EVALUATION OF *PLASMODIUM FALCIPARUM* RESISTANCE TO SULFADOXINE-PYRIMETHAMINE IN HUMAN IMMUNODEFICIENCY VIRUS (HIV) - INFECTED AND NON-INFECTED PREGNANT WOMEN IN A MALARIA-ENDEMIC REGION OF WESTERN KENYA

BY

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ABSTRACT

Malaria and Human immunodeficiency virus (HIV) co-infection in pregnancy is a major cause of anaemia, low birth weight (LBW) abortion and infant mortality in malaria-endemic countries of sub-Saharan Africa. Intermittent preventive therapy (IPTp) with Sulfadoxine-pyrimethamine (SP) is recommended by the World Health Organization (WHO) for malaria control during pregnancy. However, widespread resistance of *Plasmodium falciparum* to SP is a threat to the IPTp strategy. Therefore, there is an urgent need to evaluate the extent of parasite resistance to SP, specifically in pregnant women. The overall objective of this study was to evaluate *P. falciparum* resistance to SP in HIV-infected and non-infected pregnant women using real-time PCR. Samples used in this study were obtained from 94 women enrolled in a retrospective study conducted in Siaya and Bondo district hospitals in western Kenya, to investigate the effectiveness of daily cotrimoxazole (CTX) in preventing malaria during pregnancy. The enrolled women were categorized into four treatment arms on the basis of the drug used during pregnancy: SP, cotrimoxazole (CTX), SP and CTX and neither SP nor CTX. After delivery, peripheral and placental blood was collected and thick and thin smears made and stained with 10% Giemsa stain for *P. falciparum* detection using a light microscope. Unigold™ and Determine™ rapid tests were used for HIV testing. Parasite-positive peripheral and placental dried blood spots, obtained at delivery, were analyzed for the presence of specific mutations in the *P. falciparum* dihydrofolate reductase (*Pf/dhfr)* and dihydropteorate synthase *(Pf/dhps*) genes associated with SP resistance using real-time PCR. Prevalence and profiles of *Pf/dhfr* and *Pf/dhps* mutations were compared using the chi-square test (χ^2, $P \leq 0.050$). Overall, there was high prevalence (>50%) of *Pf/dhfr* and *Pf/dhps* mutant codons (*Pf/dhfr*108**N**, 51**I**, 59**R**, and *Pf/dhps* 437**G**, 540**E**) in both peripheral and placental samples in all treatment arms. There was no significant difference in the profiles of *Pf/dhfr* (SP, P=0.934; CTX, P=0.189; SP and CTX, P=0.407; neither SP nor CTX, P=0.477) and *Pf/dhps* (SP, P=0.655; CTX, P=0.705; SP and CTX, P=0.513; neither SP nor CTX, P=0.646) mutant codons between peripheral and placental samples. The prevalence of the quintuple mutant (*Pf/dhfr* Asn-108/Ile-51/Arg-59/*Pf/dhps* Gly-437/Glu-540) associated with *in vivo* SP treatment failure was high in all the treatment arms (>50%) and was comparable in both peripheral and placental samples in the different arms (SP, P=0.350; CTX, P=0.083; SP and CTX, P=1.000; neither SP nor CTX, P=0.362). However, in HIV-positive women in the CTX treatment arm, the prevalence of the quintuple mutant was significantly higher in placental samples (90.9%) than in peripheral samples (50%) (P=0.033). This study revealed that there is very high prevalence of *Pf/dhfr* and *Pf/dhps* resistance markers. Therefore, there is need for *in vivo* trials to evaluate SP efficacy in pregnant women in western Kenya. In addition, these findings can inform the design of field surveillance and monitoring of SP resistance in pregnant women resident in malaria-endemic regions.

CHAPTER ONE

1.0. Introduction

Every year, approximately 20-50 million pregnant women, living in malaria-endemic areas of sub-Saharan Africa, are at risk from malaria infection due to *P. falciparum* (WHO, 2003; Smith and Miller, 2004). Annually, at least 440 000 cases of malaria and Human immunodeficiency virus (HIV) co-infection are reported in pregnant women in malaria-endemic areas in sub-Saharan Africa (WHO, 2003). The severity of malaria during pregnancy is determined by the level of acquired immunity which, in turn, is dependent on factors such as malaria transmission intensity (Briand *et al.*, 2007) and HIV infection status. In unstable malaria transmission areas, pregnant women have lower levels of immunity to malaria and infection during pregnancy can result in severe maternal anaemia, stillbirth, spontaneous abortion or premature delivery (Briand *et al.*, 2007). In contrast, pregnant women in areas of high malaria transmission have substantial levels of immunity and the adverse effects of malaria during pregnancy are less severe (McGregor, 2000). However, malaria infection in pregnant women in these areas can remain undetected and untreated, leading to severe maternal anaemia and the increased risk of premature delivery and low birth weight (LBW), a risk factor for neonatal mortality (Smith and Miller, 2004). Pregnant women with malaria and HIV co-infection develop placental malaria more often and have high malaria parasite densities, in addition, they are at risk of premature delivery and giving birth to low birth weight infants (Van Geertruyden *et al.*, 2008).

To address the enormous problem of malaria during pregnancy, the World Health Organization (WHO) recommends three key strategies (WHO, 2003). These include: effective case management, intermittent preventive therapy (IPTp) with sulfadoxine-pyrimethamine (SP) and the use of insecticide-treated nets (ITNs) (D'Alessandro *et al.*, 1996).

For many years, intermittent preventive treatment using SP (IPTp-SP) has been shown to be effective and safe in reducing the adverse effects of malaria during pregnancy (Smith and Miller, 2004). However, IPTp programmes are currently threatened by widespread *P. falciparum* resistance to SP, prompting urgent calls for studies to evaluate the extent of *P. falciparum* resistance to SP specifically in pregnant women and its usefulness in IPTp (Alker *et al.*, 2004). Traditionally, studies conducted in young children are used to monitor the extent of parasite resistance to antimalarial drugs in endemic areas (McGregor, 2000). However, SP efficacy studies can no longer be carried out in young children because many countries in malaria-endemic areas have switched to artemisinin combination therapies (ACTs) for the treatment of uncomplicated malaria (WHO, 2004). Therefore, there is need to explore alternative strategies such as using SP resistance molecular markers on the *P. falciparum* dihydrofolate reductase (*Pf/dhfr*) and dihydropteorate synthase (*Pf/dhps)* genes in parasite isolates from pregnant women to evaluate the extent of *P. falciparum* resistance to SP.

1.1. Problem Statement

The use of SP for the first line treatment of uncomplicated malaria has been discontinued in many malaria-endemic countries due to widespread *P. falciparum* resistance (WHO, 2004). However, SP continues to be used for IPTp in pregnant women partly due to lack of data showing the prevalence and the profiles of *P. falciparum* mutations associated with SP resistance in peripheral and placental circulation during pregnancy (Briand *et al.*, 2007). In addition, it is difficult to carry out *in vivo* studies to evaluate alternative drugs for IPTp in pregnant women (Alker *et al.*, 2004). Therefore, there is need to evaluate *P. falciparum* resistance to SP in pregnant women to provide data to justify its continued use for IPTp. Studies are also needed to investigate the influence of other drugs (such as cotrimoxazole (CTX) which is used in HIV-

infected patients) on the presence of *P. falciparum* mutations that are associated with SP resistance. Pharmacological studies have shown that CTX has a similar mode of action to SP and it is used in HIV-positive patients to clear opportunistic infections (Parise *et al.*, 1998). It is unknown how the concurrent use of SP and CTX during pregnancy could affect the proportions and the prevalence of *P. falciparum* mutations in pregnant women. In this study, molecular markers associated with SP resistance were used to evaluate the extent of *P. falciparum* resistance to SP in samples from pregnant women enrolled in a retrospective study conducted in Siaya and Bondo district hospitals to investigate the effectiveness of daily cotrimoxazole (CTX) in preventing malaria during pregnancy in HIV-infected and non-infected women. These two regions (Siaya and Bondo districts) are holoendemic for *P. falciparum* transmission (ter Kuile *et al.*, 2003).

1.2. Justification

Sulfadoxine-pyrimethamine (SP) remains the only antimalarial drug recommended for intermittent preventive therapy (IPTp) due to its safety profile, low cost and dosing regimen (WHO, 2004). However, *in vivo* clinical studies to monitor SP resistance in young children and non-pregnant adults have shown a steady increase in *P. falciparum* resistance to SP over the last decade (Briand *et al.*, 2007). Because many countries in endemic areas have adopted artemisinin combination therapies (ACTs) as a first-line antimalarial drug for treatment of uncomplicated malaria, it is unethical to continue conducting SP efficacy trials in young children as a surrogate for its effectiveness in pregnant women (Alker *et al.*, 2004). This is due to differences in host immunity, drug pharmacokinetics and the fact that non-pregnant patients do not have placental *P. falciparum* parasites (Briand *et al.*, 2007). This could affect the overall proportion of molecular markers of SP resistance which may lead to difficulties in interpretation of data

(Menendez *et al.*, 2007). Malaria in pregnancy may also influence the value of *Pf/dhfr* and *Pf/dhps* mutations because, due to specific ligand expression, *P. falciparum* sequesters in the placental intervillous space and this leads to the absence of *P. falciparum* in peripheral blood despite placental parasitemia (Mockenhaupt *et al.*, 2007). It is therefore unclear whether peripheral parasites in pregnant women represent the actual parasite population. Studies are urgently needed specifically in pregnant women to evaluate the extent of peripheral and placental *P. falciparum* resistance to SP (Wilson *et al.*, 2010) and to obtain current resistance data on specific mutations on the *Pf/dhfr* and *Pf/dhps* genes which have been linked to SP resistance to warrant its continued use in malaria control during pregnancy. Studies in malaria-endemic regions have shown that pregnant women with HIV receiving CTX to clear opportunistic infections are more likely to suffer from malaria (Van Geertruyden *et al.*, 2008), however, there is lack of current data to confirm this observation. The current study was designed to evaluate the profiles and prevalence of *Pf/dhfr* and *Pf/dhps* mutations associated with SP resistance in peripheral and placental samples using molecular markers that are associated with *P.falciparum* resistance to SP.

1.3. Research questions

a) What are the differences in the profiles of molecular markers associated with *P. falciparum* resistance to SP between peripheral and placental parasites?

b) What is the prevalence of molecular markers of *P. falciparum* resistance to SP in peripheral and placental parasites?

c) What is the prevalence of molecular markers of *P. falciparum* resistance to SP in peripheral and placental parasites from HIV-positive and HIV-negative women?

1.4. Objectives

1.4.1. Main objective

To assess the prevalence of *P. falciparum* resistance to SP in HIV-infected and non-infected pregnant women using molecular markers.

1.4.2. Specific objectives

a) To compare the profiles of molecular markers associated with *P. falciparum* resistance to SP between peripheral and placental parasites.

b) To compare the prevalence of molecular markers of *P. falciparum* resistance to SP in peripheral and placental parasites.

c) To compare the prevalence of molecular markers associated with *P. falciparum* resistance to SP between peripheral and placental parasites from HIV-positive and HIV-negative women.

CHAPTER TWO

2.0. Literature review

2.1. The burden of malaria and HIV in Africa

In malaria-endemic areas of sub-Saharan Africa, an estimated one million people die from malaria annually and most of these are children under five years old (WHO, 2003). Every year, an estimated 10,000 pregnant women and 200,000 of their infants die as a result of the adverse effects of malaria during pregnancy (McGregor, 2000). In sub-Saharan Africa at least 440,000 women had malaria infection during pregnancy attributable to Human immunodeficiency virus (HIV) (WHO, 2003). Repeated malaria infection in children not only contributes to the development of severe anaemia, but it also makes young children more susceptible to childhood illnesses, such as diarrhoea and respiratory infections, thereby contributing to mortality (Molineaux, 1997). In areas of stable malaria transmission, pregnant women are at risk of malaria morbidity and mortality. In thes e areas, adult women have significant levels of immunity against malaria, but this is impaired in the first and second pregnancies leading to a significant risk of malaria infection and its adverse effects (WHO, 2002). Malaria and HIV co-infected pregnant women are more likely to develop clinical and placental malaria, and have detectable malaria parasitaemia (Van Geertruyden *et al.*, 2008).

2.2. Malaria and HIV infection in pregnancy

Every year, an estimated 20-50 million pregnant women living in malaria-endemic areas in Africa (WHO, 2003) are at risk from malaria infection (Smith and Miller, 2004). Malaria causes up to 15% of maternal anaemia and about 35% of preventable low birth-weight (Uneke, 2007). In high transmission areas, malaria during pregnancy is usually asymptomatic (Briand *et al.*, 2007), and is associated with a high frequency and density of *P. falciparum* parasitaemia,

with high rates of maternal morbidity, including placental malaria, fever and severe anaemia, (Uneke, 2007). In addition, longitudinal studies in high malaria transmission areas in Malawi and Uganda have shown that HIV-infection increases the risk of severe malaria in pregnant women (Van Geertruyden *et al.*, 2008). Most HIV-infected pregnant women in their first or second pregnancy are at higher risk of severe or complicated malaria than during subsequent pregnancies (WHO, 2004). In areas of low transmission, *P. falciparum* infected pregnant women have low levels of immunity and are, therefore, at risk of dying from severe malarial disease or from experiencing spontaneous abortion, premature delivery or stillbirth (Brabin *et al.*, 2008). In malaria-endemic areas, HIV increases the risk of malaria infection and in low transmission areas, HIV-infected pregnant mothers are at increased risk of complicated and severe malaria anaemia, premature delivery, low birth weight infants and death (Van Geertruyden *et al.*, 2008).

2.3. Prevention and management of HIV and malaria in pregnancy

The World Health Organization (WHO) recommends that in high transmission areas, case management of malaria during pregnancy using an effective antimalarial drug should form part of routine antenatal clinic (ANC) visits (WHO, 2003). The WHO also advocates the use of insecticide-treated nets (ITNs) during pregnancy in these areas to decrease exposure to infective mosquito bites and provide protection from malaria (D'Alessandro *et al.*, 1996; Kabanywanyi *et al.*, 2008). In addition, WHO also recommends that IPTp with an effective one-dose antimalarial drug be made available to women in their first and second pregnancies in high malaria-endemic areas (WHO, 2000). Currently, SP (500mg sulfadoxine and 25 mg pyrimethamine) is the recommended drug for IPTp and the standard regimen uses at least 2 curative treatment courses given in the second and third trimesters, regardless of whether or not pregnant women are infected with malaria (WHO, 2004) and in HIV-positive pregnant women, at least three doses of

SP are needed to sufficiently clear malaria infection (Parise *et al.*, 1998). These doses are administered during routine antenatal clinic visits after foetal quickening with each course given less than one month apart and prior to the last month of pregnancy (Briand *et al.*, 2007). The current WHO-recommended schedule for ANC includes a total of four visits, including three after quickening (WHO, 2003). Following pharmaco-dynamic modeling, it is currently recommended by the division of malaria control (DOMC) in Kenya, that increasing the frequency of IPTp to at least three doses for all pregnant women, irrespective of their HIV status, would be of more practical advantage (Menendez *et al.*, 2007). Malaria treatment with SP clears asymptomatic peripheral and placental parasitaemia and provides intermittent chemoprophylaxis against malaria infection during pregnancy (Mbugi *et al.*, 2006). To prevent opportunistic infections in HIV-positive patients including all pregnant women after their first trimester, Joint United Nations Program on HIV/AIDS (UNAIDS) recommends daily intake of CTX (UNAIDS, 2000; Newman *et al.*, 2003).

2.4. Challenges to the WHO recommended methods for malaria and HIV control during pregnancy

Several studies in areas of diverse malaria transmission patterns have shown that ITNs can protect pregnant women from the adverse effects of malaria during pregnancy (D'Alessandro *et al.*, 1996; Noor *et al.*, 2007). However, according to a recent study involving pregnant women sleeping under ITNs but not on IPTp-SP in Ifakara, Tanzania, the incidence of low birth weight (LBW) was 15% and placental parasitaemia was 8% at delivery, implying that malaria in pregnancy is still a problem even in areas with high bed net coverage (Kabanywanyi *et al.*, 2008). Additionally, the use of ITNs for malaria control is threatened by sustainability and emergence of vector resistance to insecticides used in impregnating bed-nets (Noor *et al.*, 2007).

Malaria control using case management in endemic areas is also faced with challenges such as weak infrastructure, drug stock-outs and availability of qualified staff (Steketee *et al.*, 2001). In addition, laboratory services in most malaria-endemic countries are weak and incapable of adequately supporting malaria diagnosis and case management (Yartey, 2006). To prevent the adverse effects of malaria during pregnancy, the WHO recommended a weekly chloroquine chemoprophylaxis during pregnancy (300 mg per week) for self administration until the mid 1990s when the IPTp-SP policy was adopted (Denoeud *et al.*, 2007). This is because compliance with chloroquine remained low because it was associated with pruritus and patients attributed its bitter taste with medications that induce abortion (Helitzer-Allen *et al.*, 1994). In addition, there were increasing rates of *P. falciparum* resistance to chloroquine (Ridley, 2002). Therefore, SP began to be used for treatment of chloroquine-resistant malaria in Africa in the 1980s (Pearce *et al.*, 2009). In HIV-positive pregnant women with malaria, more doses of SP are needed for IPTp-SP due to impaired host immunity and drug efficacy (Parise *et al.*, 1998). However, concurrent administration of SP and CTX in HIV-positive pregnant women is inadvisable because they have same mode of action and this is likely to increase *P. falciparum* resistance to SP (ter Kuile *et al.*, 2003). Clinical treatment failure with SP was first reported in Kenya in 1988 (Certain *et al.*, 2008) and over the last decade, there has been increasing *P. falciparum* resistance to SP in sub-Saharan Africa (ter Kuile and Steketee, 2007) hence the adoption of artemisinin-based combination therapies (ACTs) as first-line therapy for treating uncomplicated *P. falciparum* malaria (WHO, 2006). The widespread parasite resistance to SP is likely to have a negative impact on malaria control in pregnancy.

2.5. *In vitro* assays to detect *Plasmodium falciparum* resistance to antimalarial

Malaria-based *in vitro* assays measure susceptibility of *P. falciparum* to antimalarial drugs (Wilson *et al.*, 2010). These assays have the advantage of not only determining drug response outside the host and, therefore, not affected by factors such as acquired immunity and pharmacokinetics but they are also safe, inexpensive and can be used in remote settings (Basco and Ringwald, 2003). However, *in vitro* assays require well equipped central laboratories and high parasite densities (Wilson *et al.*, 2010). In addition, adaptation of *P. falciparum* to laboratory environment is depended on maintenance of high sterile conditions, pH of the culture medium and concentration of gas mixtures among other factors (Rason *et al.*, 2008).

2.6. Monitoring parasite resistance to SP using molecular methods in pregnant women

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) which utilizes restriction enzymes to digest DNA and the resulting fragments are separated by gel electrophoresis based on their sizes is the most common technique for genotyping, and the assay used is highly specific. However, sensitivity might drop at very low parasitemia, particularly in polyclonal infections (detection of different species of *P. falciparum* in patient samples) and results occasionally differ from those obtained by other techniques (Mockenhaupt *et al.*, 2007). Single nucleotide polymorphisms (SNPs) associated with antimalarial drug resistance can be detected by real-time PCR (Afonina *et al.*, 2002). This technique has been used for allelic discrimination in malaria positive patients and a similar technique has been used to identify alleles that confer drug resistance in bacteria (Garcia de Viedma *et al.*, 2002; Kearns *et al.*, 2002). In this technique, minor groove binding (MGB) probes hybridize to the PCR amplicons and when the *Taq* DNA polymerase cleaves the probe from the 5′ end the fluorophore is released from the quencher, allowing it to fluoresce. The increase in fluorescence can be measured by

using a real-time PCR thermocycler (Alker *et al.*, 2004). Allelic discrimination is achieved by using two labeled probes complementary to wild type and mutant alleles in the assay (Livak, 1999). *P. falciparum* resistance to SP is caused by point mutations in the genes that encode the targets of pyrimethamine, *P. falciparum* dihydrofolate reductase (*Pf/dhfr*) and sulfadoxine, *P. falciparum* dihydropteorate synthase (*Pf/dhps*) respectively, and this leads to amino acid changes in the active site cavity of the enzyme (Plowe *et al.*, 2007). In *Pf/dhfr* a serine (Ser) at position 108 is associated with sensitivity to pyrimethamine and a change from Ser 108 to Asparagine (Asn) 108 (S108N) confers resistance to pyrimethamine. Mutations at positions 59 cysteine (Cys) to arginine (Arg), position 51 Asn to Isoleucine (ILe) and position 164 ILe to leucine enhances the resistance of *P. falciparum* to pyrimethamine (Zakeri *et al.*, 2003). For *Pf/dhps*, changes at codon 437 from alanine (Ala) to glycine (Gly), confer resistance which becomes profound with additional mutations at codon 540 Gly to Glutamine (Glu) (Doumbo *et al.*, 2000). Clinical studies and standard PCR methods in malaria-endemic countries have also shown that the *Pf/dhfr* triple mutant (Asn-108/Ile-51/Arg-59), the *Pf/dhps* double mutant (Gly-437/Glu-540), and the quintuple mutant (*Pdhfr*Asn-108/ile-51/Arg-59/*Pf/dhps*Gly-437/Glu-540) are associated with sulfadoxine-pyrimethamine treatment failure *in vivo* (Marfurt *et al.*, 2008). In a recent study in malaria-endemic areas of sub-Saharan Africa, HIV infection was found to increase the chances of a malaria infection progressing to symptomatic illness (Bwijo *et al.*, 2003). This increases contact between the parasites and the drug therefore contributing to the spread of parasite resistance by increasing drug exposure and drug pressure (Van Geertruyden *et al.*, 2008).

2.7. Profiles of peripheral and placental SP resistance molecular markers in western Kenya

In a study carried out in adults with malaria in western Kenya after the adoption of SP, the prevalence of peripheral *Pf/dhfr* mutant codons N51I, C59R, and S108N were near saturation in the baseline study and remained stable between the baseline and follow-up studies (Hamel *et al.*, 2008). In contrast, the prevalence of peripheral mutations at the *Pf/dhps* codons A437G and K540E increased implying that *Pf/dhfr* mutations were high prior to 1998, and use of SP as the first-line antimalarial in Kenya resulted in the development of high mutant *Pf/dhps* codons (Maroya *et al.*, 2010). Mutations on *Pf/dhfr* codons were derived from the use of SP as a second-line anti-malarial in Kenya prior to 1998 and the ongoing treatment of HIV-positive patients who were on treatment with CTX (Maroya *et al.*, 2010). A similar study using laboratory based Nested PCR preliminary survey involving peripheral and placental samples from pregnant women showed that all peripheral blood samples collected were wild type at *Pf/dhfr* codon 164 and were mutant at *Pf/dhfr* codon 108 and *Pf/dhps* codon 437 (Harrington *et al.*, 2009). Mixed parasite populations (both mutant and wild type) were identified at *Pf/dhfr* codons 51 and 59 and at *Pf/dhps* codon 540. Placental samples were nearly saturated at *Pf/dhfr* codons 51 and 59, and at *Pf/dhps* codon 540 (Harrington *et al.*, 2009). Despite these studies on the molecular markers, no studies have been carried out in western Kenya to compare the profiles and prevalences of molecular markers associated with *P. falciparum* resistance to SP between peripheral and placental parasites in HIV-infected and non-infected population of pregnant women. As such, the current study compared the profiles and prevalences of the molecular markers *Pf/dhfr* and *Pf/dhps* , the variants associated with *P. falciparum* resistance to

SP between peripheral and placental parasites in HIV-infected and non-infected population of pregnant women.

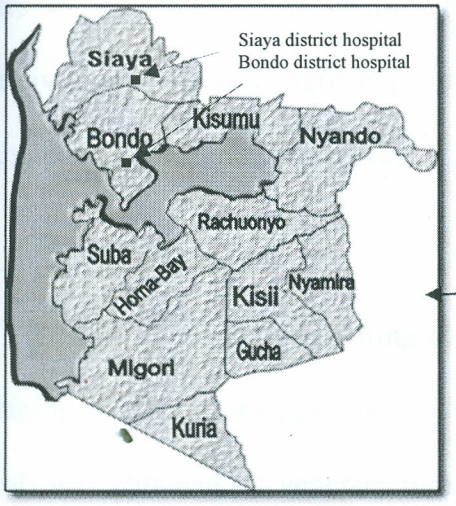
CHAPTER THREE

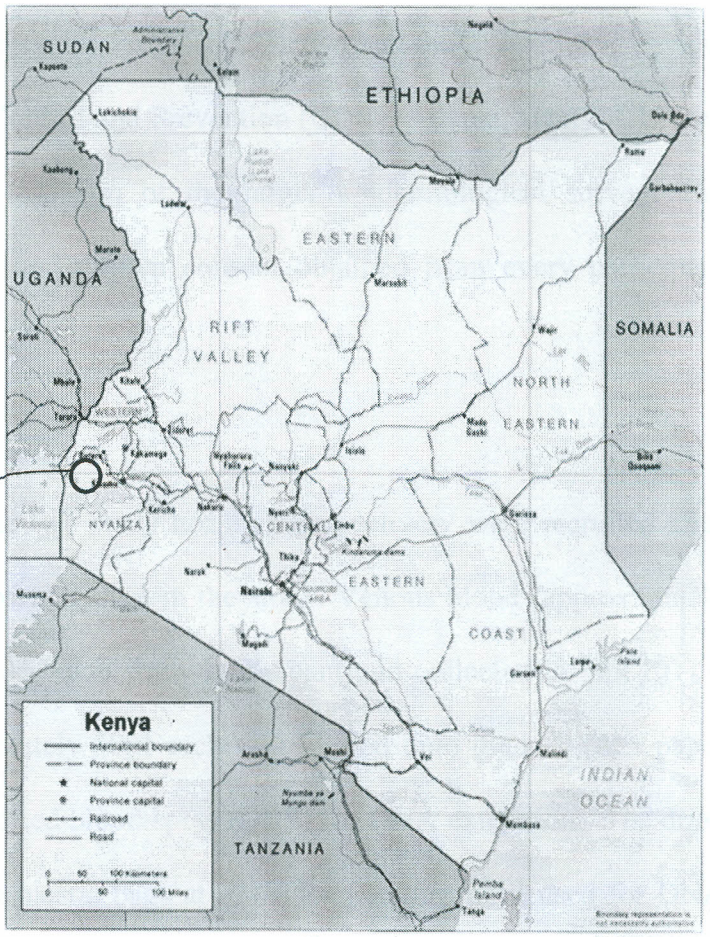
3.0. Materials and methods

3.1. Study population

The samples used in the current study were obtained from pregnant women (≥15 years) enrolled in a retrospective study conducted by KEMRI/CDC in Siaya and Bondo district hospitals (Figure 1) to investigate the effectiveness of daily CTX in preventing malaria during pregnancy. The sample size was based on the number of malaria-positive pregnant women reporting for delivery in Siaya and Bondo district hospitals for the last 6 months. In order to account for withdrawals, an additional 20% of patients were recruited. This study was done both in Bondo and Siaya which are located in a malaria-endemic region to capture recent use and adherence to the IPTp-SP policy. According to the 2009 Kenyan population census, Bondo district has a population of 271 649 and is located approximately 45 km north-east of Kisumu town while Siaya district has a population of 842, 304 and lies about 50 km north west of Kisumu town (Kenya National Bureau of Statistics, 2010). Malaria transmission in this region is intense and perennial with *P. falciparum* accounting for 98% of the total reported malaria infections (ter Kuile and Steketee, 2007). Data from the New Nyanza Provincial Hospital, the largest referral hospital in the province indicates that 20% of women reporting for delivery at Siaya and Bondo district hospitals (average for all gravidities) have peripheral malaria parasitaemia while 70% are anaemic (Hb<11.0 g/dL) (ter Kuile *et al.*, 2003). In Siaya and Bondo districts, malaria in pregnancy results to severe maternal anaemia, premature delivery and low birth weight (LBW), in addition, there is wide use of antimalarial drugs for the treatment of

malaria, it is therefore expected that the genes responsible for resistance would spread faster due to drug pressure (ter Kuile and Steketee, 2007).





Siaya district hospital
0° 3'36"N-latitude
34°17'10"E-longitude

Bondo district hospital-
0°14'19"N-latitude
34°16'9"E-longitude

www.googleimages.com
Date of access: 09 Sept 2011

Figure 1. A map of western Kenya showing the location of Bondo and Siaya district hospitals where this study was carried out.

3.2. Ethical considerations

Ethical approval was obtained from the Ethical Review Committee (ERC) of the Kenya Medical Research Institute (KEMRI) (Appendix 7), Nairobi, Kenya and the Institutional Review Board (IRB) of the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, United States of America (USA), before the initiation of the larger epidemiological survey. The objectives of the study were explained and written consent obtained from every participant before being included in the survey.

3.3. Sample collection

Pregnant women presenting at the two study hospitals for delivery were recruited after obtaining written informed consent for participation in the study. Venous blood (approximately 3.5ml) was obtained by venepuncture from each study participant and collected in 5ml EDTA tube. Thereafter, four drops of approximately 50µl each was spotted onto grade-3 filter paper (Whatman Int. Ltd, England) to prepare dried blood spots (DBS) for DNA extraction. The dried blood spots were air dried and stored in plastic bags at room temperature until used for DNA extraction. Approximately 2ml of previously collected blood in the EDTA tube was used to determine HIV status which was done using two rapid tests (Unigold® and Determine®) in a parallel algorithm according to the recommendations by the Kenyan Ministry of Health, National AIDS and STDs Control Program (NASCOP) (Hamel *et al.*, 2008). About 10µl of blood was used to prepare peripheral thick and thin smears which were stained with 10% Giemsa and stained for 10 minutes and malaria diagnosis was done by light microscopy. Using a tally counter, asexual parasites were counted per 500 white blood cells (WBCs) on a Giemsa-stained slide based on an average leukocyte count of 8000/µl as previously described (WHO, 2009). The placenta was obtained after delivery, and a small tissue ($0.5cm^3$) used to prepare thick and thin

impression smears and malaria diagnosis by light microscopy was done as previously explained. Approximately 2ml of placental blood was collected from the intervillous space using a sterile syringe and dried blood spots (DBSs) for DNA extraction were prepared as previously explained and stored at room temperature. Demographic information such as age, gravidity, fever, antimalarial drug used to prevent malaria in pregnancy and maternal haemoglobin (Hb) was collected at enrollment.

3.4. Analysis of molecular markers of SP resistance

3.4.1. DNA ext raction

The chelex method was used for DNA extraction because as compared to other methods it is fast and reliable and has the advantage of yielding genomic DNA from samples of low parasitaemia. In brief, approximately, 0.25 inch round piece (enough to fit in a 1.5ml eppendorf tube) of the dried blood spots (DBS) on grade-3 filter paper (Whatman Int. Ltd, England) were cut and incubated in 1.5ml eppendorf tubes overnight at 37°C in 0.5% saponin in phosphate buffered saline (PBS) (Invitrogen, NY, USA). The tubes were centrifuged twice at 4000 revolutions per minute (rpm) for 3 minutes to completely remove any haem residue from the samples. Saponin solution and debris was discarded leaving the blood spots in the eppendorf tubes. A total volume of 50µl of 20% homogenous chelex suspension in nuclease-free water (Life Technologies, USA) was added to each tube. Assuming that DNA was to be extracted from 10 blood spots, 100µg of chelex was weighed and suspended in 500µl of nuclease-free water, this was done to maintain 20% chelex homogeneity. A volume of 100µl of nuclease-free water was added to each eppendorf tube. The tubes were incubated for 8 minutes at 100°C on a heat block. The tubes were then centrifuged for 2 minutes at 4000 rpm and 100µl of DNA supernatant collected and transferred into labeled eppendorf tubes. Extracted DNA was then

stored at -20°C until used in genotyping SP resistance markers. A spectrophotometer was used to determine the purity of extracted DNA. An absorbance (A_{260}/A_{280}) ratio of between 1.7 and 2.0 represented pure DNA.

3.4.2. Real ti me PCR assay

Real-time PCR was carried out in duplicate in a 25µl master mix final volume containing 12.5µl of universal PCR master mix (Cat# 4305719 ABI-850 Lincoln Center, CA), 2µl of extracted DNA initially stored at -20°C (diluted 1:10 in AE buffer) (all samples tested had an initial parasitaemia of ≥16 parasites per microlitre of blood as observed under a light microscope), forward and reverse primers (CDC, Scientific Resources Program), both mutant and wild type probes (Cat# 4316034 ABI-850 Lincoln Center, CA) at various concentrations (Appendix 2). Primer and probe sequences are shown in Appendix 3 and 4, respectively. All reactions were run on a Stratagene MX3005P® instrument (Stratagene, La Jolla, CA), using thermo-cycling conditions specific for each SNP as shown in Appendix 5.

For the real-time PCR plate setup, four 10-fold serial dilutions were made in AE buffer stored at room temperature (Qiagen Biotechnology, Germany) (1:10, 1:100, 1:1000, 1:10000) of both *P. falciparum* wild type and mutant control strains (initial parasitaemia of 1%, 10 infected red blood cells in 1000 infected and non-infected red blood cells) (CDC, Scientific Resources Program) (Appendix 1) stored at -20°C were used to create individual single nucleotide polymorphism (SNP) standard curves.

3.4.3. Data interpretation

At the end of the real-time PCR reaction, positive standard curves (wild type and mutant) were compared with the amplification plots of the test samples based on the fluorescence intensity of the four 10-fold serial dilutions and used to score the test samples as either wild type

or mutant. For each reaction, the threshold bar was set above background fluorescence of positive standards in the early cycles of PCR amplification. Parasite population at specific *Pf/dhfr* and *Pf/dhps* codons was classified as either wild type or mutant. Major parasite population in a mixed infection (both wild type and mutant strains) was determined based on the Ct (threshold cycle) values (which was indirectly proportional to the amount of DNA present in the test sample) and sample ending fluorescence (tail end of the exponential phase of the real-time PCR amplification plot). The real-time PCR amplification plots of the test samples were classified according to the methods developed by Alker *et al.*, 2004. If the amplification plots for the wild type and mutant overlapped, the parasite population was considered 50% wild type and 50% mutant. If both strains amplified and the difference in the Ct value between the major and minor parasite population was less than 7, then the sample was classified as mixed. Results were recorded in an Excel spreadsheet.

3.4.4. Codon analysis and interpretation

Based on the analysis of results following real-time PCR amplification reaction, *Pf/dhfr* and *Pf/dhps* mutations for each infection were categorized as shown in Table 2 and combined to determine genotype for *Pf/dhfr* and *Pf/dhps* according to previous methods of classification of *dhfr* and *dhps* genotypes (Kublin *et al.*, 2002) (Appendix 6).

Table 1: Codon analysis for *Pf/dhfr* (1) and *Pf/dhps* (2) codon mutations based on real-time PCR results

***P. falciparum* mutants**	**2. *Pf/dhfr* codons**			
	#[a]Sample	108	51	59
Double	1	Mixed	Mixed	Wild type
	2	Mixed	Wild type	Mixed
	3	Mixed	Mixed	Mixed
	4	Mixed	Wild type	Mutant
	5	Mixed	Mixed	Mutant
	6	Mixed	Mutant	Wild type
	7	Mixed	Mutant	Mixed
	8	Mutant	Mixed	Wild type
	9	Mutant	Wild type	Mixed
	10	Mutant	Mixed	Mixed
	11	Mutant	Wild type	Mutant
	12	Mutant	Mutant	Wild type
Triple pure	1	Mutant	Mutant	Mutant
Triple mixed	1	Mixed	Mutant	Mutant
	2	Mutant	Mixed	Mutant
	3	Mutant	Mutant	Mixed

***P. falciparum* mutants**	#[a]Sample	**1. *Pf/dhps* codons**	
		437	540
Double mixed	**1**	Mixed	Mutant
	2	Mutant	Mixed
Double pure	**1**	Mutant	Mutant

[a] Indicates the expected real-time PCR assay result after amplification.

3.4.5. Data analysis

Prevalence and profiles of *Pf/dhfr/dhps* mutations present in peripheral and placental samples were compared using the chi-square test (χ^2). The mean age, peripheral and placental parasite densities in the four treatment arms were compared usin g the Kruskal-Wallis test. Statistical significance between *Pf/dhfr/dhps* mutants present in peripheral and placental samples was assessed at $P \leq 0.050$.

CHAPTER FOUR

4.0. Results

4.1. Baseline characteristics of study participants

Table 1 shows the baseline characteristics of the study participants. The following were the distribution patterns when women were stratified into four arms based on the drug used to prevent malaria during pregnancy: 65.6% of the women received sulfadoxine-pyrimethamine (SP), 14.6% were on cotrimoxazole (CTX), 7.3% had taken a combination of SP and CTX, and 10.4% were not on any treatment (Table 1). The mean age in years, parasite density and geometric mean peripheral and placental parasitaemia were comparable across the 4 different groups (P=0.529, P=0.087 and P=0.554, respectively). Similarly, the proportions of those febrile (axillary temperature), anaemic, multigravidae and primigravidae in the 4 different groups were also comparable (P=0.819, P=0.200, P=0.155 and P=0.155, respectively).

Table 2: Baseline characteristics of study participants

Parameter	Treatment arms				*P* value[c]
	SP[a] (n=63)	CTX[b] (n=14)	SP and CTX (n=7)	neither SP nor CTX (n=10)	
Mean age (yrs) (95% CI[e]	21.8	23.9	21.4	22.2	0.529[d]
Febrile (>37.0°C %)	47.6	57.1	57.1	60.0	0.819
Anaemic (Hb[f]<11 g/dl %)	60.3	85.7	85.7	70.0	0.200
Primigravidae %	61.9	28.6	57.1	60.0	0.155
Multigravidae %	38.1	71.4	42.9	40.0	0.155
GMPD[g] peripheral (95% CI)	634.5	574.4	395.7	1904.1	0.087[d]
GMPD placental (95% CI)	3342.0.	2570.4	2978.5	8699.6	0.554[d]

[a]SP, Sulfadoxine-pyrimethamine, [b]CTX, Cotrimoxazole, [c]Pearson chi square test, [d]Kruskal-Wallis test,

[f]Hb, Haemoglobin, [g]GMPD, Geometric mean parasite density, [e]CI, Confidence interval.

4.2. Profiles of *Pf/dhfr* and *Pf/dhps* mutant codons in peripheral and placental samples in the four treatment arms

Table 3 shows the percentage of *Pf/dhfr* and *Pf/dhps* mutant codons that were present in peripheral and placental samples analyzed in the SP, CTX, SP and CTX, and neither SP nor CTX treatment arms. High (>50%) proportions of *Pf/dhfr* and *Pf/dhps* mutant codons were present in all the samples analyzed in all the treatment arms, however, the lowest proportion of mutations were present in peripheral samples at the *Pf/dhfr* codon 59 in the CTX treatment arm (in bold).

Table 3. Percentage profiles of *Plasmodium falciparum* dihydrofolate reductase (*Pf/dhfr*) and dihydropteorate synthase (*Pf/dhps*) mutant codons in peripheral and placental samples

	Pf/dhfr				*Pf/dhps*		
	51	**59**	**108**	*P* value	**437**	**540**	*P* value[a]
SP							
Peripheral (n=48)	89.6	77.1	93.8	0.934	85.4	83.3	0.665
Placental (n=56)	78.6	78.6	87.5		87.5	87.5	
CTX							
Peripheral (n=12)	75.0	**58.3**	83.3	0.189	83.3	75.0	0.705
Placental (n=12)	91.7	91.7	91.7		83.3	91.7	
SP and CTX							
Peripheral (n=7)	85.7	85.7	85.7	0.407	71.4	85.7	0.513
Placental (n=7))	100.0	85.7	85.7		100.0	85.7	
Neither SP nor CTX							
Peripheral (n=9)	88.9	88.9	88.9	0.477	88.9	88.9	0.646
Placental (n=7)	100.0	100.0	100.0		100.0	100.0	

Proportions of *Pf/dhfr* and *Pf/dhps* mutant codons in peripheral and placental samples were presented as percentages after real-time PCR amplification. Percentages were obtained by expressing the number of mutant peripheral or placental samples on *Pf/dhfr* and *Pf/dhps* codons over the total number of peripheral or placental samples. [a]Statistical analysis using χ^2 test. Statistical significance was tested at $P \leq 0.050$.

4.3. Prevalence of *Pf/dhfr/dhps* mutants in peripheral and placental samples in the four treatment arms

Mutations at *Pf/dhfr* and *Pf/dhps* codons were combined to determine *dhfr* and *dhps* genotypes based on Kublin *et al.*, 2002 system of coding and classification of *P. falciparum* genotypes (Appendix 6). Table 5 shows the prevalence of *Pf/dhfr/dhps* mutants in peripheral and placental samples in the SP, CTX, SP and CTX, and neither SP nor CTX treatment arms expressed as percentages. Comparison of the double, quadruple, and quintuple mutant in peripheral and placental samples was comparable in all the treatment arms. However, comparison of the prevalence of the triple mutant in peripheral and placental samples was statistically significant (in bold).

Table 4. Percentage prevalence of *Plasmodium falciparum* dihydrofolate reductase (*Pf/dhfr*) and dihydropteorate synthase (*Pf/dhps*) mutants in peripheral and placental samples

	Pf/dhfr/dhps mutants[a]							
	Double		**Triple**		**Quadruple**		**Quintuple**	
SP		*P* value		*P* value		*P* value		*P* value
Peripheral (n=48)	89.6	0.552	77.1	0.971	87.5	1.000	66.7	0.350
Placental (n=56)	85.7		76.8		87.5		75.0	
CTX								
Peripheral (n=12)	58.3	0.059	50.0	**0.025**	58.3	0.059	50.0	0.083
Placental (n=12)	91.7		91.7		91.7		83.3	
SP and CTX								
Peripheral (n=7)	85.7	0.515	85.7	1.000	85.7	0.515	71.4	1.000
Placental (n=7)	71.4		85.7		71.4		71.4	
Neither SP nor CTX								
Peripheral (n=9)	88.9	0.362	88.9	0.362	88.9	0.362	88.9	0.362
Placental (n=7)	100.0		100.0		100.0		100.0	

Following real-time PCR amplification, the prevalence of the double, triple, quadruple and quintuple *Pf/dhfr/dhps* mutants in peripheral and placental samples was presented as percentages. Percentages were obtained by expressing the number of *Pf/dhfr/dhps* mutants over the total number of peripheral or placental samples. [a]Statistical analysis using χ^2 test. Statistical significance was tested at $P \leq 0.050$.

4.4. Prevalence of *Pf/dhfr/dhps* mutants in HIV-negative pregnant women

Table 5 shows the prevalence of *P. falciparum dhfr/dhps* (*Pf/dhfr/dhps*) mutants in peripheral and placental samples obtained from HIV-negative pregnant women in the SP, CTX, SP and CTX and neither SP nor CTX treatment arms

Table 5: Percentage of *Pf/dhfr/dhps* mutants in HIV-negative pregnant women

	Pf/dhfr/dhps mutants[a]							
	Double		**Triple**		**Quadruple**		**Quintuple**	
SP		*P* value		*P* value		*P* value		*P* value
Peripheral (n=43)	88.4	0.570	74.4	0.992	86.0	0.975	62.8	0.312
Placental (n=51)	84.3		74.5		86.3		72.5	
CTX								
Peripheral (n=0)	0	-	0	-	0	-	0	-
Placental (n=0)	0		0		0		0	
SP and CTX								
Peripheral (n=1)	100.0	-	100.0	-	100.0	-	100.0	-
Placental (n=1)	100.0		100.0		100.0		100.0	
Neither SP nor CTX								
Peripheral (n=7)	85.7	0.335	85.7	0.335	85.7	0.335	85.7	0.335
Placental (n=6)	100.0		100.0		100.0		100.0	

The prevalence of *Pf/dhfr/dhps* mutants in peripheral and placental samples was expressed as percentages. [a]Statistical analysis using χ^2 test. Percentages were obtained by expressing the number of *Pf/dhfr/dhps* mutants over the total number of peripheral or placental samples. Statistical significance was tested at $P \leq 0.050$.

4.5. Prevalence of *Pf/dhfr/dhps* mutants in HIV-positive women

Table 6 shows the prevalence of *P. falciparum dhfr/dhps* (*Pf/dhfr/dhps*) mutants in peripheral and placental samples obtained from HIV-positive pregnant women in the SP, CTX, SP and CTX and neither SP nor CTX treatment arms.

Table 6: Proportion of *Pf/dhfr/dhps* mutants in HIV-positive pregnant women

	***Pf/dhfr/dhps* mutants[a]**							
	Double		**Triple**		**Quadruple**		**Quintuple**	
SP		*P* value		*P* value		*P* value		*P* value
Peripheral (n=5)	100.0	-	100.0	-	100.0	-	100.0	-
Placental (n=5)	100.0		100.0		100.0		100.0	
CTX								
Peripheral (n=12)	58.3	**0.016**	50	**0.025**	58,3	**0.016**	50.0	**0.033**
Placental (n=11)	100.0		100.0		100.0		90.9	
SP and CTX								
Peripheral (n=6)	83.3	0.505	83.3	1.000	83.3	0.505	66.7	1.000
Placental (n=6)	66.7		83.3		66.7		66.7	
Neither SP nor CTX								
Peripheral (n=2)	100.0	-	100.0	-	100.0	-	100.0	-
Placental (n=1)	100.0		100.0		100.0		100.0	

The prevalence of *Pf/dhfr/dhps* mutants in peripheral and placental samples was expressed as percentages. [a]Statistical analysis using χ^2 test. Percentages were obtained by expressing the number of *Pf/dhfr/dhps* mutants over the total number of peripheral or placental samples. Comparison of the *Pf/dhfr/dhps* double, triple, quadruple and quintuple mutants in peripheral and placental samples was statistically significant in the CTX treatment arm ($P \leq 0.050$).

CHAPTER FIVE

5.0. Discussion

In malaria-endemic regions of sub-Saharan Africa, WHO recommends use of IPTp-SP for malaria control and prevention during pregnancy (WHO, 2004). However, reported cases of *P. falciparum* resistance to SP is a threat to the IPTp-SP strategy (Brabin *et al.*, 2008). In order to monitor emerging cases of antimalarial resistance, *in vivo* methods are considered the gold standard (Steketee *et al.*, 2001). Unfortunately, there are fears of safety on the neonates when pregnant women are involved (Brabin *et al.*, 2008). This study was designed with the main objective of investigating the prevalence of *P. falciparum* resistance to SP in HIV-infected and non-infected pregnant women using molecular methods.

Analysis of the *Pf/dhfr* 51, 59, 108 and *Pf/dhps* 437 and 540 mutant codons in all the four treatment arms showed that pregnant women with malaria have increased proportions of mutations in placental samples than peripheral samples, however, this proportions were comparable. In a similar study in West Africa involving pregnant women where samples were analyzed using PCR-restriction fragment length polymorphism (PCR-RFLP), malaria in pregnancy was shown to influence the presence of placental *Pf/dhfr* and *Pf/dhps* mutations, however, concordance was present when peripheral and placental *Pf/dhfr* and *Pf/dhps* mutations were compared (Mockenhaupt *et al.,* 2007). Stepwise selection of point mutations on *Pf/dhfr* codons 108, 51 and 59 was observed in peripheral samples obtained from pregnant women in the SP and cotrimoxazole (CTX) treatment arms. This is consistent with previous studies involving pregnant women using SP in malaria-endemic areas which have shown that *Pf/dhfr* mutations starts at codon 108, which is then followed by mutations at codon 51 and 59 (Wernsdorfer and Noedl, 2003). The characteristic stepwise pattern of *Pf/dhfr* point mutations was not evident in placental samples in all the four treatment arms. This could be attributed to the fact that

acquisition of *Pf/dhfr* mutations takes a different pathway in placental parasites. In the current study, analysis of the *Pf/dhfr* codon 164 revealed wild type parasite population in all peripheral and placental samples in pregnant women in the four treatment arms. This is consistent with previous studies in malaria-endemic areas in sub-Saharan Africa which have also reported that mutations at the *Pf/dhfr* codon 164 are not present at detectable levels where SP resistance is high (Nzila *et al.*, 2005).

Data from this study showed that, compared to peripheral samples, placental samples had a high prevalence of the *Pf/dhfr/dhps* double, triple and quintuple mutants in all the four treatment arms. A study in Mozambique which enrolled pregnant women with malaria receiving SP also showed that *Pf/dhfr/dhps* mutants were high in blood samples collected from the placenta as compared to peripheral samples because during pregnancy, immunity is altered in the placenta to support the developing foetus and *P. falciparum* parasites are cleared slowly (Menendez *et al.,* 2011) and therefore *Pf/dhfr/dhps* mutants are present at high levels in the placenta. In this study, pregnant women receiving CTX, both SP and CTX and neither SP nor CTX also had a high prevalence of *Pf/dhfr/dhps* mutants in placental samples as compared to peripheral samples, however, the prevalence of the *Pf/dhfr/dhps* triple mutant in peripheral and placental samples was statistically significant in pregnant women receiving CTX for the treatment of malaria (P=0.025), this finding was inconsistent with previous *in vitro* studies which have demonstrated that *Pf/dhfr/dhps* mutants confer resistance to CTX and therefore extensive use of CTX during pregnancy would increase the prevalence of *Pf/dhfr/dhps* mutants in peripheral blood (Mbugi *et al.*, 2006). The presence of significantly lower *Pf/dhfr/dhps* triple mutant in peripheral samples than placental samples in pregnant women receiving CTX could be due to the antimalarial activity of CTX which cleared the *Pf/dhfr/dhps* triple mutant more

effectively in peripheral samples as compared to placental samples. Poor patient compliance with antimalarial dosing schedules and the fact that patients were not on treatment for *P. falciparum* could be attributable to the presence of high *Pfdhfr/dhps* mutants in both peripheral and placental samples from pregnant women in the neither SP nor CTX arm (Nzila *et al.*, 2000).

Data from this study showed that in all the four treatment arms, HIV-negative pregnant women had a high prevalence of *Pf/dhfr/dhps* mutants in both peripheral and placental samples as compared with HIV-positive women, this finding was in contrast with previous studies in malaria-endemic areas which have shown that due to impaired immunity and poor drug absorption, HIV-positive patients with malaria have higher peripheral and placental parasite densities compared to HIV-negative patients with malaria (Parise *et al.,* 1998).

In HIV-negative pregnant women, comparison of the double, triple, quadruple and quintuple *Pf/dhfr/dhps* mutants in peripheral and placental samples was comparable in the four treatment arms, however, in HIV-positive samples, there was statistical significance when the *Pf/dhfr/dhps* mutants were compared in peripheral and placental samples in the CTX treatment arm. This finding was similar to a previous study involving pregnant women in Mali, where a single dose of CTX given for 3 consecutive days each week for 12 weeks reduced the incidence of uncomplicated *P. falciparum* peripheral malaria by 99.5% (Thera *et al.*, 2005).

5.1. Conclusions

a) The study showed that there were no differences in the profiles of molecular markers associated with *P. falciparum* resistance to SP in peripheral and placental samples.

b) The prevalence of molecular markers of *P. falciparum* resistance to SP in peripheral and placental samples was comparable, however, the *Pf/dhfr/dhps* triple mutant was significantly different in the CTX treatment arm.

c) In HIV-negative pregnant women, the prevalence of molecular markers of *P. falciparum* resistance to SP in peripheral and placental samples was comparable in pregnant women in the SP treatment arm and neither SP nor CTX treatment arm, however, in HIV-positive patients, the prevalence of molecular markers of *P. falciparum* resistance to SP in peripheral and placental samples was significantly different in pregnant women in the CTX treatment arm and comparable in the SP and CTX treatment arm.

5.2. Study recommendations

In the current study, there was high (>50%) prevalence of the *Pf/dhfr/dhps* quintuple mutant in both peripheral and placental samples obtained from HIV-infected and non-infected pregnant women in all treatment arms. The *Pf/dhfr/dhps* quintuple mutant is associated with high grade SP resistance (Bwijo *et al.*, 2003; Pearce *et al.*, 2009). This is an early warning sign of high SP treatment failure and therefore *in vivo* trials in pregnant women are needed for confirmation. In addition, the data points to an urgent need for field surveillance studies and control programmes to make concerted efforts in search of an alternative antimalarial drug to replace the use of SP in malaria control during pregnancy, particularly in malaria-endemic regions, such as Siaya and Bondo districts in western Kenya.

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