standard errors to handle repeated measures within individuals. Rates of incident parasitemia in the two study arms were calculated on the basis of person-time at risk. Time at risk included time from the date of randomisation that each participant remained parasite free, completed 1 year of follow-up, or was withdrawn from the study, whichever came first. Participants with parasitemia at enrolment did not contribute person-time to the incident parasitemia analysis. The number of distinguishable alleles for MSP-1 and MSP-2 genes was determined for each isolate and the largest of these numbers was considered the MOI. The frequency of mixed infections, defined as an MOI >1 was compared between the two study arms. All tests were 2-sided at 5% significance level. Data collected was entered into Excel spreadsheet for cleaning and all the data analysis was conducted on STATA statistical software, version 13.0 (Stata Corp, College Station, TX).

3.9. Ethical considerations

HIV-infected adults who meet the study inclusion/exclusion criteria were required to sign a written informed consent prior to enrollment. If not literate, individuals provided a witnessed thumbprint. Following consent, each participant was assigned a unique study identification number. Participants were identified using only the study identification number. The code linking the identification number to individual identifying information was kept in a separate secure location by the project coordinator. Confidentiality was maintained during all phases of the study. To increase likelihood of compliance and adequate access to care, transportation reimbursements of 200 Kenyan shillings (approximately $2) was covered for each scheduled and sick visit.

Study approvals was obtained from School of Graduate Studies (SGS) of Maseno University, University of Washington Institutional Review Board (IRB), Walter Reed Army Institute of Research (WRAIR), USAMRMC ORP HRPO (United States Army Materials and Research Medical Command, Office of Research Protections, Human Research Protection Office) (WRAIR# 1983) and the KEMRI Ethical Review Board (ERC) (KEMRI SSC#2077).This study protocol was approved by Kenya Medical Research Institute (KEMRI) Ethical Review Committee (KEMRI SSC#2077), the Walter Reed Institute of Army Research (WRAIR) Human Subject Protection Committee (WRAIR# 1983),University of Washington (UW) Institutional Review Board and the School of Graduate Studies (SGS) of Maseno University.

CHAPTER FOUR

RESULTS

4.1 Recruitment and follow-up visits.

Among enrolled subjects, 72% were women, 64% were married, 68% had primary school or less education, and 78% had a monthly income of less than 5000 Kenyan shillings. Median age at enrollment was 40 years, median CD4 count was 595 cells/mm^3, and median ART duration was 4.5 years. These demographic characteristics are representative of the population seeking care at the clinic. Randomization resulted in baseline characteristics which were generally well-balanced between the two arms (Table 4.1).

Table 4.1: Table showing clinical and demographic characteristics of the study participants

Characteristic	CTX N (%) or Median (IQR) N= 250	STOP CTX N (%) or Median (IQR) N= 250
Female gender	184 (73.6)	177 (70.8)
Age, years	41 (34, 48)	40 (34, 48)
Marital status		
Married	156 (62.4)	165 (66.0)
Divorced/separated/widowed	90 (36.0)	77 (30.8)
Single	4 (1.6)	8 (3.2)
Education (highest completed)		
Less than primary	15 (6.0)	18 (7.2)
Primary school	156 (62.4)	149 (59.6)
Secondary school	73 (29.2)	73 (29.2)
Vocational school	3 (1.2)	6 (2.4)
University	3 (1.2)	4 (1.6)
Estimated monthly income (Kenyan Shillings)		
<5,000	201 (80.4)	187 (74.8)
≥ 5,000	49 (19.6)	63 (25.2)
Enrollment CD4, cells/mm^3	598 (487, 695)	591 (515, 719)
Duration of ART at enrollment, years	4.5 (3.1, 6.3)	4.5 (3.2, 6.1)

Recruitment commenced in February 2012 and was complete by August 2012 and the study was completed a year later (August 2013). The number of subjects enrolled for the study at each month (M0) and those who reported at subsequent quarterly follow-up visits (M3, M6, M9 and M12) were similar in the CTX and STOP-CTX arms (Table 2). For analysis, the numbers at enrollments (M0) and at subsequent quarterly visits (M3, M6, M9 and M12) were consolidated.

Table 4.2: Number of study participants at enrollment and at subsequent quarterly visits by calendar months.

The number of subjects enrolled at each month and those who reported for evaluation at subsequent quarterly visits were similar. Shaded periods demonstrate period of high malaria transmission intensity. Enrolment and follow-up included periods of high and low malaria intensity.

	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Total
M0 STOP-CTX	28	30	30	36	37	52	37													**250**
M0 CTX	29	31	26	41	36	46	38													**250**
M3 STOP-CTX				26	32	29	37	36	48	40	1									**249**
M3 CTX				29	28	26	43	29	53	39	2									**249**
M6 STOP-CTX						5	25	28	35	24	23	46	42	21						**249**
M6 CTX						5	25	28	31	26	16	47	49	18						**247**
M9 STOP-CTX									5	16	18	30	34	33	46	57	6			**245**
M9 CTX									5	18	18	30	33	25	49	55	12			**245**
M12 STOP-CTX													12	26	23	49	41	45	49	**245**
M12 CTX													17	27	27	43	31	51	49	**245**

4.2 Comparison of Parasitemia prevalence over time in the study cohorts.

At enrollment, 95% (473/500) of the study population were free of malaria. The distribution of 5% population that had parasitemia between the study arms at enrolment did not differ significantly (11/250, 4%) in the discontinue CTX arm and (14/250, 6%) p= 0.53. As shown in Figure 7, within 3 months of CTX discontinuation, parasitemia prevalence increased in the arm that discontinued CTX, increasing steadily during the year to >5-fold: parasitemia prevalence was 4% (11/248) at M3, then increased to 8% (21/249) at M6, 14% (33/244) at M9 and 22% (54/245) at M12. In comparison, the arm that continued CTX had parasitemia prevalence of <1% (1/248) at M3, 2% (5/247) at M6, 2% (4/245) at M9 and 4% (10/245) at M12 ($P \leq 0.0034$ for each of the four comparisons).

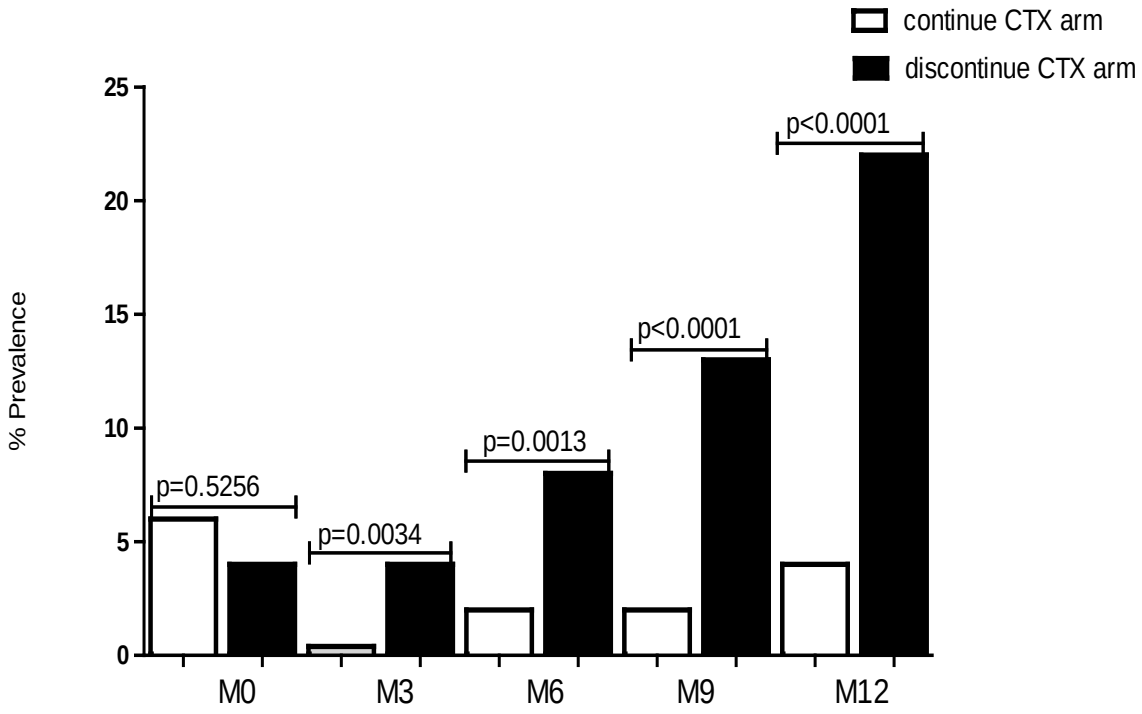


Figure 4.1: Figure showing malaria parasite point prevalence in the discontinue CTX and continue CTX study arms. Chi-square was used to test for differences between study arms in parasitemia prevalence over time.

4.3 Parasitemia incidence rates following CTX discontinuation

Over the 12 month follow up period, 474 study subjects gave 232 person-years in the continue CTX arm and 215 person-years in the discontinue CTX arm. Following enrollment, 23 new infections developed in the continue CTX arm compared to 90 in the discontinue CTX arm. The rate of parasitemia in the continue CTX arm was 9.9 per 100 person years compared to 42.0 per 100 person years in the discontinue CTX arm, giving an incidence rate ratio of 4.3 (95% CI: 0.14 - 0.37; $p < 0.0001$). In both arms, new infections peaked during the malaria transmission seasons that peaked in September 2012, March, May and August 2013 after the rains (Figure 4.2).

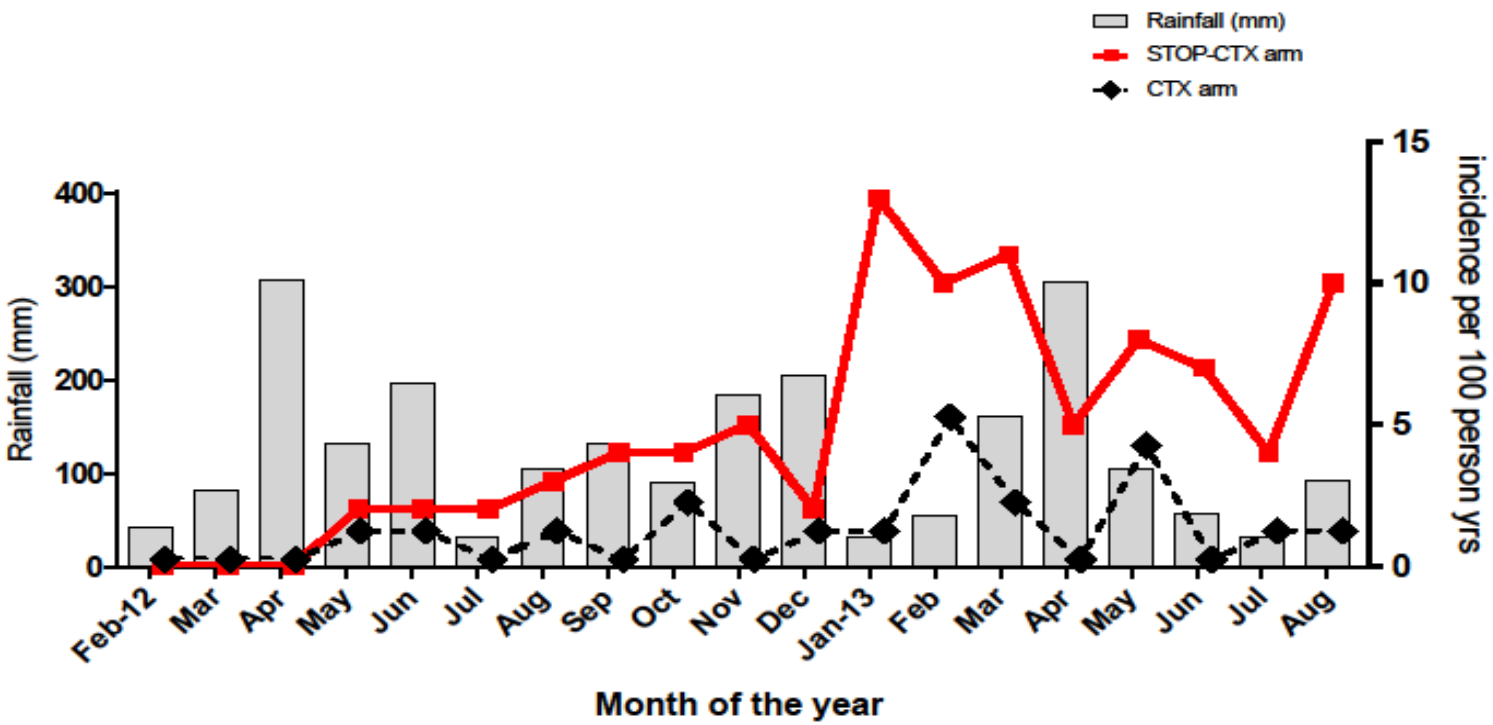


Figure 4.2: Malaria parasitemia incidence rates versus rainfall by calendar month in individuals enrolled in the discontinue CTX arm (continuous red line) and continue CTX study arm (broken black line).

4.4 CTX discontinuation was associated with a high burden of malaria parasites

In those with prevalent parasitemia at baseline, mean log10 parasite density did not differ between discontinue CTX (2.42 log10 parasites/mL) and continue CTX (2.37 log10 parasites/mL) arms, p=0.90. Figure 9 shows parasite density for all visits where parasites were detected. The discontinue CTX arm had higher mean values of log10 parasite density than the continue CTX arm: 4.98 vs. 4.92 log10 parasites/mL at month 3 (M3), 4.91 vs. 4.19 log10 parasites/mL at month 6 (M6), 4.85 vs. 3.61 log10 parasites/mL at month 9 (M9) and 3.85 vs. 2.45 log10 parasites/mL at month 12 (M12) and 5.01 vs 2.44 log10 parasites/mL at sick visits due to malaria (Figure 4.3). When all post-enrolment visits were combined, the mean log10 parasite density was 1.29 (95% CI: 0.74-1.85) log10 parasites/mL higher in the discontinue CTX arm compared to the continue CTX arm, a statistically significant difference ($p<0.001$).

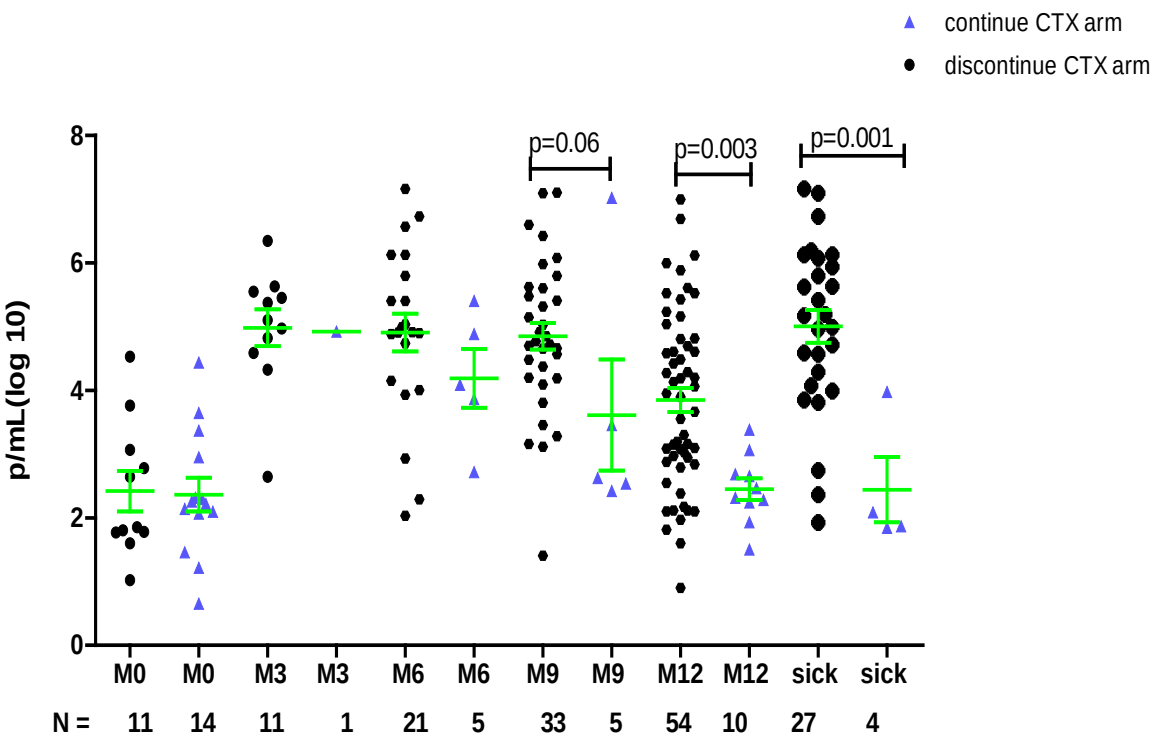
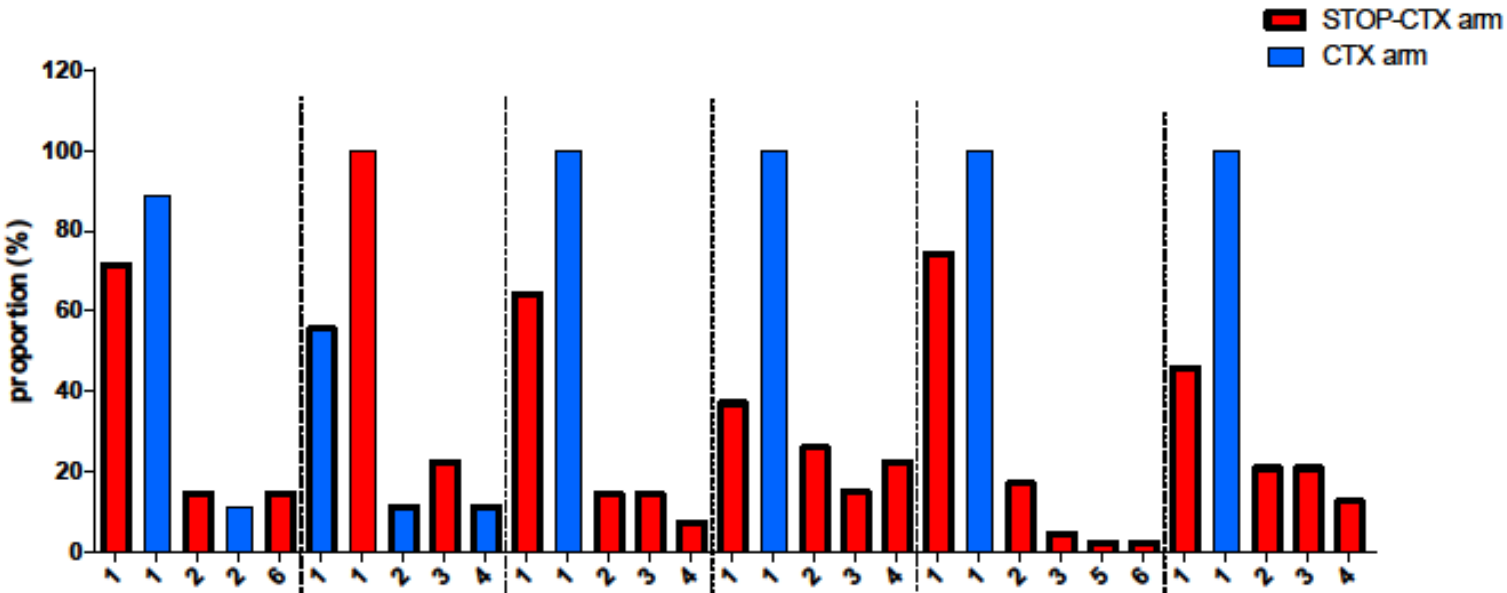


Figure 4.3: Scatter plot showing malaria parasite density at enrolment, quarterly visits and at sick visits in the discontinue and continue CTX arms, among those with parasites detected. At all visits post enrolment, the STOP-CTX arm had more malaria infected subjects with higher mean parasite density than the CTX arm. Differences in parasite density between arms over the follow-up period was tested using GEE model with identity link, independence working correlation structure, and robust standard errors to handle repeated measures within individuals. Error bars represent mean with standard error mean (SEM).

4.5 Multiplicity of Infections

Of the *Plasmodium* positive blood samples, genotyping was successful in 7/11 (64%) at M0, 8/9 (89%) at M3, 14/17 (82%) at M6, 27/29 (93%) at M9, 47/53 (89%) at M12, 24/27 (89%) for sick visits in the discontinue CTX arm. For the continue CTX arm, genotyping was less successful: 9/14 (64%) at M0, 1/1 (100%) at M3, 2/5 (40%) at M6, 1/4 (25%) at M9, 7/10 (70%) at M12 and 2/4 (50%) for sick visits. The reduced success rate in the continue CTX arm is probably due to low parasite density compared to discontinue CTX arm (Figure 4.3).

The number of distinguishable alleles for *msp-1* and *msp-2* genes was determined for each isolate and the largest of these numbers was considered the "multiplicity of infection" (MOI) of that sample. The distribution of MOI at enrolment, at quarterly visits and during malaria sick visits is shown in Figure 4.4. The frequency of mixed infections, defined as MOI >1 increased steadily throughout the year in the discontinue CTX arm: 28% (2/7) at enrolment to 50% (4/9) at M3, 36% (5/14) at M6, 52% (15/29) at M9, 26% (12/47) at M12 and was highest (54%, 13/24) at sick visits. In comparison to the continue CTX arm, mixed infections were only detected at enrolment 11% (1/9), and all subsequent visits including the sick visit had single infections. MOI, defined as the highest number of distinct MSP-1 and MSP-2 alleles per isolate was identified (Waitumbi *et al.*, 2009). The mean MOI was significantly different between the two study cohorts on consolidating all time points (stop-CTX Arm =1.8 vs CTX Arm =1.1 p = 0.002).



Stop-CTX	Enrolment	Month 3	Month 6	Month 9	Month 12	Sick Visits
Mono infections	5/7 (71%)	4/8 (50%)	9/14 (64%)	10/27 (37%)	35/47 (74%)	11/24 (46%)
Mixed infections	(2/7) 29%	4/8 (50%)	5/14 (36%)	17/47 (63%)	12/47 (26%)	13/24 (54%)
CTX	**Enrolment**	**Month 3**	**Month 6**	**Month 9**	**Month 12**	**Sick Visits**
Mono infections	8/9 (89%)	1/1 (100%)	2/2 (100%)	1/1 (100%)	7/7 (100%)	2/2 (100%)
Mixed infections	1/9 (11%)	0	0	0	0	0

Figure 4.4: Multiplicity of infections at enrollment, quarterly visits and at malaria sick visits. Red filled bar represents the discontinue CTX arm and the blue filled bar continue CTX arm.

CHAPTER FIVE

DISCUSSION

In Africa, infections such as malaria and bacterial pneumonia remain common in both the general and HIV-infected population (Flateau *et al.*, 2011). Therefore, CTX is recommended at higher $CD4^+$ counts to decrease mortality and morbidity in HIV-infected individuals (WHO, 2006). However following immune reconstitution following ART, CTX prophylaxis may not be required. Additionally, the high efficacy of cotrimoxazole in the prevention of clinical malaria has raised the question of the effect discontinuation would have on malaria in HIV infected individuals restored immunity. This study demonstrates increased parasitemia prevalence, quantity and multiplicity of infection among ART-treated HIV-infected adults who discontinued CTX compared to those who continued CTX.

At enrolment, 95% of the study population had no malaria parasites, illustrating the ability of CTX prophylaxis to control parasitemia. This is in line with earlier studies that demonstrated up to 92% protective efficacy of CTX prophylaxis (Mermin *et al.*, 2004; Watera *et al.*, 2006). It's not clear why CTX prophylaxis was unable to achieve 100% control of malaria parasitemia. The 5% of individuals with parasitemia may not have been taking their CTX or may have had parasite populations that were resistant to CTX. Resistance to CTX by *Streptococcus pneumonia* attributable to the same genes that code for antifolate resistance in malaria parasites (dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) has been reported in Europe (Schmitz *et al.*, 2001) and Uganda (Wilén *et al* ., 2009). This data suggest that the initial fear of possible diminished CTX efficacy in regions where antifolate resistance to malaria is high, such as western Kenya is unwarranted (Juma *et al.*, 2014; Kamya *et al.*, 2007; Laufer *et al.*, 2006).

HIV infection is associated with an increased prevalence of clinical malaria, in regions of stable malaria transmission, (Flateau *et al*, 2011; Francesconi *et al.,* 2001). This is because HIV infection may increase the incidence and severity of malaria in adults by compromising acquired immunity to malaria. However, with the antimalarial activity of CTX, continuous use in the context of malaria points out to a prolonged conferred protection against malaria attacks. In this study post enrolment, malaria parasitemia prevalence, and incidence increased among ART-treated HIV-infected adults who discontinued CTX compared to those who continued CTX. Despite immune recovery following ART, by one year post CTX cessation over 20% of individuals had malaria parasitemia. A similar study conducted in Uganda was stopped just 4 months after initiation, and failed to determine whether or not the increased malaria incidence seen upon discontinuing CTX prophylaxis was due to short transient rebound or would continue for a longer period (Campbell *et al.,* 2012). This study clearly shows the increased incidence following CTX discontinuation, is not short lived but would continue following discontinuation.

Parasitemia increased steadily from quarter to quarter with marked increases noted by 9 months post-cessation. HIV disease itself impairs acquired immunity to malaria. At enrolment, the mean parasites densities in both cohorts were sub-microscopic, again illustrating the ability of CTX prophylaxis to control both the parasitemia prevalence and density. Within 3 months of CTX discontinuation, mean parasite density had risen significantly when compared to the group that continued CTX. Additionally, there were more clinical malaria cases with higher parasite density in the CTX discontinuation arm compared to the CTX continue arm. The risk of malaria requiring hospitalization, which was twice as high among participants who stopped receiving CTX as it was among those who continued to receive CTX, consistent with previous studies showing that CTX prevents parasitemia and clinical malaria (Mermin *et al.,* 2004; Mermin *et al.,* 2006). Environmental and climatic

conditions have direct influence on the occurrence and distribution of plasmodia parasite and malaria in general (Ssegwanyi *et al.,* 1987; WHO, 1998). In this study, the rate of malaria parasitemia corresponded to the rainfall pattern. Three peaks are discernible, and are especially well-defined in the discontinue CTX arm: In September 2012, March, May and August 2013. These peaks correspond to the long rainy seasons of late March through June and the short rains of August to October that increase the population of anopheles mosquito (Usaid, 2013).

Advances in genotyping techniques have revealed that in a malaria infection, hosts are infected with more than one genotype (clone) of the same pathogen (Kyabayinze *et al.,* 2008). Multiclonal infections are thought to arise from infection with genetically diverse inoculum or reinfection before an existing infection is cleared (Read *et al.,* 2001). Such multiple clone infections are believed to play an important role in the development of clone specific immunity. Size polymorphisms in the MSP-1 and MSP-2 genes were used to calculate MOI as described previously (Waitumbi *et al.,* 2009). In agreement with other parameters of measuring malaria burden (Figures 4.1- 4.3), the number of samples with mixed infections were skewed to the cohort that stopped prophylaxis. At enrolment, only 29% (2/7) of subjects in discontinue CTX had mixed infections. This number increased steadily, reaching 63% (17/27) at M9. In comparison, post enrolment, all infections in the continue CTX arm were mono infections. Withdrawal of prophylaxis results in loss of the long-term conferred protection, consequently a higher MOI as these individual's become more susceptible to multiple parasite clones including the clones they were previously protected against.

The strength of this study included the long study follow-up as opposed to other studies with similar study design. The limitation of this study was the long period between follow ups (three months) this did not allow classification of recurrent malaria parasites that occurred

during the follow-up as recrudescence's or new infections. This long follow-up precludes us from proper characterization of the effect of withdrawal of prophylaxis on plasmodium genetic diversity. We can assume that observed parasitemia events self-cured. Climate has an influence on the transmissibility and occurrence of malaria, the long recruitment and follow-up period that were consolidated for analysis, as opposes to recruiting all individuals at one time and following them may have influenced the outcome.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

In summary, CTX discontinuation among ART-treated adults in a region with endemic malaria results in increased malaria prevalence, incidence, parasite density and MOI despite immune reconstitution. The implications are broad, and this study suggests that malaria endemicity should be considered when making decisions about whether to stop CTX after ART-driven immune reconstitution.

6.2 Conclusion

By all measurements (parasitemia prevalence, parasite density, parasitemia incidence and multiplicity of infection) over the 12 months observation period, the subjects who discontinued CTX prophylaxis had higher malaria burden compared to the subjects on CTX prophylaxis. Therefore, despite immune reconstitution following ART, discontinuation of CTX prophylaxis in individuals with HIV in malaria endemic regions results in;

1. Increased parasitemia prevalence. The increased parasite incidence following discontinuation of prophylaxis is not due to a short-lived rebound, but continues for a longer period.

2. High parasite burden. Over the 12 months observation period discontinuation of CTX prophylaxis in individuals with HIV while on ART resulted in increased burden of subclinical malaria parasitemias as well as increased episodes of clinical malaria

3. Higher Multiplicity of Infection (MOI), this may be due to the loss of the long-term conferred protection, as these individual's become more susceptible to multiple parasite clones including the clones they were previously protected against.

6.3 Recommendations from the current study

Despite immune reconstitution following ART, discontinuation of CTX prophylaxis in individuals with HIV would be ill advised especially in areas with high malaria endemicity. The prevalence of asymptomatic malaria in those who discontinue CTX over the one year observation in this study mirrors those of the general population in western Kenya. However, basing on the increasing parasitemia trend exhibited following CTX discontinuation, there is a possibility of even higher prevalence to be seen over time if CTX is discontinued despite immune reconstitution in HIV infected individuals.

6.3.1 Recommendations for future research

I. It remains to be determined whether, the benefits of continuing CTX prophylaxis out ways the concerns raised by proponents of stopping CTX prophylaxis, i.e. prevention of drug toxicities, decrease of the pill burdens and decrease to the risk of microorganism's resistance.

II. More comprehensive studies to determine if any immunological differences exist in terms of antibodies to antigens known to be involved in malaria immunology, in individuals who develop clinical and subclinical malaria in the arm discontinued CTX.

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APPENDIX

Appendix 1: *Plasmodium genus* primers and probe

PLASMODIUM GENERIC PRIMERS/PROBE

FORWARD PRIMER:	PLU3F	GCTCTTTCTTGATTTCTTGGATG
REVERSE PRIMER:	PLU3R	AGCAGGTTAAGATCTCGTTCG
PROBE:	PLU3P	ATGGCCGTTTTTAGTTCGTG

Appendix 2: MSP-1 and MSP-2 genotyping primers and probe

Primers for Primary PCR

Gene	Primer Name	Sequence
MSP-1	M1-O Fwd	5'- AAGCTAGAAGCTTTAGAAGATGCAGTATTGAC-3'
	M1-O Rev	5'- CTTAAATAGTATTCTAATTCAAGTGGATCA - 3'
MSP-2	Fwd (1)	5'- ATGAAGGTAATTAAAACATTGTCTATTATA - 3'
	Rev (4)	5'- ATATGGCAAAAGATAAAACAAGTGTTGCTG - 3'

Primers for nested PCR

Gene	Allelic Family	Primer Name	Sequence
MSP-1	K1	M1-K Fwd	5'- AAATGAAGAAGAAATTACTACAAAAGGTGC-3'
		M1-KRev	5'- GCTTGCATCAGCTGGAGGGCTTGCACCAGA-3'
	MAD20	M1-MFwd	5'- AAATGAAGGAACAAGTGGAACAGCTGTTAC-3'
		M1-MRev	5'- TGAATTATCTGAAGGATTTGTACGTCTTGAATTACC-3'
	RO33	M1-RFwd	5'-TAAAGGATGGAGCAAATACTCAAGTTGTTGCAAAGC-3'
		M1-RRev	5'- CATCTGAAGGATTTGCAGCACCTGGAGATCT-3'
MSP-2	IC3D7	Fwd A1	5'- GCAGAAAGTAAGCCTTCTACTGGTGCT -3'
		Rev A2	5'- GATTTGTTTCGGCATTATTATGA -3'
	FC27	Fwd B1	5'- GCAAATGAAGGTTCTAATACTAATAG -3'
		Rev B2	5'- GCTTTGGGTCCTTCTTCAGTTGATTC -3'

Appendix 3: Research Approvals

KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1 **July 18, 2011**

TO: **BENSON SINGA,**
PRINCIPAL INVESTIGATOR

THRO': **DR. JUMA RASHID,**
THE DIRECTOR, CCR,
NAIROBI

RE: **SSC PROTOCOL NO. 2077 (*INITIAL SUBMISSION*): DISCONTINUATION OF TRIMETHOPRIM-SULFAMETHOXALE PROPHYLAXIS IN ADULTS ON ANTIRETROVIRAL THERAPY IN KENYA: A RANDOMIZED TRIAL.**

This is to inform you that during the 191st meeting of the KEMRI/ERC meeting held on 12th July 2011, the above study was reviewed.

The Committee notes that the above referenced study aims to determine whether continued TMP/SMZ prophylaxis confers benefits in decreasing morbidity in individuals with reconstituted immune systems.

Due consideration has been given to ethical issues and the study is hereby **granted approval** for implementation effective this **18th day of July 2011,** for a period of twelve (12) months.

Please note that authorization to conduct this study will automatically expire on **17th July 2012.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **17th May 2012**.

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the ERC prior to initiation. You may embark on the study.

Yours sincerely,

Caroline Kithinji,
FOR: SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

Appendix 4: Consent Form

CTX RCT consent ***v 20 January 2013***

PARTICIPANT INFORMATION SHEET

University of Washington & Kenya Medical Research Institute,
Centre for Clinical Research

Discontinuation of trimethoprim-sulfamethoxazole prophylaxis in adults on antiretroviral therapy in Kenya: a randomized clinical trial.

CONSENT FOR ENROLLMENT
Randomized Clinical Trial Cohort

STUDY STAFF TO PROVIDE THIS FORM FOR POTENTIAL PARTICIPANTS TO READ
IF PARTICIPANT IS UNABLE TO READ OR UNDERSTAND, STUDY STAFF IS TO READ THIS FORM ALOUD (IN THE LANGUAGE OF THE PARTICIPANTS CHOOSING).

Investigator	Title	Institution	Telephone Contact
Benson Singa	MBChB, MPH	Research Scientist, Kenya Medical Research Institute	0725234844
Christina Polyak	MD, MPH	Visiting Scientist, Kenya Medical Research Institute/ Department of Medicine, University of Washington; **Research Physician, Walter Reed Army Institute of Research**	0701648519
Juma Rashid	MBChB, MMed	Ag, Director, Center for Clinical Research; Chief Research Officer, KEMRI	0723726526
Judd Walson	MD, MPH	Visiting Scientist, Kenya Medical Research Institute/ Department of Medicine, University of Washington	0721165696
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Jacob Johnson	PhD	**Executive Officer, United States Army Research Unit-Kenya**	0717727461
Edwin Kamau	**PhD**	**Director WRP, Malaria Drug Resistance Lab, WRP Kisumu**	**0708285289**

Emergency telephone number staffed 24 hours a day:
Dr. Benson Singa: 0725234844
Ethical Review Committee Secretary: (020) 2722541 or 0722205901 or 0733400003

Researcher's Statement

We are asking you to volunteer for a research study. Before you decide whether to take part in the study, we would like to explain the purpose of the study. We also want to explain the risks and benefits, and what would be expected of you if you agree to be in the study. This process is called 'informed consent'. It is important that you understand that your participation in this study is voluntary. This form will help you decide if you want to take part in the study. Once you understand the study, you can choose to be a part of the study or not. If you choose to be a part of this study, we will ask you to sign your name or make your mark on this form with your thumbprint. We will give you a copy of this form