EVALUATION OF THE EFFECTIVENESS OF ANTIMALARIAL INTERVENTIONS DURING PREGNANCY IN BLANTYRE, MALAWI

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I56F/21877/2010

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October, 2014
DECLARATION

I declare that this thesis is my original work and has not been presented for a degree in any other university or for any award.

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DEDICATION

To my father, Dr. Matthews Fulakeza, Mom Bertha, brothers Khalani and Steve and my girlfriend Fanny Malikebu. Without your love, care and support, I would not have reached this far.
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<table>
<thead>
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>ACT</td>
<td>Artemisinin-based Combination Therapy</td>
</tr>
<tr>
<td>ANC</td>
<td>Antenatal Clinic</td>
</tr>
<tr>
<td>CSA</td>
<td>Chondroitin Sulphate A</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold Cycle</td>
</tr>
<tr>
<td>CQ</td>
<td>Chloroquine</td>
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<tr>
<td>DHFR</td>
<td>Dihydrofolate Reductase</td>
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<tr>
<td>DHPS</td>
<td>Dihydropteroate Synthase</td>
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<tr>
<td>DNA</td>
<td>Deoxy-ribonucleic Acid</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular Adhesion Molecule 1</td>
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<tr>
<td>IPT</td>
<td>Intermittent Preventive Therapy</td>
</tr>
<tr>
<td>IPTp</td>
<td>Intermittent Preventive Therapy for malaria in pregnancy</td>
</tr>
<tr>
<td>IRS</td>
<td>Indoor Residual Spraying</td>
</tr>
<tr>
<td>ITNs</td>
<td>Insecticide Treated Nets</td>
</tr>
<tr>
<td>LBW</td>
<td>Low Birth Weight</td>
</tr>
<tr>
<td>LLINs</td>
<td>Long Lasting Insecticide Treated Nets</td>
</tr>
<tr>
<td>MIS</td>
<td>Malaria Indicator Survey</td>
</tr>
<tr>
<td>MoH</td>
<td>Ministry of Health</td>
</tr>
<tr>
<td>NMCP</td>
<td>National Malaria Control Programme</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PfEMP1</td>
<td><em>Plasmodium falciparum</em> Erythrocyte Membrane Protein 1</td>
</tr>
<tr>
<td>PMI</td>
<td>President’s Malaria Initiative</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction/ Real Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SP</td>
<td>Sulfadoxine-Pyrimethamine</td>
</tr>
<tr>
<td>VSA</td>
<td>Variant Surface Antigen</td>
</tr>
<tr>
<td>WHO/AFRO</td>
<td>World Health Organization Regional Office for Africa</td>
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<td>WHO</td>
<td>World Health Organization</td>
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ABSTRACT

Due to an increased risk of malaria during pregnancy, WHO recommends a strategic framework for malaria prevention and control during pregnancy in areas of stable transmission in Africa such as Malawi. One of the central policies is intermittent preventive treatment in pregnancy (IPTp) with sulfadoxine-pyrimethamine (SP). However, the emergence of SP resistance calls into question its effectiveness for IPTp. It has recently been demonstrated that 12 years after the withdrawal of chloroquine (CQ) use in Malawi, CQ susceptible malaria parasites now predominate and CQ is regaining its efficacy. To explore the potential role of CQ for the prevention of malaria during pregnancy, a randomized, controlled clinical trial of CQ chemoprophylaxis versus intermittent preventive therapy compared to standard IPTp with SP to prevent malaria in pregnancy in Blantyre, Malawi was conducted. The trial provided a platform to investigate the prevalence and incidence of malaria infection during pregnancy in three treatment arms namely CQ chemoprophylaxis, CQ-IPTp and SP-IPTp. Filter paper specimens from 240 patients (80 from each of the three treatment arms of the clinical trial) were used to determine and compare the incidence of malaria, the time to the first occurrence of malaria after treatment and compare baseline prevalence against the prevalence at delivery. A real-time PCR malaria detection assay technique was used to detect presence of malaria parasites from specimens collected at regular monthly visits and unscheduled sick visits from women who participated in the study and whenever they were ill. The occurrence of malaria infection among women receiving the three different interventions (CQ chemoprophylaxis, CQ-IPT and SP-IPT) was compared using Poisson regression. Kaplan-Meier survival analysis was used for overall comparison of time to the occurrence of malaria infection across the three treatment arms. McNemar test was used to compare the prevalence of malaria infection at baseline and at delivery in each treatment arm. The incidence of malaria infection were 1.98 (95% CI: 0.50, 8.04) per 100 person months in the CQ-IPTp arm and 0.34 (95% CI: 0.04, 3.33) per 100 person months in the CQ prophylaxis as compared to 0.99 per 100 person months in the standard SP-IPTp arm (p>0.05). No statistically significant difference was observed on pairwise comparison of the CQ prophylaxis arm and the CQ-IPTp interventions to the standard SP-IPTp. Mean time in months to the first occurrence of malaria infection (survival times in months) were 7.59 (95% CI: 7.38-7.78), 7.76 (95% CI: 7.67-7.86) and 7.60 (95% CI: 7.38-7.83) for the SP-IPTp, CQ-Prophylaxis and CQ-IPTp arms respectively and overall comparison by log rank did not show statistical significance (p>0.05). Whilst the CQ prophylaxis arm and the CQ-IPTp interventions reduced the prevalence of malaria infection to none at delivery, malaria parasitaemia was detected at delivery in the SP-IPTp arm. Nonetheless there was a significant difference (p<0.05) between the prevalence of parasitaemia at baseline and delivery in the SP-IPTp arm. This study shows non-inferiority of CQ-IPTp and CQ-prophylaxis over SP-IPTp for the prevention of pregnancy associated malaria. If these findings are validated in different settings, it could therefore imply that CQ-IPTp and CQ-prophylaxis could be re-introduced for control of malaria during pregnancy.
CHAPTER ONE
INTRODUCTION

1.1 Background

Malaria is a mosquito borne disease caused by protozoan parasites of the genus *Plasmodium*. Five species are known to infect humans: *P. falciparum*, *P. ovale*, *P. malariae*, *P. vivax*, and the recently established *P. knowlesi*, previously thought to infect non-human primates only (Cox-Singh *et al.*, 2008). *P. falciparum* causes most infections and malaria related deaths and is the most common cause of malaria cases in Africa (National Institute of Allergy and Infectious Diseases, 2007). Infection may be asymptomatic, without any sign of illness, or symptomatic, associated with a wide range of symptoms including fever, chills, malaise, and headache. Globally, it is estimated that there are as many as 3.4 billion people who are at risk of malaria. WHO estimates that 207 million cases of malaria occurred globally in 2012 (uncertainty range 135–287 million) and 627 000 deaths (uncertainty range 473 000–789 000). Most cases (80%) and deaths (90%) occurred in Africa, and most deaths (77%) were in children under 5 years of age (WHO, 2013a).

Apart from children less than five years old, other high-risk groups include non-immune people moving into malarial regions, and populations with repressed immune systems, including pregnant women especially the primigravidae and people suffering from HIV-AIDS (Verhoeff *et al.*, 1999; Diagne *et al.*, 2000).
In areas where malaria is highly endemic, protective semi-immunity against *P. falciparum* is acquired during the first 10-15 years of life such that the majority of malaria-related morbidity and mortality happens in young children under the age of five (Riley *et al.*, 1989). However, Brabin (1983) observed that pregnant women in endemic areas are highly susceptible to malaria with both high frequency and severity.

Parasite antigens that serve as ligands for adhesion of infected erythrocytes in the placenta are different from corresponding antigens in non-placental *P. falciparum* infections (Rogerson *et al.*, 2007a). These are collectively known as variant surface antigens (*var* proteins) and are targets of protective immunity that is gradually developed in response to repeated episodes of malaria in non-pregnant individuals (Marsh *et al.*, 1989; Bull *et al.*, 1998). The variant surface antigens are responsible for the increased vulnerability of pregnant women to malaria because the immune system does not recognize them on their first encounter; and this explains why in subsequent pregnancies, women develop immunity against these parasite surface antigens and clear them by immune response (Rogerson *et al.*, 2007a). At least 50 million pregnant women live in malaria endemic areas (WHO/AFRO, 2004), more than 3 million pregnant women are affected by malaria in the developing countries, where it causes serious adverse effects including abortion, low birth weight and anaemia (Brabin, 1983; Menendez *et al.*, 2000a; Menendez *et al.*, 2000b)


1.2 Prevention and control of malaria

There are several methods through which prevention and control of malaria can be achieved. These include insecticide-treated bed nets (ITNs), indoor residual spraying (IRS), vaccines and chemotherapy.

1.2.1 Insecticide-treated bed nets (ITNs)

An insecticide-treated net (ITN) is a mosquito net that repels, disables and/or kills mosquitoes coming in contact with an insecticide on the netting material (WHO/GMP, 2007). There are two categories of ITNs namely conventionally treated nets and long-lasting insecticidal nets (LLINs) (Ministry of Health and Child Welfare, 2007). Conventionally treated nets are mosquito nets that have been treated by dipping in a WHO-recommended insecticide. Pyrethroids are the only insecticide that can be used on bed nets (Palmquist et al., 2012; WHO, 2013b). The insecticides used for this purpose belong to the class of synthetic pyrethroids which include permethrin, deltamethrin, lambdacyhalothrin and cypermethrin and share the property of a relatively long residual activity when kept out of daylight but break down rapidly under influence of UV-radiation (Takken, 2002). To ensure its continued insecticidal effect, the net should be re-treated after three washes, or at least once a year (Ministry of Health and Child Welfare, 2007).
On the other hand, LLINs are factory-treated mosquito nets made with netting material that has an insecticide incorporated within or bound around the fibres (WHO/GMP, 2007). The net must retain its effective biological activity without retreatment for at least twenty WHO standard washes under laboratory conditions and three years of recommended use under field conditions (WHO Global Malaria Programme, 2007). Despite a previous study that demonstrated a relatively modest coverage of ITN use (around 60%) of all adults and children, a recent study has shown that the ITN use remains far below international targets, despite fairly high rates of attendance at antenatal clinics (Killeen et al., 2007; van Eijk et al., 2013). However, mosquitoes have developed resistance to insecticides of all approved classes. Such complete resistance, which includes exceptionally strong phenotypes, presents a major threat to malaria control (Edi et al., 2012).

1.2.2 Indoor residual spraying (IRS)

Indoor residual spraying (IRS) involves spraying houses with insecticides to kill mosquitoes. Indoor residual spraying has helped to eliminate malaria from great parts of Asia, Russia, Europe and Latin America, and successful IRS programmes have also been run in parts of Africa (Pluess et al., 2010). Despite its initial widespread use and contribution to the success of malaria eradication and control efforts, in recent years, the use of IRS has declined. This is due in part to lack of government commitment and financing to sustain these efforts over the long term and to concerns about insecticide resistance and community acceptance (WHO, 2006). Insecticides recommended by WHO for indoor residual spraying include the
organochlorine (dichloro-diphenyl-trichloroethane, DDT), organophosphates (fenitrothion, malathion and pirimiphos-methyl), carbamates (propoxur and bandiocarb) and pyrethroids (alpha-cypermethrin, cyfluthrin, deltamethrin, etofenprox, lambda-cyhalothrin and bifenthrin) (WHO, 2006; 2013b). Although DDT was the insecticide of choice for IRS during the Global Malaria Eradication Campaign (1955-1969), the efforts did not achieve its objective entirely though it did eliminate malaria from several areas and sharply reduced the burden of malaria disease in others (Najera et al., 2011). Concerns over the environmental impact of DDT led to the introduction of other, more expensive insecticides. The Stockholm Convention of 2001 listed DDT as one of the twelve persistent organic pollutants recommended for phase-out and eventual elimination though the chemical was never banned for disease vector control (Sadasivaiah et al., 2007). However, the recent success of IRS using DDT in reducing malaria cases in South Africa by more than 80% has revived interest in this insecticide (Razavi et al., 2009).

1.2.3 Vaccines

Despite malaria vaccines being an area of extensive research, no practical or effective vaccine has been introduced to clinical practice to date. Various vaccines have reached the clinical trials stage but most of them have demonstrated insufficient immunogenicity (Barry and Arnott, 2014). SPf66 was tested extensively in endemic areas in the 1990s, but clinical trials showed it to be insufficiently effective (Graves and Gelband, 2006a). Other vaccine candidates, targeting the blood-stage of the parasite life cycle, have also been insufficient on their own (Graves and Gelband,
Several potential vaccines targeting the pre-erythrocytic stage are being developed, with RTS,S by GlaxoSmithKline (GSK) showing the most promising results so far (Graves and Gelband, 2006c). RTS,S is a recombinant antigen expressed in *Saccharomyces cerevisiae* and consists of two proteins RTS and S. RTS is a hybrid polypeptide consisting of a portion of the circumsporozoite protein (CS), a sporozoite surface antigen of the malaria parasite *P. falciparum* strain NF54, fused to the amino-terminal end of the hepatitis B virus S protein. S designates the surface antigen of Hepatitis B virus and is the same antigen used in GSK Biological's licensed Hepatitis-B vaccines (Glaxosmithkline, 2009). In an 18 month long clinical trial, the vaccine reduced the amount of cases amongst young children by almost 50% and among infants by around 25% and WHO states that it will recommend the use of RTS,S for clinical use starting in 2015, provided it gets approval (Glaxosmithkline, 2014).

### 1.2.4 Chemotherapy

Drugs are widely used to prevent and treat infection. Drugs listed and used for malaria chemotherapy include artemisinin derivatives (including artesunate artemisinin, artemether and dihydroartemisinin), chloroquine (CQ), pyrimethamine, sulfonamide derivatives and antibiotics (Ibezim and Odo, 2008). Generally, these antimalarial drugs are classified based on the stage of the parasite they act on such as tissue schizonticides, sporonticides, blood schizonticides and gametocytocides or how they act. For instance, there are drugs used for prophylaxis and for clinical cure (Tracy and Webster, 1996; Ibezim and Odo, 2008).
For decades, CQ was the mainstay of malaria chemotherapy until the 1980s when its sensitivity rapidly declined throughout the tropics (Winstanley and Ward, 2006). After the withdrawal of CQ as the WHO recommended first-line treatment for uncomplicated malaria, no drug has promised to survive long as evidenced by the replacement of its successor, sulfadoxine-pyrimethamine (SP), with artemisinin combination therapy (ACT) (Whitty et al., 2004). Malawi switched from CQ to SP as the first-line treatment of uncomplicated malaria in 1993, becoming the first country in sub-Saharan Africa to do so (Bell et al., 2008).

1.3 Epidemiology and burden of malaria in pregnancy

1.3.1 Global epidemiology and burden of malaria in pregnancy

A demographic study by Dellicour et al. (2010), estimated that approximately 125 million women living in malaria-endemic countries throughout the world become pregnant every year, of which over 30 million live in tropical areas of Africa where there is intense transmission of Plasmodium falciparum. This included 77.4 million pregnancies in the countries falling under the WHO regional offices for South-East-Asia (SEARO) and the Western-Pacific (WPRO) combined, 30.3 million in AFRICA (AFRO), 13.1 million in Europe and the Eastern Mediterranean (EURO/EMRO) and 4.3 million in the Americas/USA (AMRO). According to Marchesini and Crawley (2004), the overall global prevalence of maternal anaemia caused by malaria in pregnancy is estimated to be as high as 66%, and it is the major cause of maternal mortality which is also as high as 675 deaths per 100 000 live births (UNICEF, 2011).
Figure 1.1 below is a malaria risk map for *P. falciparum* showing the total number of pregnancies at risk of malaria and the corresponding number of live-births born to pregnancies at risk of malaria (in parentheses) in each continent in 2007 (Dellicour *et al.*, 2010).

**Figure 1.1:** Malaria risk map for *P. falciparum* showing the total number of pregnancies at risk of malaria and the corresponding number live-births born to pregnancies at risk of malaria (in parentheses) in each continent in 2007. doi:10.1371/journal.pmed.1000221.g001 (Adapted from, Dellicour *et al.*, 2010).

### 1.3.2 Epidemiology and burden of malaria during pregnancy in Africa

Sub-Saharan Africa has the largest burden of malarial disease, with over 90% of the world’s malaria-related deaths occurring in this region (Schantz-Dunn and Nour, 2009). Nigeria is the most populous country in Africa, with an estimated population of over 168 million and therefore has the largest population of persons exposed to malaria infection in sub-Saharan Africa (World Bank, 2012). Every year, at least 30 million pregnancies occur among women in malarious areas of Africa, most of these women reside in areas of relatively stable malaria transmission (Awosan, 2013). Estimates by light microscopy indicate that approximately one in four pregnant
women in areas of stable transmission in Africa have evidence of malaria infection at
the time of delivery (Desai et al., 2007). This is an underestimation as low-grade
submicroscopic parasitaemia is often missed but is detectable using more sensitive
methods such as polymerase chain reaction (PCR) and placental histology (Anchang-
Kimbi, 2009). Although direct fatal consequences of malaria in pregnancy are rare,
these consequences are still severe and include severe maternal anaemia and low
infant birth weight. Anaemia is the most common consequence of *P. falciparum*
infection (Uneke, 2008).

### 1.3.3 Epidemiology and burden of malaria during pregnancy in Malawi

In Malawi, just like other sub-Saharan African countries, malaria is endemic and
each year approximately half a million women become pregnant and are therefore at
a risk of malaria (Launiala, 2010). The whole population of Malawi is at risk of
malaria, 97% at endemic risk and 3% at epidemic risk (USAID, 2010). The
President’s Malaria Initiative (PMI) Malaria Operation Plan (2014) estimates that the
number of pregnant women in Malawi in 2015 will be 815,522 (5% of the
population). The 2012 Malaria Indicator Survey for Malawi by the (National Malaria
Control Programme and ICF International, 2012) found that more than half (54%) of
pregnant women received IPTp, that is, at least two doses of SP with at least one
dose received during an antenatal care visit, which occurred during the most recent
pregnancy. This percentage is a slight decline from the 2010 Malaria Indicator
Survey (MIS) of 60%. This is also below the 80% goal set by the Ministry of Health
(MoH) (President’s Malaria Initiative, 2014). Awareness campaigns and provision of
LLINs through antenatal clinics (ANCs) support the use of LLINs during pregnancy. In the 2012 MIS in Malawi, 51% of pregnant women reported sleeping under an ITN the night before. This goal is also well below the 80% coverage level set by the Malawi’s MoH (National Malaria Control Programme and ICF International, 2012). The national estimate of prevalence of malaria in pregnant women in Malawi is 19% (Launiala, 2010). In Malawi, low birth weights associated with malaria in pregnancy result in approximately 20% of babies born below 2500 grams which follows the finding that at the time of delivery, up to 40% of primigravidae and secundigravidae have placental malaria (Rogerson et al., 2000).

1.4 Management of malaria during pregnancy

As early as the 1950s, the WHO had released its first recommendations on the prevention of malaria during pregnancy, which consisted of weekly or bimonthly chemoprophylaxis with CQ, SP or dapsone-pyrimethamine for the duration of pregnancy (Briand et al., 2008). The WHO recommended that all pregnant women resident in areas of moderate or high malaria transmission should receive chemoprophylaxis with CQ throughout the second and third trimesters of pregnancy (Greenwood, 2004). Prophylaxis is aimed at preventing, suppressing or eradicating malaria parasites and has been investigated through the 1980s followed by its modification to intermittent preventive treatment since the 1990s (Garner and Gulmezoglu, 2009). Malaria prophylaxis involves frequent, regular use of an antimalarial drug given at less than the therapeutic dose with the aim of suppressing blood stage infection (US Centers for Disease Control and Prevention, 2009). This
strategy was initially recommended by WHO so that pregnant women in malaria-endemic areas would receive a full antimalarial treatment on their first contact with antenatal care followed by weekly chemoprophylaxis (Gillespie et al., 2007). CQ was the drug most commonly used before the policy became challenged due to factors such as spread of resistance to CQ, poor compliance with the weekly regimen throughout pregnancy and adverse effects, especially pruritus associated with CQ (WHO, 1994; WHO/AFRO, 2004).

A modification of the chemoprophylaxis strategy, intermittent preventive treatment (IPT) has been used where women are treated for malaria presumptively at fixed times during pregnancy usually with a long half-life drug such as sulfadoxine-pyrimethamine (Garner and Gülmezoglu, 2009). The choice of drug, dosing, and dose spacing for IPT should be based on a better understanding of pharmacokinetics and pharmacodynamics (White, 2005). Intermittent preventive treatment requires just two or three doses during pregnancy, compared to prophylaxis regimens that may be daily (e.g. with proguanil) or weekly (e.g. with CQ) (Garner and Gülmezoglu, 2009). Prophylaxis and IPT are administered in addition to good care practices during pregnancy, which include prompt treatment of women when they present clinically with fever or anaemia, use of ITNs/LLINs and IRS already discussed in section 1.2.

1.4.1 Chloroquine prophylaxis

The efficacy, tolerability and safety of CQ in pregnancy made the drug the cornerstone of antimalarial drugs until the 1980s (Castelli et al., 2010). The 4-
aminoquinoline interferes with parasite haem detoxification (WHO, 2010). When administered orally, blood peak concentration is reached in 1-6 hours with a half-life of 3-6 days initially, which becomes 12-14 days with protracted intake (White, 1985). Chloroquine can induce several side effects, usually mild including insomnia, nausea, headache, dizziness, blurred vision and itching (Petersen et al., 2000).

As earlier noted, malaria chemoprophylaxis is the administration of an antimalarial to suppress blood stage infection, but at a dose that is less than the curative dose (Bijker and Sauerwein, 2012). The strategy may allow persistence or recurrence of low level parasitaemia that could lead to placental sequestration in pregnant women since no chemoprophylactic regimen confers complete protection (Castelli et al., 2010). On the other hand, IPTp with an efficacious drug involves the use of a full curative dose which clears infections but allows recurrent peripheral and placental infection during intervals in high transmission areas such as Malawi (Bijker and Sauerwein, 2012).

Antimalarial chemoprophylaxis with CQ used to be recommended for pregnant women in malaria endemic areas before its usefulness became limited due to poor adherence to the weekly drug regimen and increasing levels of *P. falciparum* resistance to the drug (Brabin et al., 1990; Heymann et al., 1990; Sirima et al., 2003). The first case of *P. falciparum* resistance to CQ occurred in Thailand and Cambodia in the late 1950s, spreading to South America, Asia Oceania and the whole African continent since the 1980s (Klein, 2013; Awasthi and Das, 2013). As such, CQ had to be withdrawn for use as a prophylaxis regimen in pregnancy.
However, recent studies have revealed the potential of chloroquine in the control and prevention of malaria. Kublin et al. (2003) and Mita et al. (2003) demonstrated the reemergence of CQ sensitive malaria in Malawi by showing a decrease in the prevalence of the \textit{pfcrt} K76T molecular marker. The recovery of CQ sensitivity resulted from an expansion of parasites harboring the wild-type \textit{pfcrt} allele, and not from \textit{pfcrt} back-mutants (Mita et al., 2004). The findings have been confirmed in clinical trials in Malawi and Ethiopia (Laufer et al., 2006; Mekonnen et al., 2014).

\subsection*{1.4.2 Intermittent preventive treatment with sulfadoxine-pyrimethamine (SP-IPTp)}

Due to the increased risk of malaria during pregnancy and the spread of CQ resistance, WHO later recommended a three-pronged approach to malaria prevention and control during pregnancy in areas of stable transmission in Africa (WHO/AFRO, 2004) such as Malawi. The first component of this approach was the use of intermittent preventive treatment (IPT), in which all pregnant women in areas of stable malaria transmission receive at least two doses of IPT after the first foetal movements in the uterus (Kiwuwa and Mafubenga, 2008). Recently, the most effective drug for IPT has been SP because of its efficacy, safety for use during pregnancy, and feasibility for use in programmes as it can be delivered as a single-dose treatment under observation by a health worker (The Nigerian Academy of Science, 2004). The second component is the use of insecticide treated bed nets (ITNs) which should be provided as early as possible during pregnancy and their use should be encouraged throughout pregnancy and postpartum period (WHO/GMP, 2007). The third and last in the framework is effective case management of malaria.
illness and anaemia with iron supplementation for anaemia as part of routine antenatal care (Gomez et al., 2014). It is recommended that pregnant women be screened for anaemia, and those with moderate to severe anaemia be managed according to national reproductive health guidelines (WHO, 2006). Malawi adopted this WHO three-pronged approach as one of the first countries in the sub-Saharan region in 1993, the same year the first-line drug for the treatment of uncomplicated malaria was changed from CQ to SP (Launiala, 2010). However, SP resistance first reported on the Thai-Cambodian border in the mid-1960s has now spread to other regions in Southeast Asia and Africa rendering this drug clinically ineffective for malaria treatment (Bjorkman and Phillips-Howard, 1990; Wongsrichanalai et al., 2002). As such, all malaria-endemic countries had to change to the newly WHO recommended artemisinin-based combination therapy (ACT) as the first-line treatment for uncomplicated malaria. This points to the need for new alternative drugs, and further investigation into whether or not IPTp with SP is still effective.

However, reports from Muheza in Tanzania where there is high-level SP resistance suggests that SP use in pregnancy may exacerbate resistance and that SP-IPTp does not improve pregnancy outcomes (Harrington et al., 2009; Harrington et al., 2011). Apart from growing concerns over its effectiveness due to resistance, there are safety concerns with the drug. These include anaemia due to inhibition of the folate synthesis; teratogenicity, which has been seen in rat models; hypersensitivity reactions; and kernicterus in the new born (Peters et al., 2007). Parikh and Rosenthal (2010) observed that SP has not been recommended for prophylaxis because, when
provided weekly for chemoprophylaxis, SP caused rare life-threatening toxicity, including severe skin reactions and hepatitis though available information suggests that short-term use of SP to treat malaria or intermittent use in IPTp is safe.

Despite reports that preliminary data from recent observational studies have suggested reduced effectiveness of SP for IPTp in Malawi, the first country where SP-IPTp was implemented in sub-saharan Africa in 1993 (Feng et al., 2010), SP-IPTp still remains the recommended strategy for preventing the adverse consequences of malaria on maternal and foetal outcomes (WHO Evidence Review group, 2012). SP-IPTp replaced CQ chemoprophylaxis as an alternative prevention strategy and was shown to be efficacious in reducing rates of malaria low birth weight and severe anaemia during pregnancy (Schultz et al., 1994; Parise et al., 1998; Verhoeff et al., 1998; Schulman et al., 1999). However, *P. falciparum* resistance to SP has spread raising questions whether SP is still effective in IPTp, prompting search for alternatives.

### 1.4.3 Artemisinin-based combination therapy (ACT)

Following declining efficacy of SP, again WHO recommended the use of ACTs (WHO, 2006). Because resistance of *P. falciparum* to current drugs and resistance of malaria vectors to available insecticides are both spreading, alternative strategies for the prevention of malaria during pregnancy need to be explored. The safety and efficacy of new antimalarial agents and antimalarial combinations for use in pregnancy require urgent investigation. Artemisinins are a potentially valuable alternative as they are highly effective, act rapidly and are well-tolerated. In addition,
they have the potential to reduce the transmission of malaria and to slow development of resistance (WHO, 2001). In 2002, after a detailed review of published and unpublished data, a WHO expert committee concluded that artemisinin could be used during the second or third trimesters if no suitable alternative was available (WHO, 2002). However, treatment in the first trimester was not recommended unless the life of the woman was at risk because of concerns raised by animal experiments which suggested that artemisinin might be teratogenic and cause foetal resorption (Dellicour et al., 2007). Further studies have confirmed the embryotoxic effects of artemisinin and its derivatives in animals, including primates, with risk being confined to a defined period of gestation (Clark et al., 2004; Longo et al., 2006). However it remains unknown how these findings translate to man. Although a few studies of the safety and efficacy of artemisinin during pregnancy are currently underway, these will not produce data on the safety of artemisinin during the first trimester of pregnancy (Dellicour et al., 2007).

**1.5 Problem statement and justification**

Each year, approximately 50 million women living in malaria-endemic countries throughout the world become pregnant resulting into an estimated 10 000 and 200 000 deaths of women and their infants, respectively (Purohit and Mahapatra, 2009). Africa bears 90% of the world malaria burden with pregnant women and children being vulnerable to the adverse consequences of malaria caused by the most lethal parasite, *P. falciparum* (WHO/AFRO, 2004). In Malawi, malaria is the leading cause of morbidity and mortality in children under five years old and among pregnant
women (Diagne et al., 2000). Launiala (2010) estimates that in Malawi alone, half a million of women become pregnant each year and are therefore at risk of malaria and its adverse effects.

Despite the clear need for safe and effective antimalarial drugs for use in pregnancy, the pharmaceutical industry has been reluctant to develop drugs specifically for this indication, and in almost all cases in which a new drug is developed, use in pregnancy is contraindicated (Ward et al., 2007). Therefore, finding suitable drugs for pregnancy associated malaria is a challenge because not all drugs listed as antimalarials can be used during pregnancy. Drugs need to be proven safe for use in pregnant women. Safety must be proven in human beings through clinical trials which are expensive, labour intensive and risky. Kublin et al. (2003) demonstrated that a few years after the withdrawal of CQ, the prevalence of the chloroquine-resistant pfcrt genotype decreased from 85% in 1992 to 13% in 2000. Chloroquine cleared 100% of 63 symptomatic P. falciparum infections, no isolates were resistant to CQ in vitro and no infections with CQ-resistant pfcrt genotype were detected (Kublin et al., 2003). Mita et al. (2003), also reported the reemergence of CQ sensitive malaria in Malawi before establishing that, the recovery in CQ sensitivity resulted from an expansion of parasites harboring the wild-type pfcrt allele, and not from pfcrt back-mutants (Mita et al., 2004).

About twelve years after the withdrawal of CQ due to high rates of resistance, Laufer et al. (2006) also demonstrated in Malawi that CQ is now nearly 100% effective for the treatment of malaria and this has also been reported in Ethiopia (Mekonnen et
This drug is ideal for use in pregnant women because it does not induce abortion, does not interfere with normal embryonic development, and is well-tolerated. An additional benefit is its long half-life, making it useful for the prolonged prevention of malaria infection (Krishna and White, 1996). Chloroquine could once again be useful in the prevention of malaria infection and adverse outcomes during pregnancy and provide an alternative for the failing SP-IPTp.

Although IPTp replaced CQ prophylaxis, the benefits of IPTp versus those of CQ prophylaxis have not been thoroughly assessed (Schultz et al., 1994; Kayentao et al., 2005). From this understanding, CQ prophylaxis and CQ-IPTp were administered and their outcomes compared with the standard SP-IPTp outcome in pregnant women in Malawi.

1.6 Hypothesis

Chloroquine (CQ) prophylaxis during pregnancy and chloroquine intermittent preventive treatment in pregnancy (CQ-IPTp) are both inferior to the standard practice of intermittent preventive treatment in pregnancy with sulfadoxine-pyrimethamine (SP-IPTp).

1.7 Objectives

1.7.1 General objective

To evaluate the incidence of malaria in pregnant women before and after weekly chloroquine prophylaxis, chloroquine intermittent preventive treatment in pregnancy
and the standard practice intermittent preventive treatment with sulfadoxine-pyrimethamine.

### 1.7.2 Specific objectives

i. To determine the incidence of submicroscopic malaria infection in pregnant women on weekly chloroquine prophylaxis, chloroquine intermittent preventive treatment in pregnancy and the standard practice intermittent preventive treatment with sulfadoxine-pyrimethamine using real time-polymerase chain reaction.

ii. To determine the time taken to the occurrence of malaria infection upon chloroquine prophylaxis, chloroquine intermittent preventive treatment in pregnancy and the standard practice intermittent preventive treatment with sulfadoxine-pyrimethamine.

iii. To determine the baseline prevalence of malaria infection in untreated pregnant women and those on chloroquine prophylaxis, chloroquine intermittent preventive treatment in pregnancy and the standard practice intermittent preventive treatment with sulfadoxine-pyrimethamine.
CHAPTER TWO

LITERATURE REVIEW

2.1 Pathophysiology of malaria in pregnancy

The development, monitoring and evaluation of programmes to prevent malaria in pregnancy can be facilitated by a better understanding of the pathogenesis of malaria. Malaria in pregnancy is a public health concern worldwide. It is estimated that 40% of the world’s pregnant women are exposed to malaria infection (Schulman and Dorman, 2003). In areas with low or unstable transmission of malaria, women of reproductive age have relatively little acquired immunity such that all pregnant women are comparably susceptible to malaria (WHO/AFRO, 2004). In contrast, in stable transmission areas such as most of sub-Saharan Africa, women of child bearing age have a relatively high level of acquired antimalarial immunity (WHO/AFRO, 2004). However, their susceptibility to malaria varies with gravidity such that the primigravidae are more susceptible to malaria infection compared to the multigravidae (Steketee et al., 1996). This increased susceptibility is thought to be as a result of pregnancy associated immunological changes and also hormonal changes, the nature of which is subject to debate coupled with the ability of infected erythrocytes to sequester in the placenta (Raghupathy, 1997; Pearson, 2005; Rogerson et al., 2007a).

Although peripheral circulation can be free of parasites, high placental parasitisation is sometimes observed in infected mothers (Ismail et al., 2000). Erythrocytes
infected with mature *P. falciparum* trophozoites and schizonts accumulate in the maternal vascular area of the placenta (intervillous space) to much higher densities than in the peripheral circulation (Brabin *et al*., 2004). This has been linked to a variant surface antigen (VSA) mediated adhesion of infected erythrocytes to the glycosaminoglycan chondroitin sulphate A (CSA) in the placental intervillous space (Salanti *et al*., 2003). Rowe and Kyes (2004) observed that such adhesion leads to sequestration of mature infected erythrocytes in the placental blood spaces, allowing the parasites to grow and multiply whilst evading the host’s spleen-mediated killing mechanisms. The effects are detrimental as placental blood flow may be reduced causing impaired foetal growth with subsequent low birth weight and prematurity (Menendez *et al*., 2000a; Dorman *et al*., 2002).

The process of sequestration of infected erythrocytes in the placenta differs in important ways from sequestration in other organs like the brain, in which close apposition of infected erythrocytes to endothelial cells is mediated by receptors such as CD 36 and ICAM-1 (Turner *et al*., 1994; Grau *et al*., 2003). In *vitro*, placental infected erythrocytes can adhere to CSA and hyaluronic acid (HA), and not ICAM-1 and CD 36 (Fried and Duffy, 1996; Beeson *et al*., 2000). Rogerson *et al*., (2007b) observes that, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) encoded by the var gene family is the principal ligand which mediates CSA adhesion leading to placental sequestration. A gene belonging to a highly conserved var gene subfamily VAR2CSA has been reported to be markedly up-regulated in several *P. falciparum* isolates after selection of CSA in *vitro* (Salanti *et al*., 2003). This gene has many
characteristics expected of variant surface antigens involved in pregnancy associated malaria suggesting its pivotal role during malaria in pregnancy (Rogerson et al., 2007a).

2.2 Diagnosis of malaria in pregnancy

The accurate diagnosis of malaria infection during pregnancy remains challenging because of low parasite densities and placental sequestration of *P. falciparum* (Mayor et al., 2012). Although microscopy remains the gold standard for malaria diagnosis, polymerase chain reaction (PCR)-based methods are much more sensitive than microscopy and detect cases of malaria in pregnant women missed out by microscopy. Mockenhaupt et al. (2000) found that 32% of pregnant Ghanaian women were smear positive while 69% were parasite positive by PCR. Similarly, 29% of samples from pregnant Senegalese women were positive by microscopy compared to 85% by PCR (Schleiermacher et al., 2001). Therefore, a large number of pregnant women harbor submicroscopic parasitaemia which is detected by PCR. Submicroscopic parasitaemia may not be detected by microscopy but has adverse effects on the mother and the foetus. Evidence suggest that submicroscopic parasitaemia at delivery is likely to be associated with deleterious maternal and foetal health outcomes (Rantala et al., 2010). A study by Adenigka et al. (2006) found that women with submicroscopic levels of *P. falciparum* infection (detected by real-time PCR) at delivery had a 13-fold increased risk of delivering a child with low birth weight compared with non-infected women. In Mozambique, Mayor et al. (2012) assessed *P. falciparum* infections in 272 women at delivery by microscopy,
placental histology, quantitative polymerase chain reaction (qPCR), detection of the histidine-rich protein 2 (HRP-2) in plasma by enzyme-linked immunosorbent assay (ELISA) and a rapid diagnostic test. Microscopy, placental histology, and HRP-2 diagnostic methods failed to identify the majority of *P. falciparum* infections detected by qPCR in peripheral and placental blood. The study also found an association between microscopy undetected infections and maternal anaemia. A cross-sectional survey in Cameroon screened *P. falciparum* in peripheral blood, placental blood and placental tissue sections by microscopy. Placental histological examination was the most sensitive indicator of malaria infection at delivery. Microscopically detected parasitaemia was associated with increased risk of maternal anaemia at delivery, but not low-grade parasitaemia detected by placental histology only (Anchang-Kimbi *et al.*, 2009).

However this method missed the opportunity to compare microscopy, histology and qPCR. In Colombia, Arango *et al.* (2013) found that molecular detection of malaria at delivery by qPCR, revealed a high frequency of submicroscopic infection and associated placental damage. These infections had low parasite DNA, and 79% were submicroscopic. Submicroscopic infections were associated with placental villitis and intervillitis demonstrating that the overall frequency of *Plasmodium* infection at delivery in Colombia was much higher than previously reported. A study aimed at comparing qPCR and conventional microscopy for the detection of *P. falciparum* infection from dried blood spots and blood smears collected from peripheral blood (Rantala *et al.*, 2010) found that only 2.3% were positive by microscopy as
compared to 10.7% by qPCR. This suggests that although microscopy remains the most appropriate method for malaria diagnosis in the field settings, molecular diagnostic methods such as real-time PCR offer a more reliable means of detecting malaria parasites.

In this study, real-time PCR was employed to detect the presence of malaria parasites in pregnant women.

2.3 The prevalence of sulfadoxine-pyrimethamine resistance, uncertainty with artemisinin-based combination therapy, and the value of chloroquine

2.3.1 The prevalence of sulfadoxine-pyrimethamine resistance

Sulfadoxine-pyrimethamine resistance has increased in Africa rendering the search for alternative interventions to the SP-IPTp an issue of utmost importance. Sulfadoxine-pyrimethamine is a combination of antifolates namely sulfadoxine which targets the parasite dihydropteroate synthase (DHPS), and pyrimethamine which targets dihydrofolate reductase (DHFR) (Lumb et al., 2011). Single point mutations accumulate in an ordered pattern in dhfr and then dhps conferring resistance (Gregson and Plowe, 2005). Sulfadoxine-pyrimethamine resistance in *P. falciparum* is predominately conferred by mutations in the gene encoding *dhfr*. Mockenhaupt et al. (2008) demonstrated that the prevalence of the *dhfr* triple mutation among pregnant women doubled to almost 75% during a period of only 8 years in Ghanaian women between 1998 and 2006. A study conducted by Harrington et al. (2011), in an area of widespread resistance, hypothesized that as resistance continues to accumulate, IPTp may begin to fail. Indeed, the findings showed that
SP-IPTp did not improve overall pregnancy outcomes in such an area with widespread drug resistance. Bertin et al. (2011) demonstrated a high prevalence rate of mutant parasites in women taking SP-IPTp though there was no evidence of correlation between the resistant genotypes and lack of efficacy in IPTp. Since the relation between protective efficacy of IPTp, and the clinical and molecular markers of resistance has not been established, Briand et al. (2008) proposed a continuous monitoring of the efficacy and effectiveness of SP-IPTp and the need to evaluate alternatives.

2.3.2 The uncertainty with artemisinin-based combination therapy

Artemisinin-based combination therapies are the current WHO recommended first-line treatment for uncomplicated malaria and are effective against drug-resistant parasites. If proven safe and effective in pregnancy, they could be a candidate to replace SP for IPTp. However, the frequent exclusion of pregnant women from pharmaceutical trials, including the original studies of artemisinins and different types of ACTs, has limited the availability of data for this vulnerable population (Manyando et al., 2012). WHO has recommended the use of some ACTs, including artemether-lumefantrine (AL) as first-line treatment for uncomplicated malaria during second and third trimesters (WHO, 2010). Artemisinin based combination therapies have not been recommended in the first trimester due to limited clinical safety data (WHO, 2010) and evidence of embryo lethality and developmental abnormalities in animal studies following artemisinin exposure early in pregnancy, at times equivalent to the first trimester in humans (Clark et al., 2008; White and
Clark, 2008). A large four year multi-centre clinical trial (PREGACT) to determine the safety and efficacy of four ACTs (amodiaquine-артесунат, дихидротермисинин-артенсона, артеме-лумэфантрин and мелфоquine-артесунат) when administered to pregnant women is currently underway in Malawi, Zambia, Burkina Faso, Ghana and Belgium (National Institute of Health, 2014).

Early reports of artemisinin-resistant malaria have been documented in South East Asia (Dondorp et al., 2009; Amaratunga et al., 2012; Phyo et al., 2012), which is regarded as the epicentre of the development of resistance (Wiwanitkit, 2010). This creates an uncertainty with ACTs as a potential candidate to replace SP in prevention of malaria in pregnancy and underlines the fact that alternative interventions with the potential to replace SP in the control of malaria in pregnancy need to be investigated.

### 2.3.3 The value of chloroquine

In 1993, Malawi stopped treating patients with CQ for *P. falciparum* malaria because of high treatment failure rate. Continued monitoring of the prevalence of the K76T mutation known to confer resistance to CQ revealed a significant decline since CQ withdraw to 2% in 2000 suggesting that the withdrawal may result in the recovery of CQ efficacy (Mita et al., 2003). Kublin et al. (2003) also reported a significant decrease in the prevalence of the chloroquine-resistant *pfcrt* genotype from 85% in 1992 to 13% in 2000. This return of CQ-sensitive *P. falciparum* malaria has been as a result of an expansion of a wild type allele rather than back mutation in *pfcrt* (Mita et al., 2004; Shaviya et al., 2012).
Twelve years after the withdrawal of CQ in Malawi, a clinical trial by Laufer et al. (2006) also demonstrated that CQ is once again efficacious making it a candidate for IPTp. This return of chloroquine-sensitive *Plasmodium falciparum* to a limited area of Blantyre, Malawi has been well demonstrated in several studies.

A recent follow up study (Frosch et al., 2014) aimed to characterize this return of chloroquine susceptibility over a wide geographic area in Malawi. Children age 6-59 months were selected using two-stage cluster sampling in eight Malawian districts. Pyrosequencing of the *pfcr* gene codon 76 region was performed for children with asexual parasitemia. Of 7145 children, 1150 had microscopic asexual parasitemia and 685 were sequenced. Of these, only one had a chloroquine-resistant genotype.

In this study, systematic countrywide sampling demonstrated that chloroquine-sensitive *pfcr* genotype has reached near-fixation, raising the possibility of re-introducing chloroquine for malaria prevention and treatment.

Similar findings have been demonstrated by Ndiaye et al. (2012) in Senegal where CQ was withdrawn in 2006 and in Ethiopia where unlike in Malawi, CQ was partially withdrawn and was still the drug of choice for the treatment of uncomplicated *P. vivax* (Mekonnen et al., 2014). Chloroquine was recommended for the prevention of malaria during pregnancy before being withdrawn due to growing resistance and poor adherence (Briand et al., 2008). There is evidence from studies in pregnant women where CQ given in normal therapeutic doses is generally safe and well tolerated even during the first trimester (Wolfe and Cardero, 1985; Steketee
et al., 1996) as compared to most drugs whose safety in pregnancy has not been demonstrated and if recommended cannot be used in the first trimester and are limited to the second or third trimesters for fear of toxicity to the foetus.

In an attempt to explore alternative and more effective methods of preventing pregnancy-associated malaria, a randomized, controlled clinical trial of chloroquine (CQ) for chemoprophylaxis versus intermittent preventive therapy to prevent malaria in pregnancy was done in Blantyre, Malawi. Samples collected from this trial between February 2012 and May 2013 were analyzed in this study to evaluate the effectiveness of different prevention strategies by assessing the incidence of malaria infection in pregnant women enrolled and randomized into three treatment arms namely CQ prophylaxis, CQ-IPTp and SP-IPTp. Pregnant women before week 28 of their gestation were recruited into the study and were randomized for CQ prophylaxis (weekly doses of CQ until delivery), CQ-IPTp (therapeutic doses of CQ administered twice during pregnancy) and SP-IPTp, the current standard of care (therapeutic doses of SP administered twice during pregnancy).

It is hoped that the findings of this study would inform whether or not CQ chemoprophylaxis and CQ-IPTp could be reintroduced for prevention of malaria in pregnancy.
CHAPTER THREE
MATERIALS AND METHODS

3.1 Study area and population

The study was done in Ndirande, a peri-urban township on the outskirts of Blantyre, Malawi. Blantyre is the commercial capital city of Malawi, located 366 km to the south of Lilongwe, the administrative capital city of Malawi. Ndirande Health Centre is located 3 km north-east of Blantyre city centre and serves a population of approximately 200 000. The majority of the population comprise of low to middle income earners working in the industries surrounding the area or those in self-employment plying their trade in the Ndirande market and other markets and streets within Blantyre city. Houses are mostly clustered together, built with bricks and roofed with iron sheets. There are no proper sewage systems and latrines are constructed very close to the houses hence stagnant water and sewage pools are a common sight. Figure 3.1 below shows a map of Malawi showing the location of Blantyre city with respect to the capital Lilongwe and Figure 3.2 shows the location of Ndirande Health Centre in Blantyre and part of surrounding settlements it serves. The trial sites were selected based on the level of malaria transmission, local CQ and SP resistance rates and rigorous pretrial site assessments by the monitoring and audit teams.
Figure 3.1 Map of Malawi showing the location of Blantyre city from the capital Lilongwe. Adapted from Google Earth (2013).
Figure 3.2: Aerial view of Ndirande showing Ndirande Health Centre and part of the surrounding community it serves. Adapted from Google Earth (2013).

3.2 Inclusion criteria

Pregnant women who met the following inclusion criteria were enrolled in the clinical trial and filter paper specimens collected were used for subsequent analysis in the study:

i. Pregnant women who had not reached the end of the 27th week of gestation period;

ii. Pregnant women who were in their first or second pregnancy;

iii. Pregnant women who anticipated to remain in Blantyre until 14 weeks after delivery;
iv. Pregnant women who agreed to deliver at the Ndirande Health Centre or Queen Elizabeth Central Hospital (QECH);

v. Pregnant women who gave consent to participate in the study.

3.3 Exclusion criteria

i. Pregnant women who had chronic use (>14 days) of any medication with an antimalarial or antifolate activity;

ii. Pregnant women with HIV infection;

iii. Pregnant women with known high-risk pregnancy requiring regular supervision of an obstetrician;

iv. Pregnant women with allergy to any of the study drugs.

3.4 Sample size considerations

A sample size of 80 women in each treatment arm was estimated based on calculations for statistical superiority in randomized controlled trials outlined by Zhong (2009).

\[
N = \frac{1}{2} x \left( \frac{Z_{\alpha}^2 + Z_\beta}{\arcsin \sqrt{p} - \arcsin \sqrt{P_0}} \right)^2
\]

Where;

N=Size per group; p=the response rate of standard treatment group; \(P_0\)= the response rate of new drug treatment group; \(Z_\alpha\)= the standard normal deviate for a one or two sided x;

Assuming p= 40% (risk of infection in the SP IPTp arm),
$P_0 = 20\%$ (Target in the experimental arm in order to have a 20% difference from the SP IPTp arm)

$Z_{\alpha/2} = 1.96; Z_{\beta} = 0.845$

$$N = \frac{1}{2} \times \left( \frac{1.96 + 0.845}{\arcsin\sqrt{0.4} - \arcsin\sqrt{0.2}} \right)^2$$

$N = 80.49518$

The figure was rounded up to a cost effective minimum of 80 patients per treatment arm. With 80 women in each of the three arms, the study aimed to detect a 20% difference when comparing the intervention arms (CQ prophylaxis or CQ IPT) to SP IPTp for a 2 sided 2.5% significance level having a power of 80%, assuming a 40% risk of infection in the SP IPTp arm.

### 3.5 Ethical considerations

The proposed study was conducted within the approved clinical trial “A Randomized, Controlled Clinical Trial of Chloroquine as Chemoprophylaxis Versus Intermittent Preventive Therapy to Prevent Malaria in Pregnancy in Malawi” which was jointly reviewed and approved by the University of Malawi College of Medicine Research and Ethics Committee (COMREC) and the University of Maryland Institutional Review Board. Refer to ethical approval letter in Appendix I.

### 3.6 Study design

The study was a randomized, open label clinical trial conducted in Ndirande, a peri-urban township on the outskirts of Blantyre, Malawi. Two hundred and forty
pregnant women in their first or second pregnancy who presented to the Ndirande Antenatal Clinic (ANC) on the grounds of the Ndirande Health Centre were recruited for enrollment. They were randomly assigned to one of the three treatment arms: CQ chemoprophylaxis, CQ-IPTp, or the standard regimen of SP-IPTp.

**Study treatment:** Two hundred and forty participants were randomly assigned to one of three treatment arms:

1. **CQ prophylaxis:** A loading dose of CQ (base) 600 mg (2 tablets) at first administration followed by 300 mg of CQ base (1 tablet) every week until delivery.

2. **CQ-IPTp:** A therapeutic dose of CQ (1,500 mg given over 3 days, 2 tablets on Day 0, 2 tablets on Day 1, 1 tablet on Day 2) was administered twice during pregnancy.

3. **SP-IPTp:** A therapeutic dose of SP (1500 mg sulfadoxine and 75 mg pyrimethamine; 3 tablets) was administered twice during pregnancy.
Assess for prevalence and incidence of Malaria

Figure 3.3 is a schematic diagram of the study design.
3.7 Sample collection

At enrollment, finger prick blood was obtained for filter paper specimen collection. Participants were asked to return to the clinic every 4 weeks for collection of blood for filter paper specimens and were encouraged to return to the clinic any time they were ill. At delivery, peripheral and placental blood was collected on filter paper specimens filter papers well labeled with barcodes.

The filter paper specimens were air dried and stored at room temperature for analysis. Each sample was stored in a separate peel bag with a desiccant to protect it from moisture. Filter papers were cut into two, one was kept at the site in Ndirande as a backup and the other transported by road to the molecular laboratory at College of Medicine (approximately 5km from the site) for analysis.

3.8 DNA extraction and analysis

DNA was extracted as described by Bereczky et al. (2005). In brief, blood spots were cut from each filter paper using a clean pair of scissors into 1.5 ml well labeled microcentrifuge tubes. The pair of scissors was sterilized after cutting each blood spot by dipping in DNAOUT (G Biosciences) and then in 99% methanol (Glassworld) and then wiped with a clean disposable paper towel. Methanol (125µl) was added followed by incubation at room temperature for 15 minutes. Methanol was then removed by gently bloating the tubes on a clean paper towel whilst avoiding losing the filter papers. The samples were air dried overnight before adding 65µl of distilled water. The samples were heated at 97°C for 15 minutes whilst
vortexing every 5 minutes. A real-time PCR malaria detection assay described by Rantala et al. (2010) was used to detect presence of malaria parasites at the molecular laboratory of the Department of Biochemistry of the University of Malawi, College of Medicine in Blantyre, Malawi. The assay was targeting the *P. falciparum* lactate dehydrogenase gene (*pfldh*). A volume of 1.0 µl of eluted DNA was amplified using *P. falciparum* LDH forward (ACG ATT TGG CTG GAG CAG CAG AT), *P. falciparum* LDH reverse (TCT CTA TTC CAT TCT TTG TCA CTC TTT C) oligonucleotide primers and TaqMan probe, *P. falciparum* LDH (FAM-AGT AAT AGT AAC AGC TGG ATT TAC CAA GGC CCC A-TAMRA) as outlined by Rantala et al. (2010). No DNA quantitation was done as these were samples from low grade parasitaemia that required no dilution. To optimize the detection, the number of cycles was increased from 40 to 50 as most of the samples were low parasitaemia so that amplification happened late in the reaction.

Reaction volumes were 25µl each, consisting of 12.5µl universal PCR master mix (Applied Biosystems), 1 µl of template, forward and reverse primers at 250 nM each, probe at 300nM, and molecular grade water. The master mix contained a premix of AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure), Uracil-N glycosylase (UNG), deoxynucleotide triphosphates (dNTPs) with deoxyuridine triphosphate (dUTP), ROX™ Passive Reference and optimized buffer components.

All reactions were run in triplicates on ABI 7300 real-time PCR system (Applied Biosystems). Cycling conditions were 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 sec followed by 60°C for 1 min. Each reaction plate included
*P. falciparum* 3D7 genomic DNA as a positive control with a detection limit of 2.7 parasites per microlitre and a negative control with molecular grade water in place of DNA, all in triplicate. Samples were considered *P. falciparum* positive if at least two of the three amplification curves reached the threshold line (Ct value range of 20-40). Reactions in which only one amplification curve reached the threshold line were repeated.

### 3.9 Data management and statistical analysis

All raw data was recorded into a lab note book and entered into Microsoft excel spreadsheet. The data was then exported into respective software packages for analysis. The primary outcome measure of interest was the occurrence of malaria infection among the women who received the two different interventions as compared to women who received the standard SP-IPTp. Incidence rates were calculated using Stata version 10.1 by dividing the total number of malaria episodes in each arm by the total follow-up time in months and multiplying the quotient by 100 to convert to incidence rate per 100 person months. The incidence rates were used to do pairwise comparison between the two experimental interventions and the standard SP-IPTp arm. Pairwise comparison was done using a Poisson regression model and p-values at 95 % confidence intervals were reported. Incidence rates were preferred because multiple infections within individuals were accounted for in the analysis and rates were expressed per 100 person months of pregnancy within which pregnant women were exposed to malaria. Infections in the same individual detected less than 28 days apart were considered as the same episode of malaria infection
regardless of whether there was a negative PCR result in between some two infections. Infections occurring 28 days or more apart were considered as multiple infections if there was a negative test in between, otherwise they were considered the same recurrent episode if they persisted within consecutive visits.

To check whether the calculated incidence of malaria infections was affected by the differences in the total number of visits per treatment arm, Kruskal-Wallis rank test was used to test for equality in the median total number of visits. Overall comparison across the three treatment arms was done by means of Kaplan–Meier survival analysis using SPSS version 16.0. The number of events occurring in each treatment arm were calculated by means of survival tables and expressed as percentages. For each patient, survival time was calculated as the time from randomization entry until occurrence of malaria infection for those who had malaria and those who did not get malaria were censored at the date of the last clinical encounter. Participants that were lost to follow-up were censored at their last follow-up visit. Mean survival times were calculated and overall comparison was done by Log Rank (Mantel-Cox) Chi-Square analysis. Survival functions were derived by plotting the cumulative survival against time in months.

McNemar test was done in SPSS version 16.0 to measure the effect of each intervention on the prevalence of malaria infection at baseline (before drug administration) and at the endpoint of delivery (after drug administration). Since the test uses only the discordant data where change has occurred before and after intervention, McNemar exact binomial test was used instead of the asymptotic
McNemar test since the totals of discordant pairs were not more than or equal to 10 as assumed by the latter. P-values less than 0.05 were considered to indicate statistical significance.
CHAPTER FOUR

RESULTS

4.1. Real time PCR amplifications

Real time amplification plots for samples that were malaria positive ranged between Ct values of 20 to 40 whilst 3D7 controls amplified around a mean Ct value of 28. Figures 4.1 to 4.4 illustrate amplification curves for representative samples from each treatment arm and one 3D7 control amplification.

![Graph](image)

Figure 4.1: Representative patient sample amplification plot from CQ-prophylaxis arm
Figure 4.2: Representative patient sample amplification plot from the CQ-IPTp arm

Figure 4.3: Representative patient sample amplification plot from the SP-IPTp arm
4.2. Incidence of malaria infection

The incidence of malaria infection were 1.98 (95% CI: 0.50, 8.04) per 100 person months in the CQ-IPTp arm and 0.34 (95% CI: 0.04, 3.33) per 100 person months in the CQ prophylaxis arm as compared to 0.99 per 100 person months in the standard SP-IPTp arm. The differences in incidence rates were not statistically significant after pairwise comparison between the experimental interventions and the standard SP-IPTp (P-value=0.32 and P=0.36 for the CQ-IPTp and CQ-Prophylaxis respectively). Table 4.1 below summarises the results of the incidence of malaria infection detected by PCR for each of the three treatment arms.
Table 4.1: Incidence of PCR detected malaria infection.

<table>
<thead>
<tr>
<th>Treatment arm</th>
<th>Number of Episodes</th>
<th>Person-time (months)</th>
<th>Incidence of Infection per 100 person months</th>
<th>Incidence Rate Ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-IPTp</td>
<td>3</td>
<td>304.50</td>
<td>0.99</td>
<td>1 (Reference)</td>
<td></td>
</tr>
<tr>
<td>CQ Prophylaxis</td>
<td>1</td>
<td>293.03</td>
<td>0.34</td>
<td>0.34 (0.04, 3.33)</td>
<td>0.36</td>
</tr>
<tr>
<td>CQ-IPTp</td>
<td>6</td>
<td>302.97</td>
<td>1.98</td>
<td>2.01 (0.50, 8.04)</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Note: CI = 95% Confidence Interval

The 95% CI and p-values based on Poisson regression

There was no significant difference in the total number of visits by participant across the three treatment arms after testing for equality in the median total number of visits using Kruskal-Wallis rank test (p=0.40). The box plot in Figure 4.5 below shows the distribution of the total number of visits across the three arms by participant.

Figure 4.5: Distribution of total number of visits by participants
4.3. Time to event survival analysis

The total number of events occurring in each treatment arm and their percentages are summarized in Table 4.2 below.

Table 4.2: Case processing summary of malaria infection

<table>
<thead>
<tr>
<th>Treatment Arm</th>
<th>Total Number</th>
<th>Total Number of Events (peripheral + Placental)*</th>
<th>Censored</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME-SP-IPTp</td>
<td>799</td>
<td>29 (0)</td>
<td>770</td>
</tr>
<tr>
<td>CQ-Prophylaxis</td>
<td>868</td>
<td>24 (1)</td>
<td>844</td>
</tr>
<tr>
<td>CQ-IPTp</td>
<td>829</td>
<td>23 (0)</td>
<td>806</td>
</tr>
<tr>
<td>Overall</td>
<td>2496</td>
<td>76 (1)</td>
<td>2420</td>
</tr>
</tbody>
</table>

*The value in parentheses indicate number of placental cases

Mean survival times in months were 7.59 (95% CI: 7.38-7.78), 7.76 (95% CI: 7.67-7.86) and 7.60 (95% CI: 7.38-7.83) for the SP-IPTp, CQ-Prophylaxis and CQ-IPTp arms respectively. Overall comparison by Log Rank (Mantel-Cox) showed no significant difference, (p=0.34). Figure 4.6 below is the survival function derived by plotting cumulative survival against time in months.
4.4. Comparison of prevalence of malaria infection at baseline and delivery

McNemar test for analysis of correlated or dependent dichotomous variables was used to measure the effect of each intervention on the prevalence of malaria infection at baseline (before drug administration) and at the endpoint of delivery (after drug administration). Although both peripheral and placental samples were collected and analysed at delivery, only one case, which was in the CQ prophylaxis arm was positive for placental malaria infection. One woman (1.25%) had placental infection in the CQ prophylaxis arm as compared to none in the CQ-IPTp and the standard SP-IPTp arms. This showed no statistical significance at p<0.05. Results showed that there was a significant difference between the proportions of malaria infections at baseline as compared to the proportion of malaria infections at delivery (p=0.02) in the standard SP-IPTp arm. P-values could not be calculated for the experimental CQ-
IPTp and CQ-prophylaxis arms as there were no discordant pairs. However, the two experimental interventions had reduced the prevalence of malaria to none at delivery.
CHAPTER FIVE
DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussions

Currently, WHO recommends among other strategies the use of SP-IPTp for pregnant women living in areas where malaria is endemic (WHO/AFRO, 2004). Several studies (Schultz et al., 1994; Parise et al., 1998; Verhoeff et al., 1998; Shulman et al., 1999; Rogerson et al., 2000; Njagi et al., 2003; Sirima et al., 2003; Kayentao et al., 2005) have demonstrated the protective efficacy of SP in IPTp providing support for its continued use by pregnant women living in malaria endemic areas. Most of these studies were observational and aimed to evaluate the efficacy of SP-IPTp. This makes the current study unique because it compares two different interventions against SP-IPTp as tools for preventing pregnancy associated malaria using qPCR (Figures 4.1-4.4). Evidence of growing SP resistance has prompted the search for an alternative in case SP becomes completely ineffective for IPTp. The current study compared the effectiveness of two trial interventions using CQ against the standard SP-IPTp as tools for prevention of malaria during pregnancy.

In this randomized trial, pairwise comparison of each experimental arm (CQ prophylaxis and CQ-IPTp) and the standard intervention show that CQ IPTp arm had a slightly higher incidence of malaria infection than the standard SP-IPTp whilst CQ-prophylaxis arm had a lower incidence of malaria infection than the standard SP-
IPTp; 1.98 per 100 person months in the CQ-IPTp arm and 0.34 per 100 person months in the CQ prophylaxis arm as compared to 0.99 per 100 person months in the standard SP-IPTp arm (Table 4.1). However, the differences were not statistically significant in both comparisons (p>0.05). The calculated incidences of malaria infections were not affected by the differences in the total number of visits per treatment arm after Kruskal-Wallis rank test was used to test for equality in the median total number of visits (Figure 4.5). The results suggest that there is no difference in terms of the efficacy of the two experimental interventions against the WHO recommended standard, SP-IPTp, with respect to protection of pregnant women from malaria infection. This is in contrast to results from a study done in Burkina Faso (Tiono et al., 2009), which proved SP-IPTp to be superior as compared to CQ-IPTp and CQ chemoprophylaxis. However, the Burkina Faso study was done at the time when resistance was widespread and CQ was still the regimen recommended by the National Malaria Control Programme.

Similar studies in Mali (Kayentao et al., 2005) and Benin (Briand et al., 2008), showed SP-IPT to be more efficacious than weekly CQ chemoprophylaxis. However, these studies were done in areas where SP was still efficacious enough and CQ resistance was widespread (Briand et al., 2008). Two studies by Harrington et al. (2009 and 2011) in Muheza, Tanzania, an area of widespread SP resistance found reduced efficacy of SP-IPTp. In the first prospective delivery cohort (Harrington et al., 2009), the effects of SP-IPTp on parasite resistance coding alleles, parasite diversity, level of parasitaemia and inflammation in the placenta were examined. Use
of IPTp was associated with an increased fraction of parasites carrying the resistance coding alleles at DHPS codon 581, an increase in the level of parasitaemia and more intense placental inflammation. The findings support a model of parasite release and facilitation, whereby the most highly resistant parasites out-compete the less fit parasite populations and overgrow under drug pressure. As such, use of partially effective anti-malarial agents for IPTp may exacerbate malaria infection in settings of widespread resistance. A follow up cross-sectional study in the same area (Harrington et al., 2011), determined SP-IPTp use by individual report and by plasma sulfa measurements and its effects on maternal and foetal delivery outcomes. Results demonstrated that in this cohort, SP-IPTp was not associated with decreased odds of placental malaria or with increased mean maternal hemoglobin or mean birth weight.

Unexpectedly, IPTp was associated with decreased cord blood hemoglobin level and increased risk of foetal anaemia which may be related to in utero SP exposure. It was concluded from this cohort that SP-IPTp does not improve overall pregnancy outcomes in this setting where SP-resistant parasites predominate and may increase the odds of foetal anaemia. As parasite resistance increases in a community, the overall effect of IPTp may transition from net benefit to neutral or net harm (Harrington et al., 2011). Unlike these studies, the current study did not measure maternal and foetal delivery outcomes. However, although SP-IPTp arm had higher number of malaria infections, 29 cases (Table 4.2) in comparison to the other
treatment arms albeit not statistically significant (p>0.05), there was no cases of placental malaria detected.

Overall comparison of mean survival time (time to the first occurrence of malaria infection) show no significant difference across the three treatment arms (p=0.34), Table 4.2. However, Kaplan-Meier survival plots of cumulative survival against time in months suggest that the cumulative survival for CQ prophylaxis remains consistently higher than both SP-IPTp and CQ-IPTp (Figure 4.6). As explained in details earlier in the study design, CQ prophylaxis involved a loading dose of CQ at first administration followed by CQ base every week until delivery, whereas CQ-IPTp and SP-IPTp involved a therapeutic dose administered twice during pregnancy. This could explain why the weekly CQ prophylaxis may consistently maintain a higher cumulative survival than both IPTp interventions considering the duration and frequency of drug exposure.

Very few studies have compared the three interventions namely CQ prophylaxis, CQ-IPTp and SP-IPTp. In Mali, where levels of CQ resistance are low, Kayentao et al. (2005), found that IPT with SP was more efficacious than CQ prophylaxis in preventing malaria during pregnancy. In Burkina Faso, IPT with SP had shown clear superiority in reducing adverse effects at delivery compared with IPT with CQ and classical chemoprophylaxis with CQ. However, this study was done at a time when \textit{P. falciparum} resistance to CQ was on the increase (Tiono et al., 2009). Also, both studies did not attempt to compare time for first occurrence of malaria infection and
show trends of cumulative survival over time using survival analysis across the treatment arms.

Although the baseline prevalence of malaria infection was comparable across the arms, there was a reduction in the prevalence measured at delivery in all treatment arms. The prevalence of peripheral malaria infection at delivery was higher in the SP-IPTp arm compared to the two experimental arms where peripheral prevalence was reduced to undetectable levels (no peripheral malaria positive case was detected at delivery for both CQ experimental interventions). This suggests that all the three interventions SP-IPTp, CQ-IPTp and CQ prophylaxis were effective in preventing placental malaria although in the SP-IPTp arm, episodes of parasitaemia could still be detected at delivery. In a similar study, Nyunt et al. (2010) found that 1 of the 98 pregnant women on SP-IPTp became parasitaemic during follow up, none developed clinical malaria and none tested positive for *P. falciparum* on assessment of placental parasitaemia.

In this study, only one case, in the CQ prophylaxis arm, tested positive for *P. falciparum* upon assessment of the placenta, Table 4.2. This prevalence of placental infection did not differ significantly from that observed in the other intervention arms (*p* > 0.05). Offianan et al. (2012) noted that women with microscopically detectable placental *P. falciparum* malaria had a higher risk of delivering a low birth weight baby than those without detectable placental *P. falciparum*. This supports a strong association between *P. falciparum* malaria and risk of low birth weight. As
such, even a single case of placental infection found by this study is a cause for concern because the greatest risk of poor pregnancy outcomes during malaria occurs in women with placental infection (Menendez et al., 2000a). These findings are in agreement with Kayentao et al. (2005) who showed that women on CQ-IPTp and weekly CQ are more likely to have placental malaria than those on SP-IPTp.

However, the findings of the current study on SP-IPTp are in conflict with findings from two studies conducted in Muheza, Tanzania, an area with high levels of SP resistance. The first study by Harrington et al. (2009), suggested that women who received SP-IPTp were more likely to experience chronic infections with placental inflammation compared to women who did not. In that study, SP-IPTp was associated with a 5.4% increase in the prevalence of parasitaemia, increased prevalence of SP-resistance alleles, and decreased parasite density in women who were on SP-IPTp than those who were not. A subsequent study from the same area showed lack of beneficial pregnancy outcomes from SP-IPTp (Harrington et al., 2011). Data from Malawi show that the effectiveness of SP-IPTp has been decreasing over time and no longer appears to provide any benefit (Feng et al., 2010). In the findings, the relationship between IPT use and parasitaemia, anaemia and low birth weight changed over time (Feng et al., 2010). Between 1997 and 2001, SP-IPTp was associated with decreased placental malaria, maternal anaemia and low birth weight (LBW), but these associations were lost from 2002-2006. If this trend continued beyond 2006, then the results from this study are surprising because SP-IPTp would be expected to have an even more compromised efficacy over time.
Although it was predicted elsewhere (Wargo et al., 2007; Harrington et al., 2009) that administration of SP-IPTp in the presence of resistant parasites would be associated with greater degrees of pregnancy associated morbidity, Taylor et al. (2012) found the contrary. In their longitudinal study, SP-IPTp did not worsen pregnancy associated malaria morbidity despite the increasing prevalence and fixation of SP-resistant *P. falciparum* haplotypes. This supports findings from this study and several explanations have been put forward. Epidemiologically, an overall decline in parasite prevalence among pregnant women could potentially reduce the ability to detect differences (Taylor et al., 2012). Pharmacologically, in the setting of partial host immunity, SP is capable of clearing infections comprising resistant parasites (Cravo et al., 2001). Genetically, a key difference between the parasite populations in Malawi and Tanzania is the absence of *dhps* 581 mutation (Plowe et al., 1997; Nkhoma et al., 2007; Harrington et al., 2009). This mutation is not prevalent in Malawi, whereas its promotion in pregnant women was suggested to be a key consequence of SP exposure in Tanzania.

### 5.2 Conclusions

CQ chemoprophylaxis and CQ-IPTp were as effective as the standard SP-IPTp in preventing malaria in pregnancy. The two CQ interventions were equally as good as SP-IPTp in preventing the incidence of malaria in pregnant women. Both CQ chemoprophylaxis and CQ-IPTp were equally as good as SP-IPTP in terms of the time taken to the first malaria infection whilst women were on protective treatment. The two interventions also were equally as good as SP-IPTp in terms of the
reduction of the prevalence of malaria infection before and after treatment. The WHO Methods for surveillance of antimalarial drug efficacy, 2009 protocol (WHO, 2009) states that “comparative randomized studies of two or more antimalarial agents are relevant to programmes that are considering the introduction of a specific replacement medicine where the efficacy of the first line treatment is less than 90%.” For treatments once considered highly effective, it is more appropriate to show that a treatment is “non-inferior” or not worse than the standard treatment. This study demonstrated that CQ-IPTp and CQ prophylaxis treatment outcomes are not inferior from the standard SP-IPTp in prevention of malaria infection during pregnancy.

As such the hypothesis that CQ prophylaxis in pregnancy and CQ-IPTp are both inferior to the standard practice of SP-IPTp is rejected.

There is need to validate these findings in other malaria settings.

5.3 Recommendations

5.3.1 From this study, CQ interventions seem to be non-inferior to SP-IPTp in pregnant women. It is important to monitor SP-IPTp closely whilst alternative drugs such as CQ are also being considered so that when SP is rendered ineffective, a replacement should be available for use during pregnancy.

5.3.2 From this study, CQ prophylaxis and CQ-IPT demonstrated positive impact in prevention of malaria in pregnant women and were just as good as the
standard SP-IPTp. CQ would be a potential replacement for SP in IPTp especially now that it has regained its efficacy both for treatment and IPTp.

5.3.3 More clinical studies in other settings where CQ resistance has declined should be carried out to assess efficacy of CQ-IPTp. This will help provide solid evidence in support of the reintroduction of CQ-IPTp and CQ chemoprophylaxis against pregnancy associated malaria.

5.4 Limitations

5.4.1 The sample size may have been small to detect a significant difference in prevalence of pregnancy associated malaria between interventions because published baseline data for the risk of malaria infections in pregnant women was not available in the population. As such, the 40% used for sample size calculation was higher than the prevalence detected at baseline in all three treatment arms.

5.4.2 The methanol/water DNA extraction protocol used is very crude and may not yield good quality DNA for such important trials. This may have lowered chances of the procedure detecting low parasitaemia cases. The method was used because it very affordable and minimized the cost of running volumes of samples that were collected considering the multiple number of visits per participant.
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APPENDIX I: ETHICS COMMITTEE APPROVAL LETTER

Principal
K.M. Maleta MBBS PhD

Our Ref.: COMREC/10
Your Ref.: P.06/10/943

1st February 2013

Dr M. Laufer
Blantyre Malaria Project
P.O Box 32256
Blantyre 3

Dear Dr Laufer,

RE: P.06/10/943 – A Randomized Controlled Clinical trial of Chloroquine as Chemoprophylaxis versus Intermittent Preventive Therapy to Prevent Malaria in Pregnancy in Malawi

I write to inform you that COMREC reviewed the progress report for the above mentioned proposal which you submitted. I am pleased to inform you that COMREC approved continuation of the study with effect from 1st March 2013.

This renewal is subject to continued adherence to the College of Medicine requirements for all COMREC approved research studies.

On the other hand, COMREC approved the following document which was submitted along with the report:

1. DSMB report

Yours Sincerely,

Dr. G. Kalanda
CHAIRPERSON - COMREC

Approved by
College of Medicine

1 FEB 2013

(COMREC)
Research and Ethics Committee