Malaria in Pregnancy and Drug Therapies in Malawi

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Epidemiology

Chapel Hill
2006

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ABSTRACT

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Malaria in Pregnancy and Drug Therapies in Malawi
(Under the direction of Prof Steven R. Meshnick)

In this dissertation, I investigated the effect of timing and frequency of *Plasmodium falciparum* infection during pregnancy on the risk of low birth weight and maternal anemia. I also investigated the efficacy of sulfadoxine-pyrimethamine combined with azithromycin or artesunate compared with sulfadoxine-pyrimethamine monotherapy as treatment for uncomplicated *Plasmodium falciparum* infection in pregnant women.

We conducted a prospective observational study recruiting 2,462 pregnant women in Malawi. Malaria was assessed during follow-up and delivery. Birth weight and hemoglobin concentration were measured at delivery. The prevalence of low birth weight increased with the number of malaria episodes: [1 episode (prevalence ratio [PR] =2.17; 95% C.I. 1.22-3.86), and 2 episodes (PR=3.68; 95% C.I. 1.81-7.47)]. The prevalence of anemia increased with the number of episodes of malaria: [1 episode (PR= 1.16; 95% C.I. 0.77-1.77) and 2 episodes (PR= 1.82 95% C.I. 1.04-3.19)]. The prevalence of low birth weight was higher with infection in the second trimester (PR=2.97; 95% CI 1.60-5.53) than in the third trimester (PR=1.42; 95% CI 0.63-3.22). The prevalence of maternal anemia was higher with infection in the third trimester (PR=1.44; 95% CI 0.90-2.29) than

in the second trimester (PR=0.94; 95% CI 0.51-1.71). Timing and frequency of infection affect the risk of maternal anemia and low birth weight.

We conducted a randomized open-label clinical trial, recruiting 141 pregnant women. They were randomly allocated to 3 treatment groups: sulfadoxine-pyrimethamine (SP; 3 tablets, 500mg sulfadoxine and 25mg pyrimethamine per tablet); SP plus azithromycin (1g/day x 2 days); or SP plus artesunate (400mg/day x 3 days). All treatment regimens were well tolerated. Two women vomited soon after ingesting azithromycin. Recrudescent episodes of malaria were less frequent with SP-azithromycin [Hazard Ratio 0.14 (95% confidence interval 0.02 to 0.78)] and SP-artesunate [Hazard Ratio 0.13 (95% confidence interval 0.03 to 0.53)] compared with SP monotherapy. There were more abortions in the SP-azithromycin group, and more stillbirths in the SP-artesunate groups, but they were probably unrelated to treatment. A larger study is needed to determine its safety and efficacy in preventing poor birth outcomes**.**

ACKNOWLEDGEMENTS

I would like to thank my academic supervisor and dissertation committee chair, Professor Steve Meshnick, for helping me get funding and his mentorship. I would also like to thank my other committee members, William Miller, Jennifer Smith, Annelies Van Rie and Stephen Rogerson for their invaluable comments and guidance.

I am grateful to Mr Ntolo, Mrs E Chaluluka, Mr Mofolo and Dr Chaponda for the help and support they provided with the datasets that I used for this dissertation. Thanks to Nancy Colvin and Carmen Woody who were always willing to help no matter how trivial the matter. To Julius Atashili for all the help, patience and feedback on the dissertation.

I would like to thank my family for their love and support. To Lindani, I am so grateful for the support, patience and for being there all the time when things were good or bad. To mom and dad for having faith in me all the time, and for all your support. Thanks dad for going through every page of my dissertation to make sure I have written everything correctly. Jerome and Zohra for always being there for me.

I am grateful for the funding from the Forgarty-CDC Training Fellowship Award

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ARG	Arginine
ASN	Aspargine
CQ	Chloroquine
CSA	Chrondroitin sulfate A
DNA	Deoxyribonucleic Acid
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DHS	Demographic health survey
DSMB	Data safety management committee
EMM	Effect measure modification
G6PD	Glucose-6-phosphate dehydrogenase
GLU	Glutamine
GLY	Glycine
HA	Hyaluronic acid
HIV	Human immunodeficiency virus
HRP-2	Histidine-rich protein-2
HTA	Heteroduplex tracking assay
ICAM	Intercellular adhesion molecule
IES	Infected erythrocytes
IFN	Interferon
IL	Interleukin
ILE	Isoleucine

IPT	Intermittent presumptive therapy
IRB	Institution review board
ITNS	Insecticide treated bed nets
IUGR	Intrauterine growth retardation
LBW	Low birth weight
LYS	Lysine
MIF	Migratory inhibitory factor
MOHP	Ministry of Health and Population
MRNA	Messenger ribonucleic acid
MG	Multigravidae
PCR	Polymerase chain reaction
PFCRT	*Plasmodium falciparum* chloroquine resistance transporter
PFEMP-1	*Plasmodium falciparum* erythrocyte membrane protein-1
PFMDR-1	*Plasmodium falciparum* multidrug-resistance gene-1
PG	Primigravidae
PLDH	Parasite lactate dehydrogenase
QECH	Queen Elizabeth Central Hospital
SER	Serine
SP	Sulfadoxine-pyrimethamine
STIS	Sexually transmitted infections
THR	Threonine
TNF	Tumor necrosis factor
VSA	Variant surface antigens

WHO	World Health Organization

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1. Introduction

Each year, approximately 50 million women become pregnant in malaria-endemic areas [1]. Malaria infection during pregnancy can result in adverse pregnancy outcomes. It is estimated that each year malaria infection during pregnancy is responsible for 5 to 12% of all low birth weight (LBW) deliveries, 30% of preventable LBW deliveries, and 3 to 8% of infant deaths globally [2]. Malaria in pregnant women is also responsible for as many as 400,000 cases of severe anemia annually in sub-Saharan Africa [3].

Although the adverse effects of malaria infection during pregnancy are well known, there is limited information on the effect of timing and frequency of malaria infection during pregnancy on the risk of low birth weight and maternal anemia. A few studies that have assessed the effect of timing of infection on LBW and maternal anemia have used placental pathology at delivery to define timing of infection which could have resulted in misclassification [4-7]. One of the strategies recommended by the World Health Organization (WHO) for prevention of malaria and its adverse effects in pregnant women in malaria-endemic areas in sub-Saharan Africa is intermittent preventive therapy (IPT) [8]. Information on the effect of frequency and timing of infection on these adverse outcomes would be important for developing effective policies in the prevention of malaria in pregnant women. It would help identify the time when IPT would be most effective in preventing LBW and maternal anemia. This is especially important in malaria endemic areas where most women do not have access to antenatal services and anti-malarial drugs are usually in short supply due to lack of resources.

Sulfadoxine-pyrimethamine (SP) is currently the drug of choice for IPT in many countries in sub-Saharan African countries and has been shown to reduce the prevalence of parasitemia and anemia in pregnant women, and increase birth weight [9-11]. Resistance to SP is increasing rapidly in these areas [12]. There are at present very few alternative drugs available for use for IPT during pregnancy, because of lack of data on the safety in the fetus. As drug resistance to SP spreads and intensifies, there is an urgent need to identify other drug alternatives for treatment and prevention of malaria in pregnancy.

In this dissertation, I will use data from a prospective study that was conducted in Malawi to investigate the effect of timing and frequency of *Plasmodium falciparum* infection during pregnancy on the risk for LBW and maternal anemia (specific aim I). To identify new treatment alternatives for SP, I will use data from a clinical trial that was also conducted in Malawi to assess the efficacy and safety of SP combined with azithromycin or artesunate in treating and preventing malaria in pregnant women (specific aim II).

1.2. Malaria in Malawi

Malawi is located in South-East Africa, covering an area of 45,745 square miles (118,484km^2), with a population of 12 million [13].Women in the reproductive age (15-49 years), and children less than 5 years comprise 24% and 17% of the population respectively [13]. Malawi has a tropical continental climate with a dry season from August to October, and a rainy season from November to March. Malaria transmission occurs all year round, but peaks during the rainy (November–March). *P. falciparum* causes over 90% of all malaria infections. Malaria continues to be the leading cause of morbidity and

mortality particularly among children under the age of five years and pregnant women. It is estimated that there have been more than 8 million cases of malaria per year in Malawi, over the past 5 years [14]. Most of these cases were in children less than 5 years of age, who experience on average 9.7 malaria episodes each year [15, 16]. Forty percent of all hospital admissions, and 30% of all hospital deaths in children less than 5 years of age are due to malaria and its associated complications such as anemia. Although most infections in pregnant women are asymptomatic, parasitemia has been detected in up to 40% of pregnant women presenting for antenatal care [11, 17]. Malaria also causes 20% of the anemia cases in pregnant women [11].

Malaria also imposes substantial costs, both directly and indirectly due to potential loss of income from days of work lost and hospital and drug expenses. It is estimated that the total cost of malaria to the average Malawian household is US$35, or 7.2% of the average annual household income [18]. For very low-income households (40% of all Malawian households), the annual cost of malaria is nearly US$20, or 32% of annual household income.

Chloroquine (CQ) was used as the first line treatment for malaria, and chemoprophylaxis in pregnant women. Due to high levels of resistance, Malawi became the fist country in Africa to replace CQ with SP in 1993 as the first-line drug for treatment of uncomplicated *P. falciparum* malaria. It was also the first country to recommend that all pregnant women should receive at least two doses of SP IPT during pregnancy in the second trimester and early in the third trimester, which was shown to decrease the

prevalence of peripheral and placental parasitemia, maternal anemia and LBW [11, 19]. Malaria has been identified by women in Malawi as one of the most commonly identified maternal health problems in the ante-partum period, and the awareness for the need for SP during pregnancy is high [20,21]. However, only 79% of the pregnant women receive one dose of SP, and less than half (43%) receive the recommended two-dose regimen due to limited access to antenatal care services and lack of drugs in health facilities [14,21].

The level of SP resistance in Malawi has been steadily increasing since its introduction in 1993. A study conducted in the northern part of Malawi between 1995 and 1996 reported a high prevalence of mutations associated with SP resistance [22]. Another study conducted in the central region of Malawi in 1997, found that 78% of the samples had quintuple mutations, which are highly associated with resistance to SP [23]. *In vivo* and *in vitro* studies conducted in all three regions of Malawi from 1994 and 1998 reported clinical efficacy to SP in children less than 5 years of age ranging from 81% to 88% [24-26]. This indicates that SP resistance is high in Malawi and there is an urgent need to conduct research to identify new alternative anti-malarial therapies to replace SP for the future.

1.3. Malaria

Malaria is a vector-borne parasitic disease caused by protozoa belonging to the genus *Plasmodium*. Four species are known to infect human beings: *P. falciparum, P. ovale, P. vivax and P. malariae*. The majority of infections are caused by the two species, *P. falciparum* and *P. vivax. P. falciparum is* responsible for most severe disease [27]. The distribution of these *Plasmodium* species is dependent on ecological (temperature,

humidity, presence of breeding sites) and behavioral parameters affecting the ability of mosquitoes to transmit *Plasmodium* species [28]. *P. falciparum* malaria is widespread in the tropic regions in sub-Saharan Africa, certain areas of South-East Asia, Oceania and the Amazon Basin of South America [27] (Figure 1.1). *P. vivax* malaria has a wider geographic distribution compared to *P. falciparum* [29]. It is commonly found in most of Asia, parts of the Americas, eastern Europe and North Africa. *P. ovale* is found primarily in tropical western Africa and *P. malariae* is found only in pockets, particularly in Africa [30].

1.4. Transmission and life cycle of *Plasmodium* species

Malaria is transmitted to humans by over 40 species of female *Anopheline* mosquitoes (Figure 1.2). The infective parasites, in an active form called sporozoites, are released from salivary glands and injected into the human blood through a bite by an infected female *Anopheles* mosquito. The sporozoites rapidly invade liver cells within 30 to 45 minutes, beginning the pre-erythrocytic stage of the life cycle. In the liver cells, the sporozoites undergo asexual replication and develop into merozoites which are capable of invading red blood cells (erythrocytes). At the end of the pre-erythrocytic stage, parasites in the liver induce death and detachment of the liver cells, and budding of parasite-filled vesicles (merosomes) [31]. The merosomes migrate from the liver into the bloodstream, where they rupture, and thousands of merozoites are released into the blood stream. The merozoites attach to, and invade erythrocytes, beginning the erythrocytic cycle. The period it takes for parasites to multiply in the liver is species dependent, varying from 6 to 7 days for *P. falciparum* to 14 to 16 days for *P. malariae*. The *P. vivax* and *P. ovale*

parasites may remain dormant in the liver for months to years as hypnozoites before infecting red blood cells, and causing a relapse after the initial event [32].

In the erythrocytic stage, merozoites multiply asexually in erythrocytes, and depending on the species, produce 8 to 32 progeny merozoites. The merozoites are released from the erythrocytes with the destruction of the erythrocyte, and immediately invade other erythrocytes. This asexual erythrocytic cycle continues until it is controlled by the host's immune response, therapy or until the host dies. A small percentage of the merozoites undergo sexual replication and differentiate into male and female gametocytes. *P. vivax* gametocytes develop soon after the release of merozoites from the liver, whereas *P. falciparum* gametocytes develop much later [33]. These gametocytes are eventually taken up by a female *Anopheles* mosquito during a blood feed. The gametocytes develop and mature, undergoing sexual reproduction in the stomach wall of the mosquito, producing approximately 1,000 sporozoites. The sporozoites migrate to the salivary glands where the cycle is completed when they are injected into another human being during a blood feed by the mosquito.

1.5. Malaria epidemiology

1.5.1. *Plasmodium falciparum*

Despite tremendous efforts for decades to control malaria, it remains a major public health problem [1, 34]. Between 300 and 600 million episodes of *P. falciparum* infection occur each year [27]. More than 70% of these cases occur in sub-Saharan Africa and 25% in South-East Asia [27]. There is wide geographical variability in the incidence of

P. falciparum infection, even within sub-Saharan Africa with low incidence in southern Africa and in highland areas.

Pregnant women and children under 5 years are at highest risk for severe disease. *P. falciparum* malaria kills 1.5 to 2.7 million people each year, of whom about 1 million are children under the age of 5 years in sub-Saharan Africa [1, 27, 35]. *P. falciparum* infection indirectly accounts for an additional 5 to10% of neonatal and infant deaths due to its effect on birth weight [34]. Other effects of *P. falciparum* infection include neurological sequelae such as hemiplegia following cerebral malaria, developmental abnormalities and impaired intellectual development as a result of repeated infections [36-40]. *P. falciparum* infection also exacts an economic impact on both governments and families due to loss of productivity, time spent seeking treatment, and expenses arising from treatment and prevention costs [41, 42].

1.5.2. *Plasmodium vivax*

P. vivax is the second most important species causing human malaria, accounting for 70-80 million cases of malaria annually [29]. It is responsible for over 50% of malaria cases outside Africa, notably South-East Asia, Central and South America and the Indian subcontinent. It also accounts for approximately 10% of malaria cases in eastern and southern Africa. The prevalence of *P. vivax* is very low in West Africa due to the presence of Duffy-negative blood group variants that limit erythrocyte invasion by the parasite [33]. *P. vivax* infection mainly occurs in low transmission areas. Individuals living in these areas have little or no acquired immunity, and are at risk of symptomatic disease. *P. vivax*

parasites can remain dormant in the liver as hypnozoites. Reactivation of these liver forms may cause relapses of infection months after the initial infection. Tropical *P. vivax* strains usually begin to relapse within a month after the initial infection, whereas hypnozoites of temperate strains usually have an incubation period of several months [43]. *P. vivax* exclusively invades reticulocytes (which typically constitute 1% of erythrocytes), and therefore achieves relatively low parasite densities compared to *P. falciparum*. Although rarely directly life-threatening, *P. vivax* can cause severe and debilitating febrile illness [33]. Anemia is the most frequently observed pathological consequence of *P. vivax* malaria, both in acute and chronic infections.

1.6. Symptoms of malaria

The clinical manifestations of malaria appear approximately 7 to 14 days after an infectious mosquito bite. Symptoms are caused by the asexual erythrocytic stage of the parasite and are dependent on the species of plasmodium, the age and level of host immunity, and parasite species [33]. The rupture of erythrocytes by merozoites releases toxins. These toxins directly induce the release of cytokines such as tumor necrosis factor-α (TNF- α), and interleukin-1 (IL-1) from macrophages, resulting in symptoms of malaria [33]. Sequestration of parasitized erythrocytes within small vessels also contributes to the development of symptoms. The clinical presentation of malaria ranges from asymptomatic infection to rapidly progressive fatal illness. Acute malaria infection presents with nonspecific symptoms such as fever, headache, general body pains and anorexia. In non-immune individuals, there is an increased risk for developing severe disease with prostration, confusion or drowsiness, which may evolve to loss of consciousness (cerebral

malaria), respiratory distress, anemia, severe jaundice and in rare cases, renal failure and pulmonary edema [44]. Semi-immune individuals usually have mild symptoms or may remain asymptomatic. Repeated malaria infections frequently result in anemia.

1.7. Diagnosis of malaria

Microscopy remains the standard method of diagnosis in many malaria-endemic areas. It involves the examination of Giemsa or Field stained thick and thin blood films for the presence of intracellular (erythrocytic) parasites [45]. Microscopy has the advantage of low cost, high sensitivity and specificity when used by well–trained microscopists. It also enables the differentiation of species of *Plasmodium* and quantification of parasite load. In most malaria-endemic areas where resources are scarce, diagnosis of malaria is based on clinical symptoms. Most of the symptoms and signs of malaria are nonspecific, and overlap with those of other febrile illnesses [46, 47]. Clinical diagnosis of malaria can result in misdiagnosis and misuse of anti-malarial drugs. Other laboratory methods that have been developed include: the Quantitative Buffy Coat method which fluoresces parasites after an enrichment step for the infected erythrocytes; rapid diagnostic tests that detect the presence of *P. falciparum* specific histidine-rich protein-2 (HRP-2) or species-specific parasite lactate dehydrogenase (pLDH); and polymerase chain reaction (PCR) based tests that identify parasite deoxyribonucleic acid (DNA) [48-51]. All these methods have good results, some better than microscopy, but they are too expensive to be used in most malaria-endemic areas, and therefore have not replaced microscopy as the routine method for diagnosing malaria.

1.8. *Plasmodium falciparum* malaria in pregnancy

1.8.1. Epidemiology

Every year, at least 50 million women living in malaria-endemic areas are at risk of *P. falciparum* infection during pregnancy [1, 2]. Women are at higher risk of acquiring *P. falciparum* infection when pregnant than when not pregnant, or when compared with adult males [52, 53]. In areas of stable transmission, the risk of *P. falciparum* infection and its adverse effects is greatest in the first pregnancy and decreases with subsequent pregnancies [10,52,54,55]. By contrast, in areas of low or unstable transmission, there is no difference in the risk of acquiring *P. falciparum* malaria infection, and the severity of adverse effects by parity, because women have little or no acquired immunity [56,57]. The risk of *P. falciparum* infection also varies during the course of pregnancy. The prevalence of peripheral parasitemia is highest in the first half of pregnancy, then remains constant, or declines after mid-gestation, irrespective of transmission rates [54, 56]. However, susceptibility to clinical malaria appears to be higher in both second and third trimesters of pregnancy [58]. The increased risk for both acquiring *P. falciparum* infection and developing more severe disease persists for at least 60 to 70 days postpartum [58]. Pregnant women with human immunodeficiency virus (HIV) infection have a higher prevalence and intensity of *P. falciparum* infection compared with non HIV-infected women [9, 10, 59-62]. Women with HIV infection are also more likely to have symptomatic infection, and have an increased risk of malaria-associated adverse outcomes compared with women without HIV infection, irrespective of parity.

1.8.2. Pathophysiology

Sequestration of parasites

P. falciparum infection during pregnancy is characterized by the sequestration and multiplication of a distinct population of parasites in the intervillous spaces in the placenta [63,64]. The sequestration of infected erythrocytes (IEs) is mediated by adhesive interactions between parasite antigens on the surface of IEs and host molecules present on the microvascular endothelium [65-67]. IEs isolated from placentas have a unique adhesion property that is different from parasites collected from non-pregnant individuals. They do not bind receptors such as CD36 and intracellular adhesion molecule (ICAM)-1, which are common adhesion receptors among isolates from non-pregnant hosts [33, 68]. Instead, they bind chondroitin sulfate A (CSA) [69-72], hyaluronic acid (HA) [66,70,73,74] and non-immune immunoglobulin (Ig) [75]. This uniqueness enables the parasites to evade immune responses acquired prior to the first pregnancy. The adhesion to CSA has been observed in IEs collected from infected placentae from several geographically distant sites, including Kenya, Thailand, Malawi and Cameroon [69-71, 76] suggesting that pregnant women are infected with parasites that express distinct adhesion receptors. Other processes could also be involved in the sequestration of IEs in the placenta, because histological examination of infected placenta tissue has shown that not all erythrocytes are adherent to the syncytiotrophoblast layer of the placenta [63, 64, 77].

Cellular immune response

The presence of *P. falciparum* malaria parasites in the placenta causes an infiltration of inflammatory cells in the intervillous space [77-80]. The infiltrate consists

predominantly of monocytes, lymphocytes and macrophages and less commonly polymophonuclear cells. In some cases, the mononuclear cell infiltration in the intervillous space is intense, and is called massive chronic intervillousitis [80, 81]. The placental mononuclear cells help to limit the infection in the placenta through phagocytosis of malaria parasites and the production of helper T cells (Th1) cytokines. The mechanism by which mononuclear cells are recruited into the placenta is not clearly understood. However, one study found an increased expression of messenger ribonucleic acid (mRNA) for β chemokines in malaria-infected placentae, which were positively correlated with monocyte densities in the placental intervillous spaces [82]. Another study found that malaria-infected placentae produced high levels of migration inhibitory factor (MIF), a cytokine that activates macrophages and prevents the out migration of inflammatory cells [83]. Therefore, the presence of chemokines and MIF may play a central role in the accumulation of inflammatory cells in malaria-infected placentae.

Cytokines and chemokines

Cytokines are also involved in the genesis of the inflammatory response to parasites sequestered in the placenta. The immune system is altered during pregnancy by suppression of the Th1 pathway, to accept the fetal allograft while maintaining host defenses against foreign antigens [84]. *P. falciparum* infection induces a shift towards the production of Th1-type cytokines. Studies have found increased levels of TNF-α, interferon-γ (IFN-γ), interleukin factor-8 (IL-8) and interleukin factor-2 (IL-2) in malaria-infected compared with non-infected placentae [82,85-88]. These cytokines are produced by

the infiltrating macrophages and placental tissues and they attract and activate leukocytes in malaria-infected placentae [86,87].

Humoral response

Humoral immunity also plays a critical role in the protection against *P. falciparum* infection during pregnancy. Infection with novel *P. falciparum* antigens in the first pregnancy renders the repertoire of antibodies present before pregnancy ineffective against the placental parasites [76]. Following exposure to *P. falciparum* during subsequent pregnancies, women residing in areas of stable malaria transmission develop antibodies to parasite surface antigens expressed on IEs that inhibit parasite adhesion to CSA and sequestration in the placenta [71,76,89-91]. Sera from women in Asia and Africa cross-reacts to parasites from either continent, implying that the parasite-expressed proteins mediating adhesion to CSA have conserved epitopes targeted by anti-adhesion antibodies [76]. Studies have shown that antibody responses to CSA-binding IEs are absent, delayed or present in low levels in primigravidae, but are present and in higher quantities in multigravidae [74,76,91]. These findings explain the parity specific epidemiology of malaria, the high susceptibility during first pregnancies, diminishing in subsequent pregnancies as women acquire specific antibodies against placental parasites. The presence of these antibodies has been associated with reduced prevalence [76] and density [92] of placental parasitemia among pregnant women. In Kenya, secundigravidae with serum anti-adhesion antibodies delivered babies that were on average 398g heavier, and 2 weeks more mature than babies born to secundigravidae without these antibodies [93].

Histological changes in the placenta

Placental pathology occurs secondary to parasite sequestration and deposition of hemozoin (a heme-polymer formed as a by-product of hemoglobin breakdown) in the placenta, which causes the infiltration of mononuclear cells, and induction of pro-inflammatory cytokines [77,78,80]. Pathological changes are most marked in the intervillous space and to a lesser extent in the villous tissue. During early stages of infection, only parasites are detected in the intervillous space, mostly within maternal erythrocytes but occasionally in the cytoplasm of macrophages, or free in the intervillous space. Malaria parasites have also been detected in cord blood, but not in fetal erythrocytes or fetal structures [63,77]. Chronic infection is diagnosed by the presence of hemozoin deposited within intervillous mononuclear cells, fibrin strands and trophoblasts [53,63,94]. Typical histological changes that have been identified involving the villous surfaces include excessive syncytial knotting, apoptosis and disintegration of the villi, cytotrophoblast basement membrane thickening and occasional deposition of hemozoin in the cytotrophoblast or stroma [63,77-79,95-97]. These changes may alter the placental materno-fetal exchange of oxygen and nutrients in the placenta thereby contributing to the malaria-associated effect on fetal growth[98].

1.8.3. Diagnosis of malaria in pregnant women

Malaria in pregnancy is diagnosed by microscopic examination of thick films prepared using peripheral blood samples during pregnancy, or placental blood samples at delivery, and histological examination of the placenta. Although maternal peripheral parasitemia has been used routinely to detect malaria during pregnancy, it has low

sensitivity because peripheral parasitemia may remain below levels of microscopic detection while parasites are harbored by the placenta [77,99]. Different methods have been used to prepare thick films from placental blood, including scraping of the incision margin and washing of the placental tissue, and have been found to have similar sensitivities in detecting placental parasitemia [99]. Histological examination of the placenta is the most sensitive indicator of maternal malaria infection [99,100], and enables classification of the infection as either active, past or chronic, and gives more information on placental lesions [77,99].

1.8.4. Complications of *P. falciparum* infection during pregnancy

The symptoms and complications of *P. falciparum* infection during pregnancy depend on the intensity and stability of transmission, and preexisting maternal acquired immunity [101,102]. Women living in areas where malaria transmission is low and unstable have little or no immunity. They are at risk of severe clinical disease, anemia, death, and adverse pregnancy outcomes including spontaneous abortions, still births, neonatal deaths and LBW [102,103]. In moderate to high transmission areas, women have acquired partial immunity by the time they reach reproductive age [52,53]. Therefore, they are not at risk for severe disease. The principal adverse effects of *P. falciparum* infection in these areas are maternal anemia and LBW.

Maternal anemia

Studies have consistently shown that *P. falciparum* infection during pregnancy increases the risk of maternal anemia [4,5,7]. Each year as many as 400,000 pregnant women

in sub-Saharan Africa develop a severe anemia event as a result of *P. falciparum* infection [3]. Severe pregnancy-related anemia may result in maternal heart failure, and decreased ability to endure blood loss at delivery, with subsequent risk for death. The etiology of anemia is multi-factorial and is dependent on the age, immune status and the duration of infection [104]. It is mediated through the hemolysis and phagocytosis of IEs by macrophages and natural killer cells, and poor bone marrow response [61]. *P. falciparum* infection also induces biochemical modifications in the membranes of both infected and uninfected erythrocytes which mimic the erythrocytes' normal aging process [105]. Additionally, pro-inflammatory cytokines activate macrophages which release oxygen and nitrogen radicals that cause oxidative damage and membrane disruption to infected and uninfected erythrocytes [106]. Consequently, there is enhanced removal of both infected and uninfected erythrocytes from the circulation by the host's reticulo-endothelial system leading to anemia. The contribution of each of these factors in the development of anemia still remains unclear. Other factors that contribute to the development of anemia in women living in malaria-endemic areas include nutritional deficiencies, and infection with other parasites such as hookworm and HIV [107,108].

Low birth weight

Infection with *P. falciparum* during pregnancy also increases the risk for LBW [109,110], which is the greatest risk factor for neonatal and early infant mortality [111,112]. Malaria causes LBW through intrauterine growth retardation (IUGR) and/or premature delivery [103,113-116]. Premature birth results commonly from symptomatic and severe malaria and therefore the major cause of LBW in low-transmission areas where acquired

immunity is low, whereas IUGR is the major cause of LBW in high transmission areas [10,56,117]. *P. falciparum* infection contributes an estimated 8 to 36% of premature deliveries, 13 to 70% of IUGR, and 3 to 8% of infant deaths in malaria endemic areas [2,109,110]. The actual mechanisms by which *P. falciparum* infection causes LBW still remain uncertain. Some factors that have been cited include maternal anemia [118], inadequate trophoblast invasion in early placentation [119], placental insufficiency due to mechanical compromise of the placental circulation arising from the thickening of the trophoblastic membrane [10,77], elevated pro-inflammatory cytokine levels [85,87], poor oxygen and glucose transfer from the parasitized erythrocytes and use of nutrients by macrophages sequestered in the placenta [87].

Congenital malaria

P. falciparum–infected erythrocytes have been identified in umbilical cord blood. The prevalence of cord parasitemia varies widely according to geographical location. A survey of seven sites in Africa that examined cord blood microscopically found prevalence rates ranging from 0 to 23% [120]. Studies that used more sensitive PCR assays identified *P. falciparum* parasites in 10%–32% of cord-blood samples, obtained from women in African [121,122], suggesting that the presence of malaria parasites in cord blood occurs with greater frequency than has been previously reported. Parasite-specific IgM and IgE have been detected in 11%–25% of cord-blood samples obtained from individuals in malaria-endemic areas. This suggests that some of the infections detected in newborns at delivery are acquired in the antenatal period because IgM does not cross the placenta during gestation [123,124]. Infection also occurs during delivery, when mixing of maternal

and fetal blood takes place [125]. The presence of parasitemia in babies born to immune women is often self-limited, and does not lead to congenital malaria due to the presence of placentally transferred maternal antibodies which provide protection, and activation of the fetal immune system by maternal malaria during gestation [126]. However, congenital malaria does occur usually in babies born to non-immune women which can result in severe malaria and death [127].

1.8.5. The effect of frequency and timing of *P. falciparum* infection during pregnancy on low birth weight and maternal anemia

Studies have consistently shown that *P. falciparum* infection during pregnancy causes LBW [109,110], and maternal anemia [4,5,7]. However, it is not clearly known whether the frequency and the stage of pregnancy at infection have a differential influence on these adverse outcomes. There are no published epidemiologic studies on the effect of frequency of *P. falciparum* infection during pregnancy on LBW and maternal anemia. Studies that have investigated the effect of timing of *P. falciparum* infection on placental pathology have reported that significant histopathologic placental malaria changes were more frequent in women with evidence of peripheral blood infection close to delivery compared with infection in early in the antenatal period[128,129]. Studies that have investigated the effect of time of *P. falciparum* infection on LBW have reported conflicting results. One study found that chronic infection and past malaria infections, but not acute infection were associated with an increased risk of LBW [7]. In contrast, another study found that infants born to women who had active infection (parasites, monocyte pigment, with or without pigment in fibrin) had a higher risk of LBW than women who

had past infection [99]. Studies that have investigated the association between timing of infection of *P. falciparum* and the development of maternal anemia have found no association between severe anemia and past placental malaria, but an association with active placental infection [7,99]. These studies only diagnosed malaria at delivery, using placental histology to define the timing of infection during pregnancy, which could have resulted in misclassification of exposure. Therefore, there is need to conduct studies using more sensitive methods to identify the timing of infection during pregnancy and its effect on LBW and maternal anemia.

1.8.6. Prevention of malaria in pregnant women

Low transmission areas

Pregnant women in these areas have little or no background immunity. Therefore the majority of infections are symptomatic and some may lead to severe and lethal infections. Prompt diagnosis and treatment of pregnant women during routine screening at antenatal visit is the mainstay of management [56]. This reduces the risk of severe maternal disease and complications in the fetus.

High transmission areas

Most infections with *P. falciparum* in pregnant women in high transmission areas are asymptomatic, and are usually not diagnosed [130].The following prevention measures are, therefore, recommended: IPT, use of insecticide-treated nets, and effective case management [131].

Intermittent preventive treatment

The first recommendation for the prevention of malaria in pregnant women in high transmission areas is IPT which refers to the periodic administration of treatment doses of anti-malarial drugs during pregnancy irrespective of the presence of parasites or clinical illness. The aim is to clear the placenta of parasites during the period of maximum fetal growth (24-36 weeks), and to clear peripheral parasitemia, allowing hematological recovery in the pregnant women. The WHO currently recommends that pregnant women living in stable malaria transmission areas should receive at least two doses of SP, beginning after quickening (first noted fetal movement) as part of the routine antenatal care service [8]. Several studies have reported the advantages of providing IPT to pregnant women including reduction in the prevalence of peripheral and placental parasitemia, severe anemia in pregnant women [132-134], and increase in birth weight [9-11,17,19]. In a study conducted in Malawi, the prevalence of placental malaria at delivery in women who had received SP was 10% compared with 47% of women who had not received SP [19] . In another study conducted in Malawi, women who were prescribed at least two doses of SP delivered babies with a mean birth weight 195g higher than women who had not received SP [11]. In Kenya, IPT reduced the prevalence of peripheral parasitemia by 85%, and maternal anemia by 39% [132]. Maximum benefit is derived from two to three doses of SP. However, even a single dose is beneficial. HIV impairs immunity to malaria therefore HIV-infected women require at least three doses of SP [9,135,136]. As resistance to SP is increasing in many malaria endemic areas, concerns are being raised about its efficacy in preventing and treating malaria in pregnant women [137].

Insecticide treated bed-nets (ITNs)

In addition to anti-malarial drugs, the WHO recommends the use of ITNs throughout pregnancy. ITNs act as a barrier reducing human-vector contact by physically excluding vector mosquitoes. The insecticide also repels, inhibits or kills any mosquitoes attracted to feed. Studies have reported conflicting results on the effect of ITNs on *P. falciparum* related adverse outcomes in pregnant women. Some studies found that ITNs reduced the risk of morbidity in pregnant women and LBW in areas of both stable and unstable malaria transmission [138-140]. Other studies found that ITNs had very little impact on maternal anemia, peripheral or placental parasitemia, or birth weight in areas with high transmission [141,142]. A recent meta-analysis of trials that investigated the effect of ITNs on malaria in pregnancy found that use of ITNs was associated with significant reduction in peripheral and placental parasitemia, and LBW, and a non-significant reduction in maternal anemia [143]. The uptake and use of ITNs has been low in many malaria endemic communities [131]. The major reason for the low uptake rates in Africa has been high costs of buying and retreating the ITNs with insecticide [144-146]. In an effort to make ITNs more affordable, several African countries have now taken the initiative to reduce tariffs on ITNs [131].

Effective case management

The third recommendation for preventing malaria during pregnancy in high-transmission areas is prompt diagnosis and treatment of pregnant women with parasitemia even when women are not symptomatic [131]. Additionally, all pregnant women in high transmission areas with severe anemia are presumptively treated with anti-malarials, even

in the absence of peripheral parasitemia and other symptoms of malaria. However, the options for treatment of malaria in pregnant women are few due to safety concerns for the fetus. The spread of resistance to the easily accessible, safe and affordable drugs such as CQ and SP is making the control of malaria in pregnant women more difficult. Currently there is a huge drive to start using artemisinin-combination therapies for treatment of malaria, and to delay the development of resistance [147]. However, to date only a few studies that have investigated the safety and efficacy of these combination therapies in pregnant women [148-151].

1.9. Non-falciparum malaria in pregnancy

The role of *P. vivax* and its impact on the health of pregnant women and the fetus is not as well documented as *P. falciparum* infection. *P. vivax* malaria occurs mainly in low transmission areas (e.g. India, South America, and parts of South-East Asia) therefore women living in these areas have little or no immunity and consequently are at risk for symptomatic malaria infection and its adverse effects. The prevalence of *P. vivax* infection and parasite density increases during pregnancy due to the reticulocytosis of pregnancy (*P. vivax* exclusively invades reticulocytes) [33]. P. *vivax* is less likely to relapse in pregnant women than in non-pregnant women, and is more likely to cause disease in multigravid than primigravid women [152]. Sequestration of *P. vivax* in the placenta has not been observed. However, hemozoin has been detected in the placenta after infection [128]. *P. vivax* infection during pregnancy increases the risk for LBW and maternal anemia, but to a lesser extent than *P. falciparum* [152,153].There is little information available on the effect of *P. ovale* and *P.malariae* infection during pregnancy.

1.10. Safety of anti-malarial drugs and pregnancy

CQ was the drug of choice for treatment and prevention of malaria in pregnant women in malaria-endemic areas until a decade ago when it was abandoned due to high levels of resistance [12,154]. SP replaced CQ, and is currently the drug of choice for treatment and prevention of malaria in pregnant women in Africa. However, resistance to SP is also increasing [12] and there might be need to change to other anti-malarial drugs. Currently the options for malaria treatment and prevention in pregnant women are limited by safety concerns for the fetus. This is coupled with paucity of data on the safety of anti-malarial drugs because pregnant women have been systematically excluded from most malaria treatment trials for fear of toxicity to the fetus [155,156] (Table 1.1).

1.10.1. Drugs considered safe during pregnancy

Chloroquine

CQ is a 4-aminoquinoline that has been used extensively for the treatment and prevention of malaria in pregnant women. Studies in pregnant women have shown that CQ, given in normal therapeutic doses, is safe and well tolerated even in the first trimester of pregnancy [157-160]. In a study conducted in Malawi, among more than 2,500 women who had received CQ as weekly chemoprophylaxis throughout pregnancy, there were no reports of severe adverse outcomes such as increase in rates of abortions, stillbirths, or congenital abnormalities, only non-severe side effects such as itching, dizziness and gastrointestinal complaints were reported [115]. Another study found no differences in the prevalence of birth defects in children born to 169 non-immune women who took CQ chemoprophylaxis throughout pregnancy compared with 454 births to women who had

not received CQ [160]. Widespread resistance has now rendered CQ virtually useless against *P. falciparum* infections in most parts of the world. However, CQ is still useful for the treatment and prevention of malaria in pregnancy in places where resistance is still low such as West Africa, and for the treatment of *P. vivax* malaria.

Sulfadoxine-pyrimethamine

SP, a fixed dose combination of sulfadoxine (1500 mg) and pyrimethamine (75 mg) is currently the drug of choice for treatment and prevention of malaria in pregnant women. It is considered safe for administration during the second and third trimester of pregnancy with no reports of toxicity in pregnant women, and no reports of increased risk of spontaneous abortions, still births or congenital defects,[9,17,19,132,133]. Although there is a theoretical risk of jaundice among premature babies born to mothers given sulfa drugs late in the third trimester [161], this has not been reported in studies where SP was administered for IPT [9,17,19,132,133]. Published data on the use of SP during the first trimester of pregnancy are scarce [162]. Two large case-control studies found evidence of teratogenic effects including cardiovascular and urinary tract malformations, and cleft defects when SP was given during the first trimester [163,164]. When given weekly as prophylaxis to non-pregnant individuals, SP has been associated with rare and severe cutaneous reactions such as toxic epidermal necrolysis and Stevens–Johnson syndrome [165]. However, there is no evidence that this risk is any greater in pregnant women [166].

Quinine

Quinine is an alkaloid derived from the bark of the *Cinchona* tree. It is still the drug of choice for treatment of severe falciparum malaria in most countries [167]. It is also used as first-line treatment in areas with multi-drug-resistant malaria in South-East Asia, and as second-line treatment for patients who fail to respond to the standard first-line therapy. Quinine is considered safe during all trimesters of pregnancy when taken at normal therapeutic doses [155,157]. There was no evidence that quinine causes premature births or congenital abnormalities in Thailand in 178 women who were treated with quinine during pregnancy [168]. In the same community, a retrospective study that recruited over 2,000 pregnant found that the risk of abortions in women who were treated with quinine during pregnancy was similar to the women who were not exposed to quinine [169]. Quinine can cause adverse effects in pregnant women such as hypoglycemia [170,171], tinnitus and dizziness [168] and adherence is poor due to its bitter taste [150]. When taken at very high doses during pregnancy, quinine has been used as an abortifacient [172] and studies have reported fetal optic and auditory nerve damage [173,174].

Dapsone combinations

Dapsone has been used extensively in pregnant women with leprosy without reports of severe adverse effects [175]. Dapsone has also been used in combination with pyrimethamine (maloprim) [176-178] or chlorproguanil (Lapdap) [179] for chemoprophylaxis or treatment of malaria in pregnant women with no reports of adverse events or adverse birth outcomes in the pregnant women treated with either maloprim or Lapdap. Data is lacking on the safety of these drug combinations in the first trimester of pregnancy.

Agranulocytosis and severe hypersensitivity syndrome have been reported in non-pregnant women receiving dapsone chemoprophylaxis using twice weekly dosing which is no longer recommended [180].

1.10.2. Drugs with insufficient or questionable data on safety during pregnancy

Mefloquine

Mefloquine has been used both for treatment and prevention of malaria in pregnant women. However, the safety of mefloquine in pregnant women is still questionable because of conflicting results from different studies. Some studies have shown that exposure to mefloquine during pregnancy does not increase the risk of adverse pregnancy outcomes. In a study conducted in Malawi, the rates of stillbirths and abortions were similar between 451 pregnant women who had received 250mg of mefloquine weekly throughout pregnancy and 1,077 women who had received CQ prophylaxis [10]. Only minor side effects such as dizziness and gastrointestinal complaints were reported by women who took mefloquine. In Thailand, 339 pregnant who were given either mefloquine prophylaxis or placebo against *P. falciparum* and *P. vivax infections* only reported minor side effects including transient dizziness [181]. By contrast, other studies have found that exposure to mefloquine during pregnancy increases the risk of stillbirths. In a retrospective study of 3,587 pregnancies, of which 208 were exposed to mefloquine treatment in all trimesters of pregnancy, mefloquine was associated with an increased risk of stillbirth [169]. Stillbirths were more frequent among those treated with mefloquine (4.5%) than those treated with quinine (1.6%), but the rates of other severe outcomes (abortion, congenital malformations, and neurological deficits) were similar between the

treatment groups. Among 53 female US military personnel in Somalia who inadvertently used mefloquine early in pregnancy, there were 17 elective abortions, 12 spontaneous abortions, 1 molar pregnancy, and 23 live births suggesting toxicity in the first trimester of pregnancy [182]. As a result of these conflicting reports, the current recommendation is not to use mefloquine in pregnancy unless there is a benefit for the pregnant woman [183].

Amodiaquine

Although the WHO currently recommends that amodiaquine can be considered safe for use against malaria in pregnant women [184], there is insufficient data available to be certain about its safety during pregnancy. There is no data available on the effect of exposure to amodiaquine in the first trimester of pregnancy [183]. A review only identified six studies between 1948 and 1992 with a total of 300 pregnant women who had taken amodiaquine for prophylaxis or treatment in the second or third trimester of pregnancy [185]. Only one these studies assessed the toxic effects of amodiaquine, but the rest of the studies did not report pregnancy outcomes. There have been reports of severe side effects such as agranulocytosis, granulocytopenia and hepatitis in studies when amodiaquine was used for chemoprophylaxis in non-pregnant adults [186,187]. However, these severe adverse events have not been reported in studies when amodiaquine was used as treatment for malaria in non-pregnant adults [188].

Malarone

Malarone is a fixed combination of atovaquone and proguanil. There is limited data available on its use for treatment or prevention of malaria in pregnant women.

Clinical trials in non-pregnant patients, including areas of multi-drug resistance, have shown that malarone is well tolerated, and is highly efficacious in the prevention and treatment of uncomplicated *P. falciparum* malaria [189-193]. Only minor adverse events including nausea, vomiting, headache and abdominal pain have been reported in these studies. At present, a few pharmacokinetic trials recruiting very small numbers of pregnant women have been conducted and have indicated that malarone is safe, and well tolerated when administered in the second or third trimester of pregnancy [194,195].

Azithromycin

Azithromycin is a broad-spectrum macrolide antibiotic that has been used extensively in pregnant women to treat sexually transmitted infections (STIs) and other infections, with no reports of severe adverse events [196-200]. Azithromycin has just been recently found to have *in vivo* and *in vitro* activity against *P. falciparum* and *P. vivax* when administered alone, or in combination with other anti-malarial drugs [201-206]. There are at present no published studies that have evaluated the safety and efficacy of azithromycin in the treatment or prevention of malaria during pregnancy. Azithromycin could prove to be an effective agent in the prevention of malaria during pregnancy, as it has generally been well tolerated by pregnant women treated for other infections.

Artemisinin derivatives

Artemisinin derivatives such as artesunate and artemether are extracts of the Qinghao plant (*Artemisia annua*). Artemisinin derivatives are currently the most effective anti-malarial drugs known. They have a rapid onset therapeutic effect. Compared to other

drugs, artemisinin derivatives reduce the gametocyte carriage, and thereby lower the risk of infectiousness [207]. They have commonly been used in Asia and South America, and are increasingly being used in Africa to treat malaria in children and non-pregnant adults. There is limited human data on the use of artemisinin compounds in pregnancy, because most of the studies conducted on the safety of artemisinin derivatives have been in non-pregnant individuals. Currently, there is controversy over the safety of artemisins in pregnancy. Studies from animal data have shown that artemisinin derivatives cause developmental toxicity and lethal effects in rodent embryos if given in the first trimester of pregnancy [208,209]. There have been no reports of toxicity in pregnant women exposed to artemisinin derivatives during the first [148,210,211], second or third trimester of pregnancy [148-150,210,212-214]. However, the total number of women recruited in these studies was small and therefore the need for more studies to confirm the safety of the artemisinin derivatives when used during pregnancy.

Combination therapy

Currently, there is an increased drive to use the combination of artemisinin derivatives and other available anti-malarial drugs for the treatment and prevention of malaria in an attempt to delay the development and spread of drug resistance [147]. The hypothesis is that the probability of parasites developing resistance to two chemotherapeutic agents with independent mechanisms of action is extremely low, in the order of once in 10^{12} treatments [215]. Artemisinin derivatives rapidly decrease parasite density and gametocyte levels in treated patients, and, therefore, greatly reduce the level of infectiousness, and transmission of resistant strains [207]. Trials conducted in children and

non-pregnant adults have found that combination regimens have higher efficacy than monotherapy with drugs that have already developed resistance [216]. Among the artemisinin-based combinations, artesunate-amodiaquine and artemether-lumefantrine are the most developed, most marketed and currently most advocated for treating African children with uncomplicated *P. falciparum* malaria. Other studies have also examined the safety and efficacy of drug combinations not containing artemisinin derivatives in children with uncomplicated *P. falciparum* malaria. Combinations studied include SP-mefloquine, SP-CQ, chlorproguanil-dapsone (Lap Dap), malarone and lumefantrine-artemether (co-artemether), with varying efficacy levels [217]. However, very few trials have assessed the safety and efficacy of combination therapies in treating and preventing malaria in pregnant women [148-151]. So far, these studies have shown that combination therapies are efficacious, well tolerated and safe. However, because of the small number of women in these studies, there is still need for more trials to confirm the safety and efficacy of combination therapies in treating and preventing malaria in pregnant women before they can be recommended for general use in pregnant women.

1.10.3. Drugs contraindicated during pregnancy

Drugs that are not recommended for use during pregnancy include primaquine, tetracycline, doxycycline, and halofantrine. Tetracycline and doxycycline cause discoloration of teeth, lenses and cornea and disturbances in skeletal growth [166]. Primaquine is contraindicated in pregnancy because of the risk of hemolysis in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency [162]. Treatment with halofantrine has been associated with the lengthening of the QT interval and fatal

arrythmias in some individuals, and therefore needs electrocardiogram monitoring [166]. This is impractical in most malaria-endemic areas due to the lack of resources, and therefore halofantrine is not recommended during pregnancy.

1.11. Anti-malarial drug resistance

1.11.1. Epidemiology of *Plasmodium falciparum* drug resistance

P. falciparum has developed resistance to nearly all anti-malarials in current use except the artemisinin derivatives [218]. The prevalence of resistance to any single anti-malarial drug varies greatly according to geographical location [12]. Comparison of the current prevalence of resistance across different regions is complicated by the variability in the period of follow-up, definition of outcomes and type of subjects recruited into efficacy trials. Drug resistance poses a great challenge in the control of malaria, because there are few cheap alternative anti-malarial drugs available. It has led to rising malaria-related morbidity and mortality, especially in East Africa [219,220]. Resistance may be generated independently or spread across areas. There is molecular evidence of a single origin of resistant alleles to pyrimethamine in South-East Asia, which were imported into East Africa and are now spreading across the African continent [221,222]. Conversely, CQ resistance evolved independently in different areas, through multiple evolutionary pathways [223,224].

Chloroquine

P. falciparum resistance to CQ appeared in the late 1950s in Thailand and Cambodia, and in the 1970s in New Guinea and eastern sub-Saharan Africa. Clinically

evident resistance to CQ in *P. falciparum* is nearly universal and now predominates South-East Asia, South America and Africa [12,154], (Figure 1.3). A review of *in vivo* studies conducted between 1996 and 2002 with follow-up periods ranging from 7 to 28 days, found that parasitological failure rates varied from 10 to 98% in Africa, 38 to 91% in South-East Asia, 23 to 38% in the Pacific region and 46 to 77% in the Indian subcontinent [154]. Many countries have abandoned the use CQ as first line therapy. The only exceptions are North-West of Panama in Central America, Haiti and the Dominican Republic, and the Middle East where the magnitude of CQ resistance is still the subject of investigation [154].

Sulfadoxine-pyrimethamine

Resistance to SP, the main alternative to CQ emerged rapidly after its deployment in Asia. Currently, high-level resistance is found in a large part of South-East Asia, Southern China and the Amazon basin [12], (Figure 1.3). The parasitological failure rates reported by *in vivo* studies between 1996 and 2002, with follow-up periods ranging from 7 to 28 days, varied from 5 to 67% in Asia, and from 6 to 20% in South America [154].In Africa, resistance to SP also developed quickly soon after it was introduced in countries where CQ treatment was failing [225]. Currently resistance has been reported in many parts of Africa. The clinical failure rates with at least 14 days of follow-up have ranged from 0 to 76% [225].

Quinine

Clinical resistance to quinine monotherapy occurs in South-East Asia and Western Oceania [149,225] and is less frequent in South America [226] and Africa [227]. For example, the

clinical failure rates after treatment of acute uncomplicated *P. falciparum* malaria over 7 days with a follow-up of 14 days were 0% in Gabon, 4.8-5.5% in Equatorial Guinea and 6.3-9.3% in Sudan where follow-up was for 28 days [228-230].

Mefloquine

Mefloquine resistance was first observed on the Thai-Cambodian and Thai-Burmese borders in the late 1980s, and monotherapy is no longer effective there [231,232]. Resistance is believed to have spread from these areas to India and Bangladesh [233]. A few cases of resistance have been reported in the Amazon basin and South America [225,234], and *in vitro* studies have reported resistance in Africa [235,236]. Overall, clinical mefloquine resistance outside South-East Asia is rare.

Amodiaquine

Despite cross-resistance between amodiaquine and CQ, amodiaquine is generally more effective than CQ against CQ-resistant strains of *P. falciparum* [237]. Moderate-to-high levels of amodiaquine resistance have been reported from Papua New Guinea, East Africa and the Amazon Basin [225]. However, this drug continues to be efficacious as a single drug in most of West and Central Africa and on the northern Pacific Coast of South America where, in some countries, it is used in combination with sulfadoxine-pyrimethamine [225].

Artemisinin derivatives

Resistance to artemisinin derivatives has been induced in murine models [238-240].Confirmed resistance to the artemisinin derivatives has not been reported in humans.

However, recrudescence among patients receiving a short course (less than 5 days) of therapy has been observed [241]. Recrudescence is thought to be due to pharmacological factors such as taking inadequate doses and host metabolism. There is no convincing evidence yet that recrudescence in humans is due to parasite resistance.

Multi-drug resistance

Multi-drug resistance which is defined as resistance to three or more anti-malarial compounds from different chemical classes occurs in South-East Asia, particularly along the borders of Thailand with Burma and Cambodia, and in the Amazon basin [12]. In these areas, *P. falciparum* parasites are resistant to CQ, mefloquine, SP and to a lesser extent quinine. Countries in East Africa are at risk of multi-drug resistance because resistance to CQ and SP is already widespread.

1.11.2. Epidemiology of *Plasmodium vivax* drug resistance

Overall, *P. vivax* parasites are sensitive to most anti-malarial drugs. There have been reports of CQ resistance to *P. vivax* from many geographic locations worldwide, but they have been not been a very large scale. Resistance is prevalent and increasing in some areas, notably Oceania, India, Indonesia, Brazil, Papua New Guinea and Myanmar [32]. Monitoring of drug resistance to *P. vivax* is confounded by the relapse of *vivax* malaria. In some cases, it is not possible to distinguish clearly a recrudescence from a relapse when using available genotype methods, because strains causing relapse and recrudescence might be derived from the same initial infection [242,243]. Consequently, CQ-resistant *P. vivax* parasites might be more common than has been reported.

1.12. Molecular markers for anti-malarial drug resistance

Resistance to anti-malarial drugs occurs as a result of spontaneous mutations, or gene amplification in the parasite genome [244]. These mutations affect the structure and activity, at the molecular level, of the drug target in the malaria parasite or access of the drug to its target, thereby interfering with the parasite's susceptibility to a drug. Molecular markers for resistance have been identified for SP [245], CQ [246], mefloquine [247], and atovaquone [248]. Markers for most of the other anti-malarial drugs have not been identified because mechanisms of resistance are not yet understood at the molecular level. These molecular markers are beginning to be applied as tools for surveillance of resistance but are, at present, still primarily used as research tools.

Chloroquine

CQ acts by increasing its concentration in the parasite's digestive vacuole of the intra-erythrocytic trophozoite. In this organelle, CQ inhibits hemoglobin degradation and forms complexes with hematin. A mutation at codon 76 of the *P. falciparum* CQ-resistance (*pfcrt*) gene, which encodes for a trans-membrane protein located in the digestive vacuole, has been associated with resistance to CQ [224,246,249,250]. This mutation alters the accumulation of CQ in the digestive vacuole. Clinical studies have confirmed the presence of this mutation in individuals who experienced treatment failure, after treatment with CQ [246,251,252]. However, this mutation has also been found in CQ-sensitive isolates, indicating that other mutations may also be involved in the development of resistance [251,253]. Polymorphisms in *P. falciparum* multi-drug-resistance gene 1 (*pfmdr1*) modulate CQ resistance in mutant *pfcrt*-harboring parasites *in vitro* [254]. However, their

role in *in vivo* resistance has not been established [246]. Currently, mechanisms of *P. vivax* resistance to CQ are not well understood, but appear to differ from those for *P. falciparum* [255]. The mutation of the *pfcrt* gene that is essential for CQ resistance in *P. falciparum* is not present in *P. vivax* CQ resistant strains.

Sulfadoxine-pyrimethamine

Pyrimethamine interrupts deoxyribonucleic acid (DNA) replication in the parasite by competitively inhibiting the enzyme dihydrofolate reductase *(dhfr)* in the folate synthesis pathway, which is essential for the synthesis of pyrimidines [256]. Point mutations at codons Asn-108, Arg-50, Ile-51, Arg-59 and Leu-164 in the *dhfr* gene alter the active binding site cavity of the drug to the enzyme [256]. The point mutation at Asn-108 in the *dhfr* gene appears to be the key mutation that confers *P. falciparum* resistance to pyrimethamine [257,258]. Additional point mutations in codons Arg-50, Ile-51, Arg-59 and Leu-164 are known to progressively increase the degree of resistance [245,259]. These mutations have been strongly associated both with *in vitro* and *in vivo* resistance of *P. falciparum* to SP [22,260,261]. Long-term *in vitro* observations have suggested that in addition to these mutations, gene amplification of the *dhfr* gene is also an important mechanism of pyrimethamine resistance [262].Two mutations, Arg-58 and Ser-117, have been identified as key for *P. vivax* resistance to pyrimethamine [263].

Sulfadoxine inhibits the enzyme dihydropteroate synthase (*dhps*), which is essential in the early pathway of folate synthesis in the parasite. Mutations in the *dhps* that have been associated with *P. falciparum in vivo* and *in vitro* resistance include Ala-436,

Phe-436, Gly-437, Lys-540, Gly-581, Thr-613 and Ser-613 [22,261,264-266]. It is unclear which one is the key mutation, as a mutation at codon 108 is in *dhfr*.

To date, more than 25 different combinations of *dhfr* and *dhps* mutant alleles in *P. falciparum* have been observed in field isolates [266]. One study showed that patients infected with parasites carrying the *dhps* Gly-437/Glu-540 double mutant and the *dhfr* triple Asn-108/Ile-51/Arg-59 mutant had a high relative risk of treatment failure compared to those infected with parasites carrying the *dhfr* triple mutant alone [261]. This quintuple mutant (3 *dhfr* and 2 *dhps* mutations) has been suggested as a relevant molecular marker for failure of SP treatment of uncomplicated *P. falciparum* cases [245]. In population monitoring, the presence of mutations at codon 59 of the *dhfr* gene and codon 540 of the *dhps* gene have been strongly predictive of the quintuple mutation [267,268]. Treatment failure can, however, occur in the presence of fewer than five mutations on the *dhfr* and *dhps* genes [259,265].

Mefloquine and artemisinin derivatives

Mutations and gene amplification of the *pfmdr1* have been implicated in the resistance to several drugs. Studies in Thailand found that increased gene copy number of the *pmdfr1* gene was associated with *in vivo* and *in vitro* resistance to mefloquine [247,269]. Amplification of *pmdfr1* gene in *P. falciparum* and *P. yoelli* appears to be common genetic mechanism for *in vitro* resistance in artemisinin derivatives [238-240]. These *in vitro* mutations have not been associated with clinical failure to artemisinin derivatives [270,271].

Atovaquone

Atovaquone has been shown to inhibit the cytochrome bc_1 (CYT bc_1) complex of the electron transport chain of malaria parasites [272]. Resistance to atovaquone results from point mutations in the cytochrome b gene *(cytB)* codon [248]. This mutation induces an approximately 10,000 fold increase in the atovaquone IC_{50} *in vitro* tests.

1.13. Factors contributing to the development of drug resistance

The development and spread of drug resistance is influenced by an interaction between drug, host, parasite, vector and environmental factors.

1.13.1. Parasite characteristics

Vector capacity, in-vector recombination of genes and fitness of the mutated parasites are all important factors that influence the speed at which resistance emerges and spreads [154]. In Sri Lanka, researchers found that patients with CQ-resistant malaria infections were more likely to have gametocytemia than those with sensitive infections [273]. Gametocytes carrying resistant genes have also been shown to be more infectious to mosquitoes [274]. The species of *Plasmodium* also influences the transmission of resistance. *P. falciparum* gametocytes form after the appearance of symptoms and drug treatment, and hence favor the transmission of resistant genotypes. By contrast, early *P. vivax* gametocytogenesis allows the parasite to be transmitted before the symptomatic stage of the disease and anti-blood-stage chemotherapy. The mechanism of resistance also determines the rate of development of resistance. It develops rapidly when it is conferred by a low number of mutations, as was noted for atovaquone [189].

1.13.2. Individual characteristics

Age, irrespective of prior exposure to malaria assists in the ability to clear parasites following treatment. Studies have shown that adults from a malaria-free area of Indonesia who migrated to a malaria-endemic areas acquired immunity significantly more rapidly than children did [275]. The level of acquired immunity in individuals also plays a major role in the development of drug resistance. Individuals with immunity against malaria increase the efficacy of chemotherapy because they can clear parasites despite the presence of resistance [276-279]. However, individuals who do not have immunity generate a response that is not as effective as the specific immunity elicited by repeated infections. In addition, individuals with no immunity usually have high parasite levels and therefore require longer periods to clear the parasites [280]. This increases the survival time of parasites and facilitates the development of resistance [215]. Furthermore, infection in individuals with no prior immunity usually progresses to symptomatic disease. Therefore, these individuals are treated more often than are individuals with acquired immunity, increasing the probability of the selection of resistant genotypes [244].

1.13.3. Anti-malarial drug properties and drug-use practices

A number of behavioral and pharmacokinetic factors affect the probability of parasites encountering sub-therapeutic levels of anti-malarial drugs. Drugs that have a long elimination half-life such as mefloquine and SP exert substantial residual selection for resistant strains when the drug persists at sub-therapeutic concentrations in the plasma, especially in areas with high transmission rates where individuals are at risk of getting frequent infections [281,282]. Anti-malarial drugs such as atovaquone and lumefantrine are

lipophilic and hydrophobic and are variably absorbed such that there might be inadequate blood concentrations even after taking the full treatment dose [244,280]. Incorrect dosing, non-compliance with the duration of the dosing regimen, and use of poor drug quality all result in sub-therapeutic drug levels which increase the probability for the selection of resistance strains [233].

Pharmacodynamic properties of anti-malarial drugs also influence the rate of development of resistance. Anti-malarial drugs that reduce the parasite concentration rapidly such as artesunate reduce the parasite biomass, and the selective pressure for the emergence of resistant mutants [215]. The effect of anti-malarial drugs on gametocyte carriage rate also plays an important role in the development and spread of resistance. SP increases the gametocyte carriage rate after treatment, hence increasing the risk of transmission of resistant strains if present in the host to the mosquitoes and the community [283]. However, artemisinin derivatives reduce the gametocyte carriage, and thereby lower the risk of infectiousness and risk of transmission of resistant strains [207].

1.14. Monitoring drug resistance

Monitoring of anti-malarial drug resistance is an essential part of managing malaria at a country level. The rapid spread of anti-malarial drug resistance over the last few decades has increased the need for monitoring to allow early detection of changing pattern of resistance, and to ensure proper management of cases of malaria. There are three main ways in which drug resistance is monitored: *in vivo* tests, *in vitro* tests, and using molecular markers.

1.14.1. *In vitro* tests

Parasites obtained from patients are mixed with growth medium, and the growth in drug-exposed cultures is measured relative to a drug-free control. Several tests have been developed that measure the effect of anti-malarial drugs on the growth and development of parasites by measuring the inhibition of schizont maturation, the percentage change in infected red blood cell in the culture and the levels of pLDH or (HRP-2) [284]. The advantage of this method is that it measures the intrinsic sensitivity of *P. falciparum* parasites to anti-malarial drugs without the confounding factors such as host immunity and drug metabolism that influence *in vivo* tests [218]. However, this method requires a lot of technical expertise, and is expensive, making it difficult to be adapted into routine field work. It is also difficult to culture isolates from patients with low-level parasitemia and individuals who had prior anti-malarial therapy [218]. Drugs that require host conversion into active metabolites cannot be tested. Variations in techniques preclude comparison of results from different areas [154]. Furthermore, there have been inconsistencies reported in the correlation between the results of therapeutic efficacy and *in vitro* tests [26].

1.14.2. Molecular markers

Some mutations in the parasite genome have been associated with anti-malarial drug resistance when compared with *in vitro* and *in vivo* tests [257,261,265,285]. Molecular tests use PCR methods to indicate the presence of mutations encoding biological resistance to anti-malarial drugs. This method enables investigators to distinguish between reinfection and recrudescence, study many isolates in a short time, and the collection, storage and transportation of specimens is easier than when conducting *in vitro* studies [218]. The

method also eliminates the possible bias from host factors which arise from *in vivo* tests, and selective pressure from cultures *in vitro* tests [218]. However, PCR methods cannot distinguish gametocyte DNA from that of asexual forms present in the blood and therefore, acute malaria could be classified as recrudescence [154]. It is also hard to compare results from different laboratories due to lack of standardization in specimen collection protocols, DNA extraction, PCR amplification techniques for molecular markers and interpretation [286,287]. Furthermore, molecular markers of resistance have not been identified for all anti-malarial drugs. There is also need to validate with clinical data before the results can be correctly interpreted.

1.14.3. *In vivo* tests

The gold standard for monitoring parasite resistance is the *in vivo* test [288]. This test involves recruiting symptomatic and parasitemic patients, treating them with the drug of interest, and following them for 28-63 days for evidence of treatment failure (i.e. return of parasitemia with or without fever) [218]. The WHO has developed a standardized protocol to enable comparison of results from different countries, and to include malaria low-transmission areas [289]. Originally the follow-up period was restricted to 14 days in high transmission areas, because it was considered unlikely in such a short period to have a new infection [288]. However, this short follow-up period may underestimate the rate of recrudescence in drugs such as artesunate where the majority of treatment failures may occur after 14 days [290-292]. For treatment with drugs such as amodiaquine, CQ and SP, a 28-day follow-up is considered appropriate; follow-up periods of 42 days and 63 days are recommended for artemether-lumefantrine and mefloquine, respectively [293]. Because of

the longer follow-up periods, it is recommended that these studies should be accompanied by molecular genotyping using PCR techniques to distinguish recrudescence from reinfection [294].The advantage of *in vivo* tests is that they accurately portray the response which involves a complex interaction between the parasites, the drug and the host response, and the results are easily interpretable and appeal to policy makers [154]. However, diminished therapeutic efficacy can be masked by parasite clearance in individuals living in areas with high transmission with acquired immunity [277,295].

1.15. Distinguishing recrudescence from re-infection

Decisions regarding drug policy rely largely on the results of *in vivo* studies that assess clinical and parasitological outcomes after therapy. These studies are limited by their inherent inability to distinguish recrudescence from new infection when parasites are detected after therapy. Molecular genotyping is being used increasingly to help distinguish recrudescence from re-infection when conducting *in vivo* studies [259,290,291,296]. There are three genes that are commonly used as markers due to their great polymorphism, both in length and sequence: merozoite surface protein 1 and 2 (*msp1* and *msp2*) and glutamate-rich protein (GLURP) located on chromosomes 9, 2 and 10, respectively [294,297]. Extensive polymorphism makes it highly unlikely for a patient in an area of intense transmission to be re-infected, during the follow-up period, with a parasite identical to the infecting parasite at baseline. The probability that an isolate is identical in two randomly selected samples decreases significantly as the number and polymorphism of the genetic markers used increases [296]. If the parasites are genetically different, it is assumed that it is a new infection, and if they are identical it is assumed to be a recrudescence. However, these

methods also have limitations. Some genotypes, representing a minority of the parasite population may not be detected at recruitment, but could be detected during the follow-up if they are resistant and if all the other sensitive parasites have been eliminated by the drug action. Such an infection would be wrongly classified as a new infection, overestimating the efficacy of the study drug [154]. Conversely, in places where there are few circulating parasite genotypes, a new infection might be genotypically similar to the initial infection eliminated by the drug [297].

1.16. Summary and Justification

Malaria infection during pregnancy is a major public health problem, causing maternal anemia and low birth weight in malaria endemic areas. However, the importance of timing and frequency of *P.falciparum* infection during pregnancy on these adverse outcomes is still poorly understood. To reduce the risk of these adverse outcomes, women are treatment presumptively with two doses of SP during pregnancy. Resistance to SP is increasing in most malaria-endemic areas and information on the safety of most of the available anti-malarial drugs during pregnancy is lacking.

We used data longitudinal observational study that performed took serial measurements of peripheral parasitemia during pregnancy, and peripheral and placental parasitemia at delivery to accurately identify the timing and frequency of *P. falciparum* infection during pregnancy and investigate their effect on the risk LBW and maternal anemia.

To add to the existing body of knowledge on the safety and efficacy of anti-malarial drug treatments on pregnant women, we used data from a clinical trial that assessed the efficacy and safety of azithromycin and artesunate combined with SP as treatment for malaria in pregnant women in the second and third trimester of pregnancy.

1.17. References

(1) WHO. World Malaria Report, 2005. Accessed at http://rbm.who.int/wmr2005/.

(2) Steketee RW, Nahlen BL, Parise ME, Menendez C. The burden of malaria in pregnancy in malaria-endemic areas. Am.J.Trop.Med.Hyg. 2001 Jan-Feb;64(1-2 Suppl):28-35.

(3) Guyatt HL, Snow RW. The epidemiology and burden of *Plasmodium falciparum*-related anemia among pregnant women in sub-Saharan Africa. Am.J.Trop.Med.Hyg. 2001 Jan-Feb;64(1-2 Suppl):36-44.

(4) Shulman CE, Graham WJ, Jilo H, Lowe BS, New L, Obiero J, et al. Malaria is an important cause of anaemia in primigravidae: evidence from a district hospital in coastal Kenya. Trans.R.Soc.Trop.Med.Hyg. 1996 Sep-Oct;90(5):535-539.

(5) Matteelli A, Donato F, Shein A, Muchi JA, Leopardi O, Astori L, et al. Malaria and anaemia in pregnant women in urban Zanzibar, Tanzania. Ann.Trop.Med.Parasitol. 1994 Oct;88(5):475-483.

(6) Rogerson SJ, Pollina E, Getachew A, Tadesse E, Lema VM, Molyneux ME. Placental monocyte infiltrates in response to *Plasmodium falciparum* malaria infection and their association with adverse pregnancy outcomes. Am.J.Trop.Med.Hyg. 2003 Jan;68(1):115-119.

(7) Shulman CE, Marshall T, Dorman EK, Bulmer JN, Cutts F, Peshu N, et al. Malaria in pregnancy: adverse effects on haemoglobin levels and birthweight in primigravidae and multigravidae. Trop.Med.Int.Health 2001 Oct;6(10):770-778.

(8) WHO 1993. A Global Strategy for Malaria Control. Geneva, World Health Organisation, 1993.

(9) Parise ME, Ayisi JG, Nahlen BL, Schultz LJ, Roberts JM, Misore A, et al. Efficacy of sulfadoxine-pyrimethamine for prevention of placental malaria in an area of Kenya with a high prevalence of malaria and human immunodeficiency virus infection. Am.J.Trop.Med.Hyg. 1998 Nov;59(5):813-822.

(10) Steketee RW, Wirima JJ, Hightower AW, Slutsker L, Heymann DL, Breman JG. The effect of malaria and malaria prevention in pregnancy on offspring birthweight, prematurity, and intrauterine growth retardation in rural Malawi. Am.J.Trop.Med.Hyg. 1996;55(1 Suppl):33-41.

(11) Rogerson SJ, Chaluluka E, Kanjala M, Mkundika P, Mhango C, Molyneux ME. Intermittent sulfadoxine-pyrimethamine in pregnancy: effectiveness against malaria morbidity in Blantyre, Malawi, in 1997-99. Trans.R.Soc.Trop.Med.Hyg. 2000 Sep-Oct;94(5):549-553.

(12) Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR. Epidemiology of drug-resistant malaria. Lancet Infect.Dis. 2002 Apr;2(4):209-218.

(13) National Statistics Office of Malawi. Available at: www.nso.malawi.net. Accessed March 1, 2006.

(14) Malawi Demographic and Health Survey, 2004.

(15) Schultz LJ, Steketee RW, Chitsulo L, Macheso A, Nyasulu Y, Ettling M. Malaria and childbearing women in Malawi: knowledge, attitudes and practices. Trop.Med.Parasitol. 1994 Mar;45(1):65-69.

(16) Steketee RW, Wirima JJ, Campbell CC. Developing effective strategies for malaria prevention programs for pregnant African women. Am.J.Trop.Med.Hyg. 1996;55(1 Suppl):95-100.

(17) Verhoeff FH, Brabin BJ, Chimsuku L, Kazembe P, Russell WB, Broadhead RL. An evaluation of the effects of intermittent sulfadoxine-pyrimethamine treatment in pregnancy on parasite clearance and risk of low birthweight in rural Malawi. Ann.Trop.Med.Parasitol. 1998 Mar;92(2):141-150.

(18) Ettling M, McFarland DA, Schultz LJ, Chitsulo L. Economic impact of malaria in Malawian households. Trop.Med.Parasitol. 1994 Mar;45(1):74-79.

(19) Schultz LJ, Steketee RW, Macheso A, Kazembe P, Chitsulo L, Wirima JJ. The efficacy of antimalarial regimens containing sulfadoxine-pyrimethamine and/or chloroquine in preventing peripheral and placental *Plasmodium falciparum* infection among pregnant women in Malawi. Am.J.Trop.Med.Hyg. 1994 Nov;51(5):515-522.

(20) Rosato M, Mwansambo CW, Kazembe PN, Phiri T, Soko QS, Lewycka S, et al. Women's groups' perceptions of maternal health issues in rural Malawi. Lancet 2006 Sep 30;368(9542):1180-1188.

(21) Holtz TH, Kachur SP, Roberts JM, Marum LH, Mkandala C, Chizani N, et al. Use of antenatal care services and intermittent preventive treatment for malaria among pregnant women in Blantyre District, Malawi. Trop.Med.Int.Health 2004 Jan;9(1):77-82.

(22) Plowe CV, Cortese JF, Djimde A, Nwanyanwu OC, Watkins WM, Winstanley PA, et al. Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. J.Infect.Dis. 1997 Dec;176(6):1590-1596.

(23) Bwijo B, Kaneko A, Takechi M, Zungu IL, Moriyama Y, Lum JK, et al. High prevalence of quintuple mutant dhps/dhfr genes in *Plasmodium falciparum* infections seven years after introduction of sulfadoxine and pyrimethamine as first line treatment in Malawi. Acta Trop. 2003 Mar;85(3):363-373.

(24) Nwanyanwu OC, Ziba C, Kazembe P, Chitsulo L, Wirima JJ, Kumwenda N, et al. Efficacy of sulphadoxine/pyrimethamine for *Plasmodium falciparum* malaria in Malawian children under five years of age. Trop.Med.Int.Health 1996 Apr;1(2):231-235.

(25) Nwanyanwu OC, Ziba C, MacHeso A, Kazembe P. Efficacy of sulphadoxine-pyrimethamine for acute uncomplicated malaria due to *Plasmodium falciparum* in Malawian children under five years old. Trop.Med.Int.Health 2000 May;5(5):355-358.

(26) Takechi M, Matsuo M, Ziba C, MacHeso A, Butao D, Zungu IL, et al. Therapeutic efficacy of sulphadoxine/pyrimethamine and susceptibility in vitro of *P. falciparum* isolates to sulphadoxine-pyremethamine and other antimalarial drugs in Malawian children. Trop.Med.Int.Health 2001 Jun;6(6):429-434.

(27) Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature 2005 Mar 10;434(7030):214-217.

(28) Whitty CJ, Edmonds S, Mutabingwa TK. Malaria in pregnancy. BJOG 2005 Sep;112(9):1189-1195.

(29) Mendis K, Sina BJ, Marchesini P, Carter R. The neglected burden of *Plasmodium vivax* malaria. Am.J.Trop.Med.Hyg. 2001 Jan-Feb;64(1-2 Suppl):97-106.

(30) Ukpe IS. *Plasmodium ovale* in South Africa. Trans.R.Soc.Trop.Med.Hyg. 1998 Sep-Oct;92(5):574.

(31) Sturm A, Amino R, van de Sand C, Regen T, Retzlaff S, Rennenberg A, et al. Manipulation of Host Hepatocytes by the Malaria Parasite for Delivery into Liver Sinusoids. Science 2006 Aug 3.

(32) Pukrittayakamee S, Imwong M, Looareesuwan S, White NJ. Therapeutic responses to antimalarial and antibacterial drugs in *vivax* malaria. Acta Trop. 2004 Feb;89(3):351-356.

(33) Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. Nature 2002 Feb 7;415(6872):673-679.

(34) Hay SI, Guerra CA, Tatem AJ, Noor AM, Snow RW. The global distribution and population at risk of malaria: past, present, and future. Lancet Infect.Dis. 2004 Jun;4(6):327-336.

(35) Snow RW, Craig M, Deichmann U, Marsh K. Estimating mortality, morbidity and disability due to malaria among Africa's non-pregnant population. Bull.World Health Organ. 1999;77(8):624-640.

(36) Brewster DR, Kwiatkowski D, White NJ. Neurological sequelae of cerebral malaria in children. Lancet 1990 Oct 27;336(8722):1039-1043.

(37) Holding PA, Stevenson J, Peshu N, Marsh K. Cognitive sequelae of severe malaria with impaired consciousness. Trans.R.Soc.Trop.Med.Hyg. 1999 Sep-Oct;93(5):529-534.

(38) Murphy SC, Breman JG. Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy. Am.J.Trop.Med.Hyg. 2001 Jan-Feb;64(1-2 Suppl):57-67.

(39) Fernando SD, Gunawardena DM, Bandara MR, De Silva D, Carter R, Mendis KN, et al. The impact of repeated malaria attacks on the school performance of children. Am.J.Trop.Med.Hyg. 2003 Dec;69(6):582-588.

(40) Carter JA, Ross AJ, Neville BG, Obiero E, Katana K, Mung'ala-Odera V, et al. Developmental impairments following severe *falciparum* malaria in children. Trop.Med.Int.Health 2005 Jan;10(1):3-10.

(41) Sachs J, Malaney P. The economic and social burden of malaria. Nature 2002 Feb 7;415(6872):680-685.

(42) Chima RI, Goodman CA, Mills A. The economic impact of malaria in Africa: a critical review of the evidence. Health Policy 2003 Jan;63(1):17-36.

(43) Adak T, Sharma VP, Orlov VS. Studies on the *Plasmodium vivax* relapse pattern in Delhi, India. Am.J.Trop.Med.Hyg. 1998 Jul;59(1):175-179.

(44) Severe *falciparum* malaria. World Health Organization, Communicable Diseases Cluster. Trans.R.Soc.Trop.Med.Hyg. 2000 Apr;94 Suppl 1:S1-90.

(45) Payne D. Use and limitations of light microscopy for diagnosing malaria at the primary health care level. Bull.World Health Organ. 1988;66(5):621-626.

(46) Redd SC, Bloland PB, Kazembe PN, Patrick E, Tembenu R, Campbell CC. Usefulness of clinical case-definitions in guiding therapy for African children with malaria or pneumonia. Lancet 1992 Nov 7;340(8828):1140-1143.

(47) Luxemburger C, Nosten F, Kyle DE, Kiricharoen L, Chongsuphajaisiddhi T, White NJ. Clinical features cannot predict a diagnosis of malaria or differentiate the infecting species in children living in an area of low transmission. Trans.R.Soc.Trop.Med.Hyg. 1998 Jan-Feb;92(1):45-49.

(48) Hanscheid T. Diagnosis of malaria: a review of alternatives to conventional microscopy. Clin.Lab.Haematol. 1999 Aug;21(4):235-245.

(49) Levine RA, Wardlaw SC, Patton CL. Detection of haematoparasites using quantitative buffy coat analysis tubes. Parasitol.Today 1989 Apr;5(4):132-134.

(50) Iqbal J, Hira PR, Sher A, Al-Enezi AA. Diagnosis of imported malaria by *Plasmodium* lactate dehydrogenase (pLDH) and histidine-rich protein 2 (PfHRP-2)-based immunocapture assays. Am.J.Trop.Med.Hyg. 2001 Jan-Feb;64(1-2):20-23.

(51) Oliveira DA, Holloway BP, Durigon EL, Collins WE, Lal AA. Polymerase chain reaction and a liquid-phase, nonisotopic hybridization for species-specific and sensitive detection of malaria infection. Am.J.Trop.Med.Hyg. 1995 Feb;52(2):139-144.

(52) McGregor IA. Epidemiology, malaria and pregnancy. Am.J.Trop.Med.Hyg. 1984 Jul;33(4):517-525.

(53) Brabin BJ, Romagosa C, Abdelgalil S, Menendez C, Verhoeff FH, McGready R, et al. The sick placenta-the role of malaria. Placenta 2004 May;25(5):359-378.

(54) Brabin BJ. An analysis of malaria in pregnancy in Africa. Bull.World Health Organ. 1983;61(6):1005-1016.

(55) Menendez C. Malaria during pregnancy: a priority area of malaria research and control. Parasitol.Today 1995 May;11(5):178-183.

(56) Nosten F, ter Kuile F, Maelankirri L, Decludt B, White NJ. Malaria during pregnancy in an area of unstable endemicity. Trans.R.Soc.Trop.Med.Hyg. 1991 Jul-Aug;85(4):424-429.

(57) Luxemburger C, Ricci F, Nosten F, Raimond D, Bathet S, White NJ. The epidemiology of severe malaria in an area of low transmission in Thailand. Trans.R.Soc.Trop.Med.Hyg. 1997 May-Jun;91(3):256-262.

(58) Diagne N, Rogier C, Sokhna CS, Tall A, Fontenille D, Roussilhon C, et al. Increased susceptibility to malaria during the early postpartum period. N.Engl.J.Med. 2000 Aug 31;343(9):598-603.

(59) ter Kuile FO, Parise ME, Verhoeff FH, Udhayakumar V, Newman RD, van Eijk AM, et al. The burden of co-infection with human immunodeficiency virus type 1 and malaria in pregnant women in sub-saharan Africa. Am.J.Trop.Med.Hyg. 2004 Aug;71(2 Suppl):41-54.

(60) van Eijk AM, Ayisi JG, ter Kuile FO, Misore AO, Otieno JA, Rosen DH, et al. HIV increases the risk of malaria in women of all gravidities in Kisumu, Kenya. AIDS 2003 Mar 7;17(4):595-603.

(61) Verhoeff FH, Brabin BJ, Chimsuku L, Kazembe P, Broadhead RL. An analysis of the determinants of anaemia in pregnant women in rural Malawi--a basis for action. Ann.Trop.Med.Parasitol. 1999 Mar;93(2):119-133.

(62) Ayisi JG, van Eijk AM, ter Kuile FO, Kolczak MS, Otieno JA, Misore AO, et al. The effect of dual infection with HIV and malaria on pregnancy outcome in western Kenya. AIDS 2003 Mar 7;17(4):585-594.

(63) Walter PR, Garin Y, Blot P. Placental pathologic changes in malaria. A histologic and ultrastructural study. Am.J.Pathol. 1982 Dec;109(3):330-342.

(64) Yamada M, Steketee R, Abramowsky C, Kida M, Wirima J, Heymann D, et al. *Plasmodium falciparum* associated placental pathology: a light and electron microscopic and immunohistologic study. Am.J.Trop.Med.Hyg. 1989 Aug;41(2):161-168.

(65) Reeder JC, Cowman AF, Davern KM, Beeson JG, Thompson JK, Rogerson SJ, et al. The adhesion of Plasmodium falciparum-infected erythrocytes to chondroitin sulfate A is mediated by P. falciparum erythrocyte membrane protein 1. Proc.Natl.Acad.Sci.U.S.A. 1999 Apr 27;96(9):5198-5202.

(66) Beeson JG, Reeder JC, Rogerson SJ, Brown GV. Parasite adhesion and immune evasion in placental malaria. Trends Parasitol. 2001 Jul;17(7):331-337.

(67) Pouvelle B, Buffet PA, Lepolard C, Scherf A, Gysin J. Cytoadhesion of *Plasmodium falciparum* ring-stage-infected erythrocytes. Nat.Med. 2000 Nov;6(11):1264-1268.

(68) Ockenhouse CF, Ho M, Tandon NN, Van Seventer GA, Shaw S, White NJ, et al. Molecular basis of sequestration in severe and uncomplicated *Plasmodium falciparum* malaria: differential adhesion of infected erythrocytes to CD36 and ICAM-1. J.Infect.Dis. 1991 Jul;164(1):163-169.

(69) Fried M, Duffy PE. Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. Science 1996 Jun 7;272(5267):1502-1504.

(70) Beeson JG, Brown GV, Molyneux ME, Mhango C, Dzinjalamala F, Rogerson SJ. *Plasmodium falciparum* isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. J.Infect.Dis. 1999 Aug;180(2):464-472.

(71) Maubert B, Fievet N, Tami G, Boudin C, Deloron P. Cytoadherence of *Plasmodium falciparum*-infected erythrocytes in the human placenta. Parasite Immunol. 2000 Apr;22(4):191-199.

(72) Rogerson SJ, Chaiyaroj SC, Ng K, Reeder JC, Brown GV. Chondroitin sulfate A is a cell surface receptor for *Plasmodium falciparum*-infected erythrocytes. J.Exp.Med. 1995 Jul 1;182(1):15-20.

(73) Beeson JG, Rogerson SJ, Cooke BM, Reeder JC, Chai W, Lawson AM, et al. Adhesion of *Plasmodium falciparum*-infected erythrocytes to hyaluronic acid in placental malaria. Nat.Med. 2000 Jan;6(1):86-90.

(74) Beeson JG, Mann EJ, Elliott SR, Lema VM, Tadesse E, Molyneux ME, et al. Antibodies to variant surface antigens of *Plasmodium falciparum*-infected erythrocytes and adhesion inhibitory antibodies are associated with placental malaria and have overlapping and distinct targets. J.Infect.Dis. 2004 Feb 1;189(3):540-551.

(75) Flick K, Scholander C, Chen Q, Fernandez V, Pouvelle B, Gysin J, et al. Role of nonimmune IgG bound to PfEMP1 in placental malaria. Science 2001 Sep 14;293(5537):2098-2100.

(76) Fried M, Nosten F, Brockman A, Brabin BJ, Duffy PE. Maternal antibodies block malaria. Nature 1998 Oct 29;395(6705):851-852.

(77) Ismail MR, Ordi J, Menendez C, Ventura PJ, Aponte JJ, Kahigwa E, et al. Placental pathology in malaria: a histological, immunohistochemical, and quantitative study. Hum.Pathol. 2000 Jan;31(1):85-93.

(78) Bulmer JN, Rasheed FN, Francis N, Morrison L, Greenwood BM. Placental malaria. I. Pathological classification. Histopathology 1993 Mar;22(3):211-218.

(79) Galbraith RM, Faulk WP, Galbraith GM, Holbrook TW, Bray RS. The human materno-foetal relationship in malaria: I. Identification of pigment and parasites in the placenta. Trans.R.Soc.Trop.Med.Hyg. 1980;74(1):52-60.

(80) Ordi J, Menendez C, Ismail MR, Ventura PJ, Palacin A, Kahigwa E, et al. Placental malaria is associated with cell-mediated inflammatory responses with selective absence of natural killer cells. J.Infect.Dis. 2001 Apr 1;183(7):1100-1107.

(81) Ordi J, Ismail MR, Ventura PJ, Kahigwa E, Hirt R, Cardesa A, et al. Massive chronic intervillositis of the placenta associated with malaria infection. Am.J.Surg.Pathol. 1998 Aug;22(8):1006-1011.

(82) Abrams ET, Brown H, Chensue SW, Turner GD, Tadesse E, Lema VM, et al. Host response to malaria during pregnancy: placental monocyte recruitment is associated with elevated beta chemokine expression. J.Immunol. 2003 Mar 1;170(5):2759-2764.

(83) Chaisavaneeyakorn S, Moore JM, Othoro C, Otieno J, Chaiyaroj SC, Shi YP, et al. Immunity to placental malaria. IV. Placental malaria is associated with up-regulation of macrophage migration inhibitory factor in intervillous blood. J.Infect.Dis. 2002 Nov 1;186(9):1371-1375.

(84) Raghupathy R. Th1-type immunity is incompatible with successful pregnancy. Immunol.Today 1997 Oct;18(10):478-482.

(85) Fried M, Muga RO, Misore AO, Duffy PE. Malaria elicits type 1 cytokines in the human placenta: IFN-gamma and TNF-alpha associated with pregnancy outcomes. J.Immunol. 1998 Mar 1;160(5):2523-2530.

(86) Suguitan AL,Jr, Leke RG, Fouda G, Zhou A, Thuita L, Metenou S, et al. Changes in the levels of chemokines and cytokines in the placentas of women with *Plasmodium falciparum* malaria. J.Infect.Dis. 2003 Oct 1;188(7):1074-1082.

(87) Moormann AM, Sullivan AD, Rochford RA, Chensue SW, Bock PJ, Nyirenda T, et al. Malaria and pregnancy: placental cytokine expression and its relationship to intrauterine growth retardation. J.Infect.Dis. 1999 Dec;180(6):1987-1993.

(88) Moore JM, Nahlen BL, Misore A, Lal AA, Udhayakumar V. Immunity to placental malaria. I. Elevated production of interferon-gamma by placental blood mononuclear cells is associated with protection in an area with high transmission of malaria. J.Infect.Dis. 1999 May;179(5):1218-1225.

(89) Ricke CH, Staalsoe T, Koram K, Akanmori BD, Riley EM, Theander TG, et al. Plasma antibodies from malaria-exposed pregnant women recognize variant surface antigens on *Plasmodium falciparum*-infected erythrocytes in a parity-dependent manner and block parasite adhesion to chondroitin sulfate A. J.Immunol. 2000 Sep 15;165(6):3309-3316.

(90) Elliott SR, Brennan AK, Beeson JG, Tadesse E, Molyneux ME, Brown GV, et al. Placental malaria induces variant-specific antibodies of the cytophilic subtypes immunoglobulin G1 (IgG1) and IgG3 that correlate with adhesion inhibitory activity. Infect.Immun. 2005 Sep;73(9):5903-5907.

(91) O'Neil-Dunne I, Achur RN, Agbor-Enoh ST, Valiyaveettil M, Naik RS, Ockenhouse CF, et al. Gravidity-dependent production of antibodies that inhibit binding of *Plasmodium falciparum*-infected erythrocytes to placental chondroitin sulfate proteoglycan during pregnancy. Infect.Immun. 2001 Dec;69(12):7487-7492.

(92) Staalsoe T, Megnekou R, Fievet N, Ricke CH, Zornig HD, Leke R, et al. Acquisition and decay of antibodies to pregnancy-associated variant antigens on the surface of *Plasmodium falciparum*-infected erythrocytes that protect against placental parasitemia. J.Infect.Dis. 2001 Sep 1;184(5):618-626.

(93) Duffy PE, Fried M. Antibodies that inhibit *Plasmodium falciparum* adhesion to chondroitin sulfate A are associated with increased birth weight and the gestational age of newborns. Infect.Immun. 2003 Nov;71(11):6620-6623.

(94) Watkinson M, Rushton DI. Plasmodial pigmentation of placenta and outcome of pregnancy in West African mothers. Br.Med.J.(Clin.Res.Ed) 1983 Jul 23;287(6387):251-254.

(95) Crocker IP, Tanner OM, Myers JE, Bulmer JN, Walraven G, Baker PN. Syncytiotrophoblast degradation and the pathophysiology of the malaria-infected placenta. Placenta 2004 Apr;25(4):273-282.

(96) Leopardi O, Naughten W, Salvia L, Colecchia M, Matteelli A, Zucchi A, et al. Malaric placentas. A quantitative study and clinico-pathological correlations. Pathol.Res.Pract. 1996 Sep;192(9):892-8; discussion 899-900.

(97) Smith SC, Baker PN, Symonds EM. Increased placental apoptosis in intrauterine growth restriction. Am.J.Obstet.Gynecol. 1997 Dec;177(6):1395-1401.

(98) Okoko BJ, Enwere G, Ota MO. The epidemiology and consequences of maternal malaria: a review of immunological basis. Acta Trop. 2003 Jul;87(2):193-205.

(99) Rogerson SJ, Mkundika P, Kanjala MK. Diagnosis of *Plasmodium falciparum* malaria at delivery: comparison of blood film preparation methods and of blood films with histology. J.Clin.Microbiol. 2003 Apr;41(4):1370-1374.

(100) Mockenhaupt FP, Ulmen U, von Gaertner C, Bedu-Addo G, Bienzle U. Diagnosis of placental malaria. J.Clin.Microbiol. 2002 Jan;40(1):306-308.

(101) Beeson JG, Duffy PE. The immunology and pathogenesis of malaria during pregnancy. Curr.Top.Microbiol.Immunol. 2005;297:187-227.

(102) Nosten F, Rogerson SJ, Beeson JG, McGready R, Mutabingwa TK, Brabin B. Malaria in pregnancy and the endemicity spectrum: what can we learn? Trends Parasitol. 2004 Sep;20(9):425-432.

(103) Luxemburger C, McGready R, Kham A, Morison L, Cho T, Chongsuphajaisiddhi T, et al. Effects of malaria during pregnancy on infant mortality in an area of low malaria transmission. Am.J.Epidemiol. 2001 Sep 1;154(5):459-465.

(104) Ekvall H. Malaria and anemia. Curr.Opin.Hematol. 2003 Mar;10(2):108-114.

(105) Omodeo-Sale F, Motti A, Basilico N, Parapini S, Olliaro P, Taramelli D. Accelerated senescence of human erythrocytes cultured with *Plasmodium falciparum*. Blood 2003 Jul 15;102(2):705-711.

(106) Greve B, Lehman LG, Lell B, Luckner D, Schmidt-Ott R, Kremsner PG. High oxygen radical production is associated with fast parasite clearance in children with *Plasmodium falciparum* malaria. J.Infect.Dis. 1999 Jun;179(6):1584-1586.

(107) Dreyfuss ML, Stoltzfus RJ, Shrestha JB, Pradhan EK, LeClerq SC, Khatry SK, et al. Hookworms, malaria and vitamin A deficiency contribute to anemia and iron deficiency among pregnant women in the plains of Nepal. J.Nutr. 2000 Oct;130(10):2527-2536.

(108) van den Broek NR, Letsky EA. Etiology of anemia in pregnancy in south Malawi. Am.J.Clin.Nutr. 2000 Jul;72(1 Suppl):247S-256S.

(109) Guyatt HL, Snow RW. Malaria in pregnancy as an indirect cause of infant mortality in sub-Saharan Africa. Trans.R.Soc.Trop.Med.Hyg. 2001 Nov-Dec;95(6):569-576.

(110) Guyatt HL, Snow RW. Impact of malaria during pregnancy on low birth weight in sub-Saharan Africa. Clin.Microbiol.Rev. 2004 Oct;17(4):760-9, table of contents.

(111) McCormick MC. The contribution of low birth weight to infant mortality and childhood morbidity. N.Engl.J.Med. 1985 Jan 10;312(2):82-90.

(112) Ashworth A. Effects of intrauterine growth retardation on mortality and morbidity in infants and young children. Eur.J.Clin.Nutr. 1998 Jan;52 Suppl 1:S34-41; discussion S41-2.

(113) Brabin B. An assessment of low birthweight risk in primiparae as an indicator of malaria control in pregnancy. Int.J.Epidemiol. 1991 Mar;20(1):276-283.

(114) Okoko BJ, Ota MO, Yamuah LK, Idiong D, Mkpanam SN, Avieka A, et al. Influence of placental malaria infection on foetal outcome in the Gambia: twenty years after Ian Mcgregor. J.Health Popul.Nutr. 2002 Mar;20(1):4-11.

(115) Steketee RW, Wirima JJ, Slutsker L, Roberts JM, Khoromana CO, Heymann DL, et al. Malaria parasite infection during pregnancy and at delivery in mother, placenta, and newborn: efficacy of chloroquine and mefloquine in rural Malawi. Am.J.Trop.Med.Hyg. 1996;55(1 Suppl):24-32.

(116) Sullivan AD, Nyirenda T, Cullinan T, Taylor T, Harlow SD, James SA, et al. Malaria infection during pregnancy: intrauterine growth retardation and preterm delivery in Malawi. J.Infect.Dis. 1999 Jun;179(6):1580-1583.

(117) Menendez C, Ordi J, Ismail MR, Ventura PJ, Aponte JJ, Kahigwa E, et al. The impact of placental malaria on gestational age and birth weight. J.Infect.Dis. 2000 May;181(5):1740-1745.

(118) Verhoeff FH, Brabin BJ, van Buuren S, Chimsuku L, Kazembe P, Wit JM, et al. An analysis of intra-uterine growth retardation in rural Malawi. Eur.J.Clin.Nutr. 2001 Aug;55(8):682-689.

(119) Dorman EK, Shulman CE, Kingdom J, Bulmer JN, Mwendwa J, Peshu N, et al. Impaired uteroplacental blood flow in pregnancies complicated by *falciparum* malaria. Ultrasound Obstet.Gynecol. 2002 Feb;19(2):165-170.

(120) Fischer PR. Congenital malaria: an African survey. Clin.Pediatr.(Phila) 1997 Jul;36(7):411-413.

(121) Tobian AA, Mehlotra RK, Malhotra I, Wamachi A, Mungai P, Koech D, et al. Frequent umbilical cord-blood and maternal-blood infections with *Plasmodium falciparum, P. malariae,* and *P. ovale* in Kenya. J.Infect.Dis. 2000 Aug;182(2):558-563.

(122) Kamwendo DD, Dzinjalamala FK, Snounou G, Kanjala MC, Mhango CG, Molyneux ME, et al. Plasmodium falciparum: PCR detection and genotyping of isolates from peripheral, placental, and cord blood of pregnant Malawian women and their infants. Trans.R.Soc.Trop.Med.Hyg. 2002 Mar-Apr;96(2):145-149.

(123) King CL, Malhotra I, Wamachi A, Kioko J, Mungai P, Wahab SA, et al. Acquired immune responses to *Plasmodium falciparum* merozoite surface protein-1 in the human fetus. J.Immunol. 2002 Jan 1;168(1):356-364.

(124) Desowitz RS, Elm J, Alpers MP. *Plasmodium falciparum*-specific immunoglobulin G (IgG), IgM, and IgE antibodies in paired maternal-cord sera from east Sepik Province, Papua New Guinea. Infect.Immun. 1993 Mar;61(3):988-993.

(125) Malhotra I, Mungai P, Muchiri E, Kwiek JJ, Meshnick SR, King CL. Umbilical cord-blood infections with *Plasmodium falciparum* malaria are acquired antenatally in Kenya. J.Infect.Dis. 2006 Jul 15;194(2):176-183.

(126) Xi G, Leke RG, Thuita LW, Zhou A, Leke RJ, Mbu R, et al. Congenital exposure to *Plasmodium falciparum* antigens: prevalence and antigenic specificity of in utero-produced antimalarial immunoglobulin M antibodies. Infect.Immun. 2003 Mar;71(3):1242-1246.

(127) COVELL G. Congenital malaria. Trop.Dis.Bull. 1950 Dec;47(12):1147-1167.

(128) McGready R, Davison BB, Stepniewska K, Cho T, Shee H, Brockman A, et al. The effects of *Plasmodium falciparum* and *P. vivax* infections on placental histopathology in an area of low malaria transmission. Am.J.Trop.Med.Hyg. 2004 Apr;70(4):398-407.

(129) Cottrell G, Deloron P, Fievet N, Sow S, Gaye O, Le Hesran JY. Prediction of *Plasmodium falciparum* placental infection according to the time of infection during pregnancy. Acta Trop. 2006 Jul;98(3):255-260.

(130) Shulman CE. Malaria in pregnancy: its relevance to safe-motherhood programmes. Ann.Trop.Med.Parasitol. 1999 Dec;93 Suppl 1:S59-66.

(131) WHO 2002. Strategic Framework for Malaria Control During Pregnancy in the WHO Africa Region.

(132) Shulman CE, Dorman EK, Cutts F, Kawuondo K, Bulmer JN, Peshu N, et al. Intermittent sulphadoxine-pyrimethamine to prevent severe anaemia secondary to malaria in pregnancy: a randomised placebo-controlled trial. Lancet 1999 Feb 20;353(9153):632-636.

(133) van Eijk AM, Ayisi JG, ter Kuile FO, Otieno JA, Misore AO, Odondi JO, et al. Effectiveness of intermittent preventive treatment with sulphadoxine-pyrimethamine for control of malaria in pregnancy in western Kenya: a hospital-based study. Trop.Med.Int.Health 2004 Mar;9(3):351-360.

(134) Sirima SB, Cotte AH, Konate A, Moran AC, Asamoa K, Bougouma EC, et al. Malaria prevention during pregnancy: assessing the disease burden one year after implementing a program of intermittent preventive treatment in Koupela district, Burkina Faso. Am.J.Trop.Med.Hyg. 2006 Aug;75(2):205-211.

(135) Verhoeff FH, Brabin BJ, Hart CA, Chimsuku L, Kazembe P, Broadhead RL. Increased prevalence of malaria in HIV-infected pregnant women and its implications for malaria control. Trop.Med.Int.Health 1999 Jan;4(1):5-12.

(136) Filler SJ, Kazembe P, Thigpen M, Macheso A, Parise ME, Newman RD, et al. Randomized Trial of 2-Dose versus Monthly Sulfadoxine-Pyrimethamine Intermittent Preventive Treatment for Malaria in HIV-Positive and HIV-Negative Pregnant Women in Malawi. J.Infect.Dis. 2006 Aug 1;194(3):286-293.

(137) Nosten F, McGready R, Looareesuwan S, White NJ. Editorial: Maternal malaria: time for action. Trop.Med.Int.Health 2003 Jun;8(6):485-487.

(138) D'Alessandro U, Langerock P, Bennett S, Francis N, Cham K, Greenwood BM. The impact of a national impregnated bed net programme on the outcome of pregnancy in primigravidae in The Gambia. Trans.R.Soc.Trop.Med.Hyg. 1996 Sep-Oct;90(5):487-492.

(139) ter Kuile FO, Terlouw DJ, Phillips-Howard PA, Hawley WA, Friedman JF, Kariuki SK, et al. Reduction of malaria during pregnancy by permethrin-treated bed nets in an area of intense perennial malaria transmission in western Kenya. Am.J.Trop.Med.Hyg. 2003 Apr;68(4 Suppl):50-60.

(140) Dolan G, ter Kuile FO, Jacoutot V, White NJ, Luxemburger C, Malankirii L, et al. Bed nets for the prevention of malaria and anaemia in pregnancy. Trans.R.Soc.Trop.Med.Hyg. 1993 Nov-Dec;87(6):620-626.

(141) Shulman CE, Dorman EK, Talisuna AO, Lowe BS, Nevill C, Snow RW, et al. A community randomized controlled trial of insecticide-treated bednets for the prevention of malaria and anaemia among primigravid women on the Kenyan coast. Trop.Med.Int.Health 1998 Mar;3(3):197-204.

(142) Browne EN, Maude GH, Binka FN. The impact of insecticide-treated bednets on malaria and anaemia in pregnancy in Kassena-Nankana district, Ghana: a randomized controlled trial. Trop.Med.Int.Health 2001 Sep;6(9):667-676.

(143) Gamble C, Ekwaru JP, ter Kuile FO. Insecticide-treated nets for preventing malaria in pregnancy. Cochrane Database Syst.Rev. 2006 Apr 19;(2)(2):CD003755.

(144) Guyatt HL, Ochola SA, Snow RW. Too poor to pay: charging for insecticide-treated bednets in highland Kenya. Trop.Med.Int.Health 2002 Oct;7(10):846-850.

(145) Holtz TH, Marum LH, Mkandala C, Chizani N, Roberts JM, Macheso A, et al. Insecticide-treated bednet use, anaemia, and malaria parasitaemia in Blantyre District, Malawi. Trop.Med.Int.Health 2002 Mar;7(3):220-230.

(146) Nuwaha F. Factors influencing the use of bed nets in Mbarara municipality of Uganda. Am.J.Trop.Med.Hyg. 2001 Dec;65(6):877-882.

(147) White NJ, Nosten F, Looareesuwan S, Watkins WM, Marsh K, Snow RW, et al. Averting a malaria disaster. Lancet 1999 Jun 5;353(9168):1965-1967.

(148) Deen JL, von Seidlein L, Pinder M, Walraven GE, Greenwood BM. The safety of the combination artesunate and pyrimethamine-sulfadoxine given during pregnancy. Trans.R.Soc.Trop.Med.Hyg. 2001 Jul-Aug;95(4):424-428.

(149) McGready R, Brockman A, Cho T, Cho D, van Vugt M, Luxemburger C, et al. Randomized comparison of mefloquine-artesunate versus quinine in the treatment of multidrug-resistant falciparum malaria in pregnancy. Trans.R.Soc.Trop.Med.Hyg. 2000 Nov-Dec;94(6):689-693.

(150) McGready R, Ashley EA, Moo E, Cho T, Barends M, Hutagalung R, et al. A randomized comparison of artesunate-atovaquone-proguanil versus quinine in treatment for uncomplicated *falciparum* malaria during pregnancy. J.Infect.Dis. 2005 Sep 1;192(5):846-853.

(151) Adam I, Ali DM, Abdalla MA. Artesunate plus sulfadoxine-pyrimethamine in the treatment of uncomplicated *Plasmodium falciparum* malaria during pregnancy in eastern Sudan. Trans.R.Soc.Trop.Med.Hyg. 2006 Jan 24.

(152) Nosten F, McGready R, Simpson JA, Thwai KL, Balkan S, Cho T, et al. Effects of *Plasmodium vivax* malaria in pregnancy. Lancet 1999 Aug 14;354(9178):546-549.

(153) Singh N, Shukla MM, Sharma VP. Epidemiology of malaria in pregnancy in central India. Bull.World Health Organ. 1999;77(7):567-572.

(154) Talisuna AO, Bloland P, D'Alessandro U. History, dynamics, and public health importance of malaria parasite resistance. Clin.Microbiol.Rev. 2004 Jan;17(1):235-254.

(155) Phillips-Howard PA, Wood D. The safety of antimalarial drugs in pregnancy. Drug Saf. 1996 Mar;14(3):131-145.

(156) Guerin PJ, Olliaro P, Nosten F, Druilhe P, Laxminarayan R, Binka F, et al. Malaria: current status of control, diagnosis, treatment, and a proposed agenda for research and development. Lancet Infect.Dis. 2002 Sep;2(9):564-573.

(157) McGready R, Thwai KL, Cho T, Samuel, Looareesuwan S, White NJ, et al. The effects of quinine and chloroquine antimalarial treatments in the first trimester of pregnancy. Trans.R.Soc.Trop.Med.Hyg. 2002 Mar-Apr;96(2):180-184.

(158) Steketee RW, Wirima JJ, Slutsker L, Khoromana CO, Heymann DL, Breman JG. Malaria treatment and prevention in pregnancy: indications for use and adverse events associated with use of chloroquine or mefloquine. Am.J.Trop.Med.Hyg. 1996;55(1 Suppl):50-56.

(159) Mutabingwa TK, Malle LN, Mtui SN. Chloroquine therapy still useful in the management of malaria during pregnancy in Muheza, Tanzania. Trop.Geogr.Med. 1991 Jan-Apr;43(1-2):131-135.

(160) Wolfe MS, Cordero JF. Safety of chloroquine in chemosuppression of malaria during pregnancy. Br.Med.J.(Clin.Res.Ed) 1985 May 18;290(6480):1466-1467.

(161) Andersen DH, Blanc WA, Crozier DN, Silverman WA. A difference in mortality rate and incidence of kernicterus among premature infants allotted to two prophylactic antibacterial regimens. Pediatrics 1956 Oct;18(4):614-625.

(162) Taylor WR, White NJ. Antimalarial drug toxicity: a review. Drug Saf. 2004;27(1):25-61.

(163) Hernandez-Diaz S, Werler MM, Walker AM, Mitchell AA. Folic acid antagonists during pregnancy and the risk of birth defects. N.Engl.J.Med. 2000 Nov 30;343(22):1608-1614.

(164) Czeizel AE, Rockenbauer M, Sorensen HT, Olsen J. The teratogenic risk of trimethoprim-sulfonamides: a population based case-control study. Reprod.Toxicol. 2001 Nov-Dec;15(6):637-646.

(165) Miller KD, Lobel HO, Satriale RF, Kuritsky JN, Stern R, Campbell CC. Severe cutaneous reactions among American travelers using pyrimethamine-sulfadoxine (Fansidar) for malaria prophylaxis. Am.J.Trop.Med.Hyg. 1986 May;35(3):451-458.

(166) Newman RD, Parise ME, Slutsker L, Nahlen B, Steketee RW. Safety, efficacy and determinants of effectiveness of antimalarial drugs during pregnancy: implications for prevention programmes in *Plasmodium falciparum*-endemic sub-Saharan Africa. Trop.Med.Int.Health 2003 Jun;8(6):488-506.

(167) WHO 2000. Severe *falciparum* malaria. Transactions of the Royal Society of Tropical Medicine & Hygiene 94, 1–90.

(168) McGready R, Cho T, Hkirijaroen L, Simpson J, Chongsuphajaisiddhi T, White NJ, et al. Quinine and mefloquine in the treatment of multidrug-resistant *Plasmodium falciparum* malaria in pregnancy. Ann.Trop.Med.Parasitol. 1998 Sep;92(6):643-653.

(169) Nosten F, Vincenti M, Simpson J, Yei P, Thwai KL, de Vries A, et al. The effects of mefloquine treatment in pregnancy. Clin.Infect.Dis. 1999 Apr;28(4):808-815.

(170) Kochar DK, Kumawat BL, Kochar SK, Sanwal V. Hypoglycemia after oral quinine administration. J.Assoc.Physicians India 1995 Sep;43(9):654, 657.

(171) Looareesuwan S, Phillips RE, White NJ, Kietinun S, Karbwang J, Rackow C, et al. Quinine and severe *falciparum* malaria in late pregnancy. Lancet 1985 Jul 6;2(8445):4-8.

(172) Smith JP. Risky choices: the dangers of teens using self-induced abortion attempts. J.Pediatr.Health Care 1998 May-Jun;12(3):147-151.

(173) McKinna AJ. Quinine induced hypoplasia of the optic nerve. Can.J.Ophthalmol. 1966 Oct;1(4):261-266.

(174) Dannenberg AL, Dorfman SF, Johnson J. Use of quinine for self-induced abortion. South.Med.J. 1983 Jul;76(7):846-849.

(175) Kahn G. Dapsone is safe during pregnancy. J.Am.Acad.Dermatol. 1985 Nov;13(5 Pt 1):838-839.

(176) Greenwood AM, Menendez C, Todd J, Greenwood BM. The distribution of birth weights in Gambian women who received malaria chemoprophylaxis during their first pregnancy and in control women. Trans.R.Soc.Trop.Med.Hyg. 1994 May-Jun;88(3):311-312.

(177) Greenwood BM, Greenwood AM, Snow RW, Byass P, Bennett S, Hatib-N'Jie AB. The effects of malaria chemoprophylaxis given by traditional birth attendants on the course and outcome of pregnancy. Trans.R.Soc.Trop.Med.Hyg. 1989 Sep-Oct;83(5):589-594.

(178) Menendez C, Todd J, Alonso PL, Lulat S, Francis N, Greenwood BM. Malaria chemoprophylaxis, infection of the placenta and birth weight in Gambian primigravidae. J.Trop.Med.Hyg. 1994 Aug;97(4):244-248.

(179) Keuter M, van Eijk A, Hoogstrate M, Raasveld M, van de Ree M, Ngwawe WA, et al. Comparison of chloroquine, pyrimethamine and sulfadoxine, and chlorproguanil and dapsone as treatment for *falciparum* malaria in pregnant and non-pregnant women, Kakamega District, Kenya. BMJ 1990 Sep 8;301(6750):466-470.

(180) Bruce-Chwatt LJ, Hutchinson DB. Maloprim and agranulocytosis. Lancet 1983 Dec 24-31;2(8365-66):1487-1488.

(181) Nosten F, ter Kuile F, Maelankiri L, Chongsuphajaisiddhi T, Nopdonrattakoon L, Tangkitchot S, et al. Mefloquine prophylaxis prevents malaria during pregnancy: a double-blind, placebo-controlled study. J.Infect.Dis. 1994 Mar;169(3):595-603.

(182) Smoak BL, Writer JV, Keep LW, Cowan J, Chantelois JL. The effects of inadvertent exposure of mefloquine chemoprophylaxis on pregnancy outcomes and infants of US Army servicewomen. J.Infect.Dis. 1997 Sep;176(3):831-833.

(183) Nosten F, McGready R, D'Alessandro U,Bonell A, Verhoeff F,Menendez C, Mutabingwa T, Brabin B. Antimalarial Drugs in Pregnancy: A Review. Current Drug Safety 2006:1-15.

(184) The Use of Antimalarial Drugs. World Health Organization, Geneva WHO, 2001. Accessed at http://rbm.who.int/cmc_upload/0/000/014/923/about.htm.

(185) Thomas F, Erhart A, D'Alessandro U. Can amodiaquine be used safely during pregnancy? Lancet Infect.Dis. 2004 Apr;4(4):235-239.

(186) Bernuau J, Larrey D, Campillo B, Degott C, Verdier F, Rueff B, et al. Amodiaquine-induced fulminant hepatitis. J.Hepatol. 1988 Feb;6(1):109-112.

(187) Hatton CS, Peto TE, Bunch C, Pasvol G, Russell SJ, Singer CR, et al. Frequency of severe neutropenia associated with amodiaquine prophylaxis against malaria. Lancet 1986 Feb 22;1(8478):411-414.

(188) Olliaro P, Mussano P. Amodiaquine for treating malaria. Cochrane Database Syst.Rev. 2003;(2)(2):CD000016.

(189) Looareesuwan S, Viravan C, Webster HK, Kyle DE, Hutchinson DB, Canfield CJ. Clinical studies of atovaquone, alone or in combination with other antimalarial drugs, for treatment of acute uncomplicated malaria in Thailand. Am.J.Trop.Med.Hyg. 1996 Jan;54(1):62-66.

(190) Mulenga M, Sukwa TY, Canfield CJ, Hutchinson DB. Atovaquone and proguanil versus pyrimethamine/sulfadoxine for the treatment of acute *falciparum* malaria in Zambia. Clin.Ther. 1999 May;21(5):841-852.

(191) Bouchaud O, Monlun E, Muanza K, Fontanet A, Scott T, Goetschel A, et al. Atovaquone plus proguanil versus halofantrine for the treatment of imported acute uncomplicated *Plasmodium falciparum* malaria in non-immune adults: a randomized comparative trial. Am.J.Trop.Med.Hyg. 2000 Nov-Dec;63(5-6):274-279.

(192) Looareesuwan S, Wilairatana P, Chalermarut K, Rattanapong Y, Canfield CJ, Hutchinson DB. Efficacy and safety of atovaquone/proguanil compared with mefloquine for treatment of acute *Plasmodium falciparum* malaria in Thailand. Am.J.Trop.Med.Hyg. 1999 Apr;60(4):526-532.

(193) Sukwa TY, Mulenga M, Chisdaka N, Roskell NS, Scott TR. A randomized, double-blind, placebo-controlled field trial to determine the efficacy and safety of Malarone (atovaquone/proguanil) for the prophylaxis of malaria in Zambia. Am.J.Trop.Med.Hyg. 1999 Apr;60(4):521-525.

(194) McGready R, Stepniewska K, Edstein MD, Cho T, Gilveray G, Looareesuwan S, et al. The pharmacokinetics of atovaquone and proguanil in pregnant women with acute *falciparum* malaria. Eur.J.Clin.Pharmacol. 2003 Oct;59(7):545-552.

(195) Na-Bangchang K, Manyando C, Ruengweerayut R, Kioy D, Mulenga M, Miller GB, et al. The pharmacokinetics and pharmacodynamics of atovaquone and proguanil for the treatment of uncomplicated *falciparum* malaria in third-trimester pregnant women. Eur.J.Clin.Pharmacol. 2005 Sep;61(8):573-582.

(196) Adair CD, Gunter M, Stovall TG, McElroy G, Veille JC, Ernest JM. Chlamydia in pregnancy: a randomized trial of azithromycin and erythromycin. Obstet.Gynecol. 1998 Feb;91(2):165-168.

(197) Donders GG. Treatment of sexually transmitted bacterial diseases in pregnant women. Drugs 2000 Mar;59(3):477-485.

(198) Ogasawara KK, Goodwin TM. Efficacy of azithromycin in reducing lower genital Ureaplasma urealyticum colonization in women at risk for preterm delivery. J.Matern.Fetal.Med. 1999 Jan-Feb;8(1):12-16.

(199) Gray RH, Wabwire-Mangen F, Kigozi G, Sewankambo NK, Serwadda D, Moulton LH, et al. Randomized trial of presumptive sexually transmitted disease therapy during pregnancy in Rakai, Uganda. Am.J.Obstet.Gynecol. 2001 Nov;185(5):1209-1217.

(200) Sarkar M, Woodland CC, Koren G, Einarson AR. Pregnancy outcome following gestational exposure to azithromycin. BMC Pregnancy Childbirth 2006 May 30;6:18.

(201) Andersen SL, Ager A, McGreevy P, Schuster BG, Wesche D, Kuschner R, et al. Activity of azithromycin as a blood schizonticide against rodent and human plasmodia in vivo. Am.J.Trop.Med.Hyg. 1995 Feb;52(2):159-161.

(202) Taylor WR, Richie TL, Fryauff DJ, Picarima H, Ohrt C, Tang D, et al. Malaria prophylaxis using azithromycin: a double-blind, placebo-controlled trial in Irian Jaya, Indonesia. Clin.Infect.Dis. 1999 Jan;28(1):74-81.

(203) Dunne MW, Singh N, Shukla M, Valecha N, Bhattacharyya PC, Dev V, et al. A multicenter study of azithromycin, alone and in combination with chloroquine, for the treatment of acute uncomplicated *Plasmodium falciparum* malaria in India. J.Infect.Dis. 2005 May 15;191(10):1582-1588.

(204) Andersen SL, Oloo AJ, Gordon DM, Ragama OB, Aleman GM, Berman JD, et al. Successful double-blinded, randomized, placebo-controlled field trial of azithromycin and doxycycline as prophylaxis for malaria in western Kenya. Clin.Infect.Dis. 1998 Jan;26(1):146-150.

(205) Ohrt C, Willingmyre GD, Lee P, Knirsch C, Milhous W. Assessment of azithromycin in combination with other antimalarial drugs against *Plasmodium falciparum* in vitro. Antimicrob.Agents Chemother. 2002 Aug;46(8):2518-2524.

(206) Heppner DG,Jr, Walsh DS, Uthaimongkol N, Tang DB, Tulyayon S, Permpanich B, et al. Randomized, controlled, double-blind trial of daily oral azithromycin in adults for the prophylaxis of Plasmodium vivax malaria in Western Thailand. Am.J.Trop.Med.Hyg. 2005 Nov;73(5):842-849.

(207) Price RN, Nosten F, Luxemburger C, ter Kuile FO, Paiphun L, Chongsuphajaisiddhi T, et al. Effects of artemisinin derivatives on malaria transmissibility. Lancet 1996 Jun 15;347(9016):1654-1658.

(208) Chen LJ, Wang MY, Sun WK, Liu MZ. Embryotoxicity and teratogenicity studies on artemether in mice, rats and rabbits. Zhongguo Yao Li Xue Bao 1984 Jun;5(2):118-122.

(209) Clark RL, White TE, A Clode S, Gaunt I, Winstanley P, Ward SA. Developmental toxicity of artesunate and an artesunate combination in the rat and rabbit. Birth Defects Res.B.Dev.Reprod.Toxicol. 2004 Dec;71(6):380-394.

(210) McGready R, Cho T, Keo NK, Thwai KL, Villegas L, Looareesuwan S, et al. Artemisinin antimalarials in pregnancy: a prospective treatment study of 539 episodes of multidrug-resistant *Plasmodium falciparum*. Clin.Infect.Dis. 2001 Dec 15;33(12):2009-2016.

(211) McGready R, Cho T, Samuel, Villegas L, Brockman A, van Vugt M, et al. Randomized comparison of quinine-clindamycin versus artesunate in the treatment of *falciparum* malaria in pregnancy. Trans.R.Soc.Trop.Med.Hyg. 2001 Nov-Dec;95(6):651-656.

(212) Li GQ, Guo XB, Fu LC, Jian HX, Wang XH. Clinical trials of artemisinin and its derivatives in the treatment of malaria in China. Trans.R.Soc.Trop.Med.Hyg. 1994 Jun;88 Suppl 1:S5-6.

(213) Sowunmi A, Oduola AM, Ogundahunsi OA, Fehintola FA, Ilesanmi OA, Akinyinka OO, et al. Randomised trial of artemether versus artemether and mefloquine for the treatment of chloroquine/sufadoxine-pyrimethamine-resistant *falciparum* malaria during pregnancy. J.Obstet.Gynaecol. 1998 Jul;18(4):322-327.

(214) Bounyasong S. Randomized trial of artesunate and mefloquine in comparison with quinine sulfate to treat P. falciparum malaria pregnant women. J.Med.Assoc.Thai. 2001 Sep;84(9):1289-1299.

(215) White NJ. Delaying antimalarial drug resistance with combination chemotherapy. Parassitologia 1999 Sep;41(1-3):301-308.

(216) Adjuik M, Babiker A, Garner P, Olliaro P, Taylor W, White N, et al. Artesunate combinations for treatment of malaria: meta-analysis. Lancet 2004 Jan 3;363(9402):9-17.

(217) Hwang J, Bitarakwate E, Pai M, Reingold A, Rosenthal PJ, Dorsey G. Chloroquine or amodiaquine combined with sulfadoxine-pyrimethamine for uncomplicated malaria: a systematic review. Trop.Med.Int.Health 2006 Jun;11(6):789-799.

(218) Bloland P2. Drug resistance in malaria. WHO monograph WHO/CDS/CSR/DRS/2001.4. World Health Organization, Geneva, Switzerland.

(219) Snow RW, Trape JF, Marsh K. The past, present and future of childhood malaria mortality in Africa. Trends Parasitol. 2001 Dec;17(12):593-597.

(220) Trape JF, Pison G, Preziosi MP, Enel C, Desgrees du Lou A, Delaunay V, et al. Impact of chloroquine resistance on malaria mortality. C.R.Acad.Sci.III. 1998 Aug;321(8):689-697.

(221) Roper C, Pearce R, Bredenkamp B, Gumede J, Drakeley C, Mosha F, et al. Antifolate antimalarial resistance in southeast Africa: a population-based analysis. Lancet 2003 Apr 5;361(9364):1174-1181.

(222) Roper C, Pearce R, Nair S, Sharp B, Nosten F, Anderson T. Intercontinental spread of pyrimethamine-resistant malaria. Science 2004 Aug 20;305(5687):1124.

(223) Mehlotra RK, Fujioka H, Roepe PD, Janneh O, Ursos LM, Jacobs-Lorena V, et al. Evolution of a unique *Plasmodium falciparum* chloroquine-resistance phenotype in association with *pfcrt* polymorphism in Papua New Guinea and South America. Proc.Natl.Acad.Sci.U.S.A. 2001 Oct 23;98(22):12689-12694.

(224) Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, et al. Mutations in the *P. falciparum* digestive vacuole transmembrane protein *PfCRT* and evidence for their role in chloroquine resistance. Mol.Cell 2000 Oct;6(4):861-871.

(225) WHO 2005. Susceptibility of Plasmodium Falciparum to Antimalarial Drugs. Report on global monitoring 1996-2004. WHO/HTM/MAL/2005.1103. Accessed at http://www.who.int/malaria/includes_en/whomalariapublications19982004.htm.

(226) Zalis MG, Pang L, Silveira MS, Milhous WK, Wirth DF. Characterization of *Plasmodium falciparum* isolated from the Amazon region of Brazil: evidence for quinine resistance. Am.J.Trop.Med.Hyg. 1998 May;58(5):630-637.

(227) Jelinek T, Grobusch MP, Loscher T. Patterns of *Plasmodium falciparum* drug resistance in nonimmune travellers to Africa. Eur.J.Clin.Microbiol.Infect.Dis. 2001 Apr;20(4):284-286.

(228) Adam I, Osman ME, Elghzali G, Ahmed GI, Gustafssons LL, Elbashir MI. Efficacies of chloroquine, sulfadoxine-pyrimethamine and quinine in the treatment of uncomplicated, *Plasmodium falciparum* malaria in eastern Sudan. Ann.Trop.Med.Parasitol. 2004 Oct;98(7):661-666.

(229) Brandts CH, Ndjave M, Graninger W, Kremsner PG. Effect of paracetamol on parasite clearance time in *Plasmodium falciparum* malaria. Lancet 1997 Sep 6;350(9079):704-709.

(230) Roche J, Guerra-Neira A, Raso J, Benito A. Surveillance of in vivo resistance of Plasmodium falciparum to antimalarial drugs from 1992 to 1999 in Malabo (Equatorial Guinea). Am.J.Trop.Med.Hyg. 2003 May;68(5):598-601.

(231) Nosten F, ter Kuile F, Chongsuphajaisiddhi T, Luxemburger C, Webster HK, Edstein M, et al. Mefloquine-resistant *falciparum* malaria on the Thai-Burmese border. Lancet 1991 May 11;337(8750):1140-1143.

(232) Fontanet AL, Johnston DB, Walker AM, Rooney W, Thimasarn K, Sturchler D, et al. High prevalence of mefloquine-resistant *falciparum* malaria in eastern Thailand. Bull.World Health Organ. 1993;71(3-4):377-383.

(233) Wernsdorfer WH. Epidemiology of drug resistance in malaria. Acta Trop. 1994 Mar;56(2-3):143-156.

(234) Cerutti C,Jr, Durlacher RR, de Alencar FE, Segurado AA, Pang LW. In vivo efficacy of mefloquine for the treatment of Falciparum malaria in Brazil. J.Infect.Dis. 1999 Dec;180(6):2077-2080.

(235) Mockenhaupt FP. Mefloquine resistance in *Plasmodium falciparum*. Parasitol.Today 1995 Jul;11(7):248-253.

(236) Lobel HO, Varma JK, Miani M, Green M, Todd GD, Grady K, et al. Monitoring for mefloquine-resistant Plasmodium falciparum in Africa: implications for travelers' health. Am.J.Trop.Med.Hyg. 1998 Jul;59(1):129-132.

(237) Olliaro P, Nevill C, LeBras J, Ringwald P, Mussano P, Garner P, et al. Systematic review of amodiaquine treatment in uncomplicated malaria. Lancet 1996 Nov 2;348(9036):1196-1201.

(238) Walker DJ, Pitsch JL, Peng MM, Robinson BL, Peters W, Bhisutthibhan J, et al. Mechanisms of artemisinin resistance in the rodent malaria pathogen *Plasmodium yoelii.* Antimicrob.Agents Chemother. 2000 Feb;44(2):344-347.

(239) Xiao SH, Yao JM, Utzinger J, Cai Y, Chollet J, Tanner M. Selection and reversal of *Plasmodium berghei* resistance in the mouse model following repeated high doses of artemether. Parasitol.Res. 2004 Feb;92(3):215-219.

(240) Ferrer-Rodriguez I, Perez-Rosado J, Gervais GW, Peters W, Robinson BL, Serrano AE. *Plasmodium yoelii:* identification and partial characterization of an MDR1 gene in an artemisinin-resistant line. J.Parasitol. 2004 Feb;90(1):152-160.

(241) McIntosh HM, Olliaro P. Artemisinin derivatives for treating uncomplicated malaria. Cochrane Database Syst.Rev. 2000;(2)(2):CD000256.

(242) WHO. WHO Guidelines for the Treatment of Malaria, 2006. Accessed at www.who.int/malaria/docs/TreatmentGuidelines2006.pdf on 02-12-2006.

(243) Pukrittayakamee S, Chantra A, Simpson JA, Vanijanonta S, Clemens R, Looareesuwan S, et al. Therapeutic responses to different antimalarial drugs in *vivax* malaria. Antimicrob.Agents Chemother. 2000 Jun;44(6):1680-1685.

(244) White NJ. Antimalarial drug resistance. J.Clin.Invest. 2004 Apr;113(8):1084-1092.

(245) Kublin JG, Dzinjalamala FK, Kamwendo DD, Malkin EM, Cortese JF, Martino LM, et al. Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. J.Infect.Dis. 2002 Feb 1;185(3):380-388.

(246) Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, et al. A molecular marker for chloroquine-resistant *falciparum* malaria. N.Engl.J.Med. 2001 Jan 25;344(4):257-263.

(247) Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, et al. Mefloquine resistance in *Plasmodium falciparum* and increased pfmdr1 gene copy number. Lancet 2004 Jul 31-Aug 6;364(9432):438-447.

(248) Gil JP, Nogueira F, Stromberg-Norklit J, Lindberg J, Carrolo M, Casimiro C, et al. Detection of atovaquone and Malarone resistance conferring mutations in *Plasmodium falciparum* cytochrome b gene (cytb). Mol.Cell.Probes 2003 Apr-Jun;17(2-3):85-89.

(249) Durand R, Jafari S, Vauzelle J, Delabre JF, Jesic Z, Le Bras J. Analysis of pfcrt point mutations and chloroquine susceptibility in isolates of *Plasmodium falciparum*. Mol.Biochem.Parasitol. 2001 Apr 25;114(1):95-102.

(250) Wellems TE, Plowe CV. Chloroquine-resistant malaria. J.Infect.Dis. 2001 Sep 15;184(6):770-776.

(251) Basco LK, Ndounga M, Ngane VF, Soula G. Molecular epidemiology of malaria in Cameroon. XIV. *Plasmodium falciparum* chloroquine resistance transporter (PFCRT) gene sequences of isolates before and after chloroquine treatment. Am.J.Trop.Med.Hyg. 2002 Oct;67(4):392-395.

(252) Basco LK, Ringwald P. Analysis of the key *pfcrt* point mutation and in vitro and in vivo response to chloroquine in Yaounde, Cameroon. J.Infect.Dis. 2001 Jun 15;183(12):1828-1831.

(253) Dorsey G, Kamya MR, Singh A, Rosenthal PJ. Polymorphisms in the *Plasmodium falciparum pfcrt* and *pfmdr-1* genes and clinical response to chloroquine in Kampala, Uganda. J.Infect.Dis. 2001 May 1;183(9):1417-1420.

(254) Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature 2000 Feb 24;403(6772):906-909.

(255) Nomura T, Carlton JM, Baird JK, del Portillo HA, Fryauff DJ, Rathore D, et al. Evidence for different mechanisms of chloroquine resistance in 2 *Plasmodium* species that cause human malaria. J.Infect.Dis. 2001 Jun 1;183(11):1653-1661.

(256) Foote SJ, Cowman AF. The mode of action and the mechanism of resistance to antimalarial drugs. Acta Trop. 1994 Mar;56(2-3):157-171.

(257) Basco LK, Eldin de Pecoulas P, Wilson CM, Le Bras J, Mazabraud A. Point mutations in the dihydrofolate reductase-thymidylate synthase gene and pyrimethamine and cycloguanil resistance in *Plasmodium falciparum*. Mol.Biochem.Parasitol. 1995 Jan;69(1):135-138.

(258) Peterson DS, Walliker D, Wellems TE. Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in *falciparum* malaria. Proc.Natl.Acad.Sci.U.S.A. 1988 Dec;85(23):9114-9118.

(259) Basco LK, Tahar R, Keundjian A, Ringwald P. Sequence variations in the genes encoding dihydropteroate synthase and dihydrofolate reductase and clinical response to sulfadoxine-pyrimethamine in patients with acute uncomplicated *falciparum* malaria. J.Infect.Dis. 2000 Aug;182(2):624-628.

(260) Mendez F, Munoz A, Carrasquilla G, Jurado D, Arevalo-Herrera M, Cortese JF, et al. Determinants of treatment response to sulfadoxine-pyrimethamine and subsequent transmission potential in *falciparum* malaria. Am.J.Epidemiol. 2002 Aug 1;156(3):230-238.

(261) Nzila AM, Mberu EK, Sulo J, Dayo H, Winstanley PA, Sibley CH, et al. Towards an understanding of the mechanism of pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: genotyping of dihydrofolate reductase and dihydropteroate synthase of Kenyan parasites. Antimicrob.Agents Chemother. 2000 Apr;44(4):991-996.

(262) Thaithong S, Ranford-Cartwright LC, Siripoon N, Harnyuttanakorn P, Kanchanakhan NS, Seugorn A, et al. *Plasmodium falciparum*: gene mutations and amplification of dihydrofolate reductase genes in parasites grown in vitro in presence of pyrimethamine. Exp.Parasitol. 2001 Jun;98(2):59-70.

(263) Eldin de Pecoulas P, Basco LK, Tahar R, Ouatas T, Mazabraud A. Analysis of the *Plasmodium vivax* dihydrofolate reductase-thymidylate synthase gene sequence. Gene 1998 Apr 28;211(1):177-185.

(264) Berglez J, Iliades P, Sirawaraporn W, Coloe P, Macreadie I. Analysis in Escherichia coli of *Plasmodium falciparum* dihydropteroate synthase (DHPS) alleles implicated in resistance to sulfadoxine. Int.J.Parasitol. 2004 Jan;34(1):95-100.

(265) Kublin JG, Witzig RS, Shankar AH, Zurita JQ, Gilman RH, Guarda JA, et al. Molecular assays for surveillance of antifolate-resistant malaria. Lancet 1998 May 30;351(9116):1629-1630.

(266) Wang P, Lee CS, Bayoumi R, Djimde A, Doumbo O, Swedberg G, et al. Resistance to antifolates in *Plasmodium falciparum* monitored by sequence analysis of dihydropteroate synthetase and dihydrofolate reductase alleles in a large number of field samples of diverse origins. Mol.Biochem.Parasitol. 1997 Nov;89(2):161-177.

(267) Talisuna AO, Nalunkuma-Kazibwe A, Langi P, Mutabingwa TK, Watkins WW, Van Marck E, et al. Two mutations in dihydrofolate reductase combined with one in the dihydropteroate synthase gene predict sulphadoxine-pyrimethamine parasitological failure in Ugandan children with uncomplicated *falciparum* malaria. Infect.Genet.Evol. 2004 Dec;4(4):321-327.

(268) Dorsey G, Dokomajilar C, Kiggundu M, Staedke SG, Kamya MR, Rosenthal PJ. Principal role of dihydropteroate synthase mutations in mediating resistance to sulfadoxine-pyrimethamine in single-drug and combination therapy of uncomplicated malaria in Uganda. Am.J.Trop.Med.Hyg. 2004 Dec;71(6):758-763.

(269) Nelson AL, Purfield A, McDaniel P, Uthaimongkol N, Buathong N, Sriwichai S, et al. *pfmdr1* genotyping and in vivo mefloquine resistance on the Thai-Myanmar border. Am.J.Trop.Med.Hyg. 2005 May;72(5):586-592.

(270) Price RN, Cassar C, Brockman A, Duraisingh M, van Vugt M, White NJ, et al. The pfmdr1 gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand. Antimicrob.Agents Chemother. 1999 Dec;43(12):2943-2949.

(271) Anderson TJ, Nair S, Qin H, Singlam S, Brockman A, Paiphun L, et al. Are transporter genes other than the chloroquine resistance locus (*pfcrt*) and multidrug resistance gene (*pfmdr*) associated with antimalarial drug resistance? Antimicrob.Agents Chemother. 2005 Jun;49(6):2180-2188.

(272) Korsinczky M, Chen N, Kotecka B, Saul A, Rieckmann K, Cheng Q. Mutations in *Plasmodium falciparum* cytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site. Antimicrob.Agents Chemother. 2000 Aug;44(8):2100-2108.

(273) Handunnetti SM, Gunewardena DM, Pathirana PP, Ekanayake K, Weerasinghe S, Mendis KN. Features of recrudescent chloroquine-resistant *Plasmodium falciparum* infections confer a survival advantage on parasites and have implications for disease control. Trans.R.Soc.Trop.Med.Hyg. 1996 Sep-Oct;90(5):563-567.

(274) Robert V, Awono-Ambene HP, Le Hesran JY, Trape JF. Gametocytemia and infectivity to mosquitoes of patients with uncomplicated *Plasmodium falciparum* malaria attacks treated with chloroquine or sulfadoxine plus pyrimethamine. Am.J.Trop.Med.Hyg. 2000 Feb;62(2):210-216.

(275) Baird JK, Purnomo, Basri H, Bangs MJ, Andersen EM, Jones TR, et al. Age-specific prevalence of *Plasmodium falciparum* among six populations with limited histories of exposure to endemic malaria. Am.J.Trop.Med.Hyg. 1993 Dec;49(6):707-719.

(276) Sibley CH, Hyde JE, Sims PF, Plowe CV, Kublin JG, Mberu EK, et al. Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: what next? Trends Parasitol. 2001 Dec;17(12):582-588.

(277) Staedke SG, Sendagire H, Lamola S, Kamya MR, Dorsey G, Rosenthal PJ. Relationship between age, molecular markers, and response to sulphadoxine-pyrimethamine treatment in Kampala, Uganda. Trop.Med.Int.Health 2004 May;9(5):624-629.

(278) Djimde AA, Doumbo OK, Traore O, Guindo AB, Kayentao K, Diourte Y, et al. Clearance of drug-resistant parasites as a model for protective immunity in *Plasmodium falciparum* malaria. Am.J.Trop.Med.Hyg. 2003 Nov;69(5):558-563.

(279) Francis D, Nsobya SL, Talisuna A, Yeka A, Kamya MR, Machekano R, et al. Geographic differences in antimalarial drug efficacy in Uganda are explained by differences in endemicity and not by known molecular markers of drug resistance. J.Infect.Dis. 2006 Apr 1;193(7):978-986.

(280) White NJ. Why is it that antimalarial drug treatments do not always work? Ann.Trop.Med.Parasitol. 1998 Jun;92(4):449-458.

(281) Hastings IM, Watkins WM, White NJ. The evolution of drug-resistant malaria: the role of drug elimination half-life. Philos.Trans.R.Soc.Lond.B.Biol.Sci. 2002 Apr 29;357(1420):505-519.

(282) Watkins WM, Mosobo M. Treatment of *Plasmodium falciparum* malaria with pyrimethamine-sulfadoxine: selective pressure for resistance is a function of long elimination half-life. Trans.R.Soc.Trop.Med.Hyg. 1993 Jan-Feb;87(1):75-78.

(283) Butcher GA. Antimalarial drugs and the mosquito transmission of *Plasmodium.* Int.J.Parasitol. 1997 Sep;27(9):975-987.

(284) Noedl H, Wongsrichanalai C, Wernsdorfer WH. Malaria drug-sensitivity testing: new assays, new perspectives. Trends Parasitol. 2003 Apr;19(4):175-181.

(285) Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE. Pyrimethamine and proguanil resistance-conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. Am.J.Trop.Med.Hyg. 1995 Jun;52(6):565-568.

(286) Farnert A, Arez AP, Correia AT, Bjorkman A, Snounou G, do Rosario V. Sampling and storage of blood and the detection of malaria parasites by polymerase chain reaction. Trans.R.Soc.Trop.Med.Hyg. 1999 Jan-Feb;93(1):50-53.

(287) Farnert A, Arez AP, Babiker HA, Beck HP, Benito A, Bjorkman A, et al. Genotyping of *Plasmodium falciparum* infections by PCR: a comparative multicentre study. Trans.R.Soc.Trop.Med.Hyg. 2001 Mar-Apr;95(2):225-232.

(288) World Health Organization. 1996. World Health Organization. 1996. Assessment of therapeutic efficacy of antimalarial drugs for uncomplicated malaria in areas with intense transmission. WHO/MAL/96.1077. World Health Organization, Geneva, Switzerland.

(289) World Health Organization. 2001. Monitoring antimalarial drug resistance. Report of WHO Consultation, Geneva, Switzerland, 3-5 December 2001.

(290) Adjuik M, Agnamey P, Babiker A, Borrmann S, Brasseur P, Cisse M, et al. Amodiaquine-artesunate versus amodiaquine for uncomplicated *Plasmodium falciparum*

malaria in African children: a randomised, multicentre trial. Lancet 2002 Apr 20;359(9315):1365-1372.

(291) Dorsey G, Njama D, Kamya MR, Cattamanchi A, Kyabayinze D, Staedke SG, et al. Sulfadoxine/pyrimethamine alone or with amodiaquine or artesunate for treatment of uncomplicated malaria: a longitudinal randomised trial. Lancet 2002 Dec 21-28;360(9350):2031-2038.

(292) Stepniewska K, Taylor WR, Mayxay M, Price R, Smithuis F, Guthmann JP, et al. In vivo assessment of drug efficacy against *Plasmodium falciparum* malaria: duration of follow-up. Antimicrob.Agents Chemother. 2004 Nov;48(11):4271-4280.

(293) Ringwald P. Monitoring antimalarial drug efficacy. Clin.Infect.Dis. 2004 Apr 15;38(8):1192-3; author reply 1193-4.

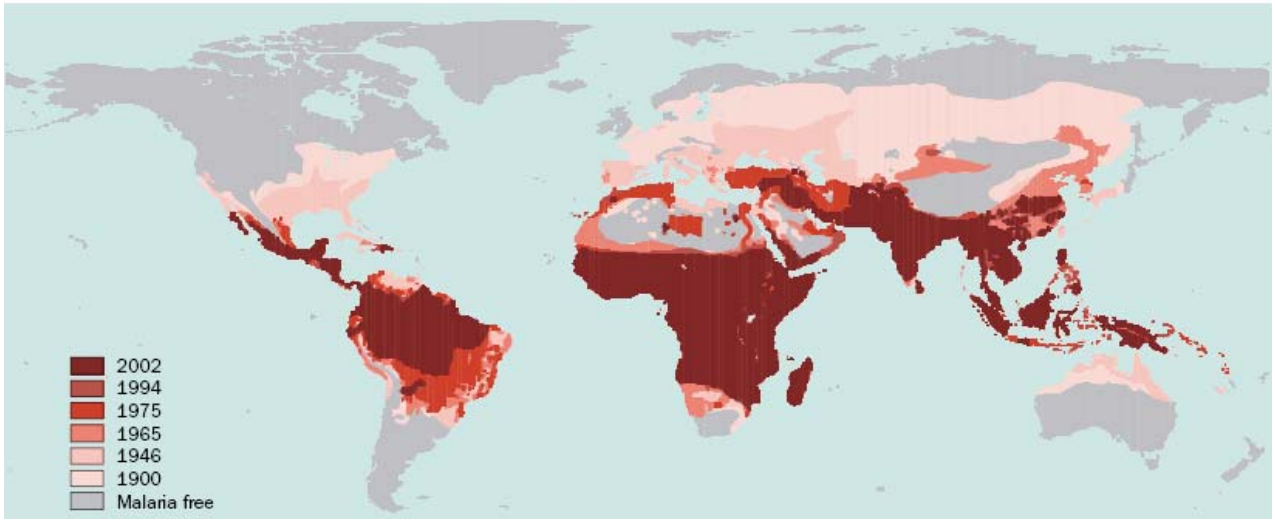
(294) Snounou G, Beck HP. The use of PCR genotyping in the assessment of recrudescence or re-infection after antimalarial drug treatment. Parasitol Today 1998(14):462-467.

(295) White NJ. Assessment of the pharmacodynamic properties of antimalarial drugs in vivo. Antimicrob.Agents Chemother. 1997 Jul;41(7):1413-1422.

(296) Cattamanchi A, Kyabayinze D, Hubbard A, Rosenthal PJ, Dorsey G. Distinguishing recrudescence from reinfection in a longitudinal antimalarial drug efficacy study: comparison of results based on genotyping of msp-1, msp-2, and glurp. Am.J.Trop.Med.Hyg. 2003 Feb;68(2):133-139.

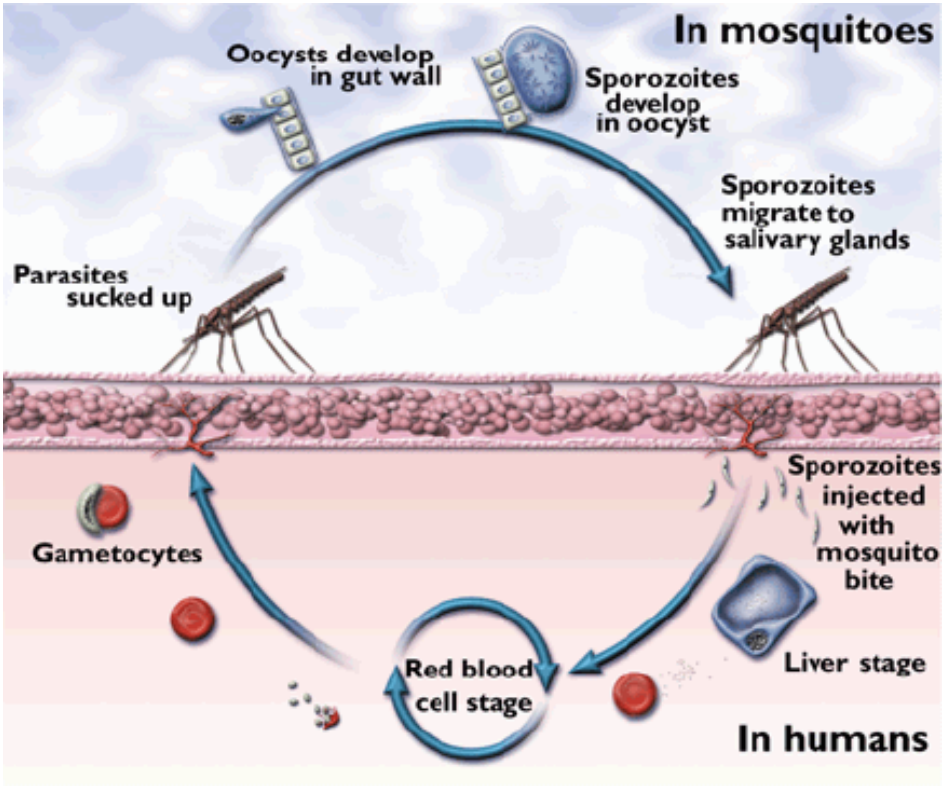
(297) Greenwood B. The molecular epidemiology of malaria. Trop.Med.Int.Health 2002 Dec;7(12):1012-1021.

Figure 1.1. The global distribution of Plasmodium species



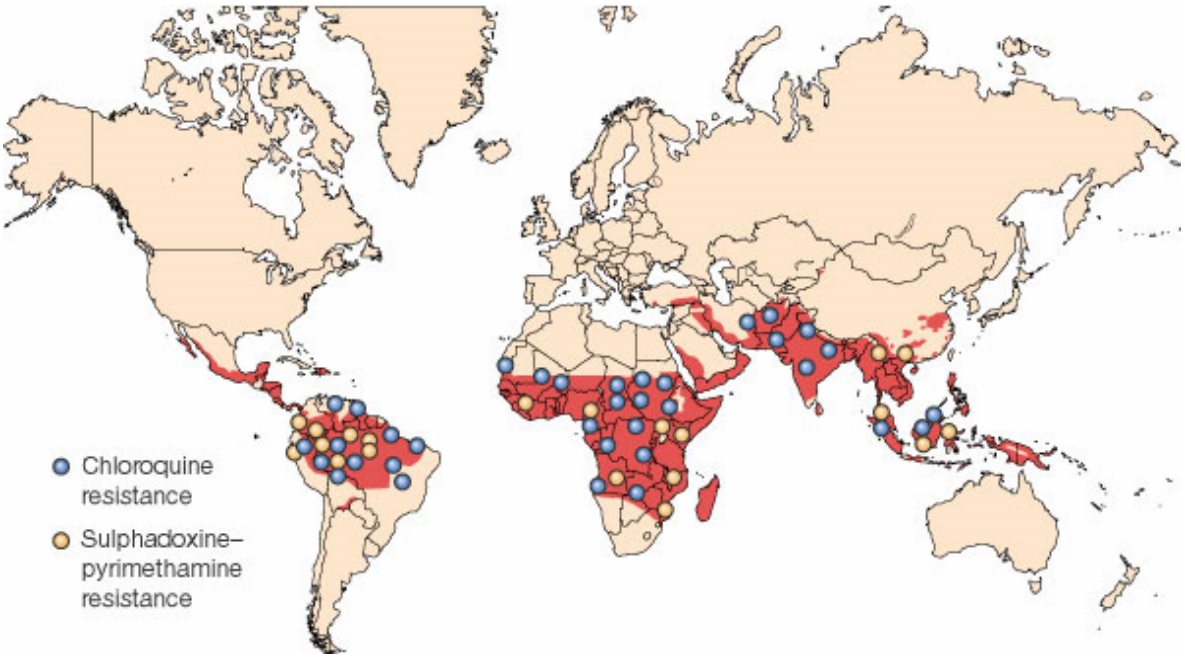
Adapted from, Lancet Infect Dis 2004; 327–36

Figure 1.2. The transmission and life cycle of Plasmodium species



Adapted from *Trends in Parasitology* (2002) 18, 411-418

Figure 1.3. The world distribution of reported resistance to chloroquine and sulfadoxine-pyrimethamine by *Plasmodium falciparum*, 2004



Source: WHO, 2004

Table 1.1.The use and safety of anti-malarial drugs during pregnancy

Drug	Treatment of malaria	Prevention of malaria	First trimester	Second trimester	Third trimester	Conclusion
Chloroquine[157-160]	✓	✓	✓	✓	✓	Safe in the all trimesters of pregnancy
Sulfadoxine-pyrimethamine[9,19,132,163,164]	✓	✓	X	✓	✓	Safe in the second and third trimester of pregnancy
Quinine [155,157]	✓	X	✓	✓	✓	Safe in all trimesters of pregnancy
Dapsone-pyrimethamine [176-178]	✓	X	Limited data available	✓	✓	Limited data available
Dapsone-chlorproguanil [179]	✓	X	Limited data available	✓	✓	Limited data available
Mefloquine [10,169,181,182]	✓	✓	Limited data available	✓/X	✓/X	Conflicting results
Amodiaquine [185]	✓	X	Limited data available	✓	✓	Limited data available
Malarone [194,195]	✓	X	Limited data available	Limited data available	Limited data available	Limited data available
Azithromycin [201-206]	✓	X	✓	✓	✓	Limited data available
Artesunate [148,148-150,208-210,210-214]	✓	X	Not known	✓	✓	Limited data available
Halofantrine [166]	X	X	X	X	X	Not safe
Tetracycline/Doxycycline [166]	X	X	X	X	X	Not safe
Primaquine [166]	X	X	X	X	X	Not safe

✓- safe: X- not safe

CHAPTER 2

OBJECTIVES, STUDY DESIGN, AND METHODS

2.1. Specific Aim I

To investigate the effect of the timing and frequency of *P. falciparum* infection during pregnancy on low birth weight and maternal hemoglobin concentration

2.1.1. Rationale

While it is clear that *P. falciparum* infection during pregnancy increases the risk for low birth weight (LBW) and maternal anemia [1-7], it is not known whether this effect depends on the time at which the infection occurs during pregnancy. It has also not been established whether the number of episodes of *P. falciparum* infection experienced during pregnancy determine the severity of these malaria-related adverse outcomes. Most previous studies have only examined the association between *P. falciparum* infection and these adverse outcomes at one point in time during pregnancy [4-6,8]. They have commonly used placental infection as a standard indicator of malaria infection during pregnancy. However, this indicator, as any single measure, may not accurately describe the evolution of infection status throughout pregnancy. Furthermore, some of the studies have limited recruitment to primigravidae [9]. Although the risk of malaria infection and its complications is highest in primigravidae [2], *P. falciparum* infection in multigravidae is not infrequent, and still poses a significant risk for severe anemia and LBW. In most malaria-endemic areas, resources are limited, women do not have frequent access to antenatal care services, and there is usually a shortage of drug supplies. The effect of frequency and timing of infection can provide information about the crucial risk period during pregnancy

relative to malaria infection when intervention would have the most important effect. This would aid health policy makers to adapt existing malaria prevention programs to be most effective in preventing the adverse effects of malaria during pregnancy.

2.1.2. Objectives

1. The primary objective was to investigate the effect of the frequency of episodes of *P. falciparum* infection experienced during pregnancy on the risk of low birth weight and maternal anemia.
2. The secondary objective was to investigate the effect of timing of *P. falciparum* infection during pregnancy on the risk of low birth weight and maternal anemia.

2.1.3. Primary hypotheses

1. The risk of LBW and maternal anemia increases with the number of episodes of *P. falciparum* infection detected during pregnancy

2. *P. falciparum* infection in the third trimester of pregnancy increases the risk of LBW and maternal anemia more than infection in the second trimester of pregnancy.

2.1.4. Study design and methods

Study site and population

The study was conducted at Mpemba and Madziabango Health Centers in Blantyre district, in the southern region of Malawi. Blantyre District covers 2,012 km^2 with a population of approximately 950,000 people [10]. Sulfadoxine pyrimethamine (SP) has been the standard treatment for uncomplicated malaria and presumptive treatment for most fevers since 1993 at all health facilities, and the private sector in Blantyre. IPT is routinely available as part of the antenatal care in Malawi. The two health centers have maternity beds for delivery of low risk pregnancies, 4 full-time and 9 part-time nurses, and a malaria microscopy laboratory with a trained medical technologist. There are 18 traditional birth attendants (TBAs) that take care of births that occur in the community. Approximately 200 pregnant women have their first antenatal visit every month at the 2 health centers. Women with high risk pregnancies are referred to Queen Elizabeth Central Hospital (QECH), a tertiary hospital located about 10 km away from the health centers for management and delivery.

Study recruitment and enrollment

All pregnant women attending routine antenatal care at the two health facilities were eligible to participate in the study. A total of 2,462 women were recruited over a period of 15 months. Written or witnessed verbal consent was sought from the women for participation in the study prior to enrolment. Women who gave consent to participate in the study were interviewed using a standardized questionnaire to obtain demographic

information, past obstetric history and factors associated with use of malaria prevention measures including use of ITNs and anti-malarial drugs during the current pregnancy. The women also received routine antenatal assessment which included abdominal examination, height, weight, temperature, blood pressure and fetal heart rate measurements, and examination for the presence of high risk factors for the pregnancy. Gestational age was estimated by a midwife using the last menstrual period and the symphysis-fundal height. A venous blood sample was collected for syphilis testing (in accordance with the Malawi government national policy), maternal hemoglobin concentration estimation and preparation of thick blood films. The women were given a dose of SP for IPT under direct observation by the antenatal clinic personnel. Antenatal records were checked before administering SP to ensure that the women had not received SP in the previous 4 weeks prior to enrolment. Study participants were given a study number to retain until delivery for easy identification during follow-up.

Follow-up

The follow-up visits were scheduled according to the normal antenatal routine care. Depending on the gestation age at enrolment, women were seen at these time points: 12 weeks, 26 weeks, 32 weeks and 36-38 weeks age and at delivery. At these scheduled visits, women received routine antenatal care. This included abdominal examination, blood pressure and fetal heart rate measurement. A standardized questionnaire was used to obtain information on history of malarial illnesses, and use of anti-malarial drugs outside the study setting. A peripheral blood sample was obtained for examination of thick films.

The women were given another dose of SP, at least 4 weeks after the initial dose according to the Malawi national policy schedule.

Delivery

The majority of women in the study area deliver at the health centers. However, there are some women who deliver in the community under the supervision of TBAs. For women who delivered at the health center, blood samples were obtained to measure maternal hemoglobin concentration, and to prepare peripheral, placental and cord thick films. Newborns were weighed within 24 hours of delivery using a digital scale to the nearest gram. A system was set up such that community nurses and TBAs were able to identify women enrolled into the study who delivered at home. TBAs were trained to prepare peripheral, placental and cord blood smears from women who delivered in the community, and to forward the samples to the clinics through community health workers. Newborns delivered in the community were weighed using color coded scales that indicated green if the baby weighed more than 2500 grams and red if the baby weighed less than 2500 grams.

Laboratory procedures

Diagnosis of malaria

Malaria diagnosis was performed using microscopy, which remains the standard for laboratory confirmation of malaria. Thick films were prepared from blood obtained from the subjects at enrollment, subsequent visits and at delivery. The slides were air dried and then flooded with buffered water (pH 7.2) for 3-5 minutes to allow the lyses of red blood cells. This was followed by staining by flooding the slide with 10% phosphate-

buffered Giemsa for 30 minutes, and then rinsing with tap water and air-drying [11]. Slides were examined under a microscope using a magnification of 100 x oil immersion objective to detect and quantify parasitemia. Assuming a standard white blood cell count of 6,000 per microliter, the number of parasites present until 200 leucocytes were counted, and multiplied by 30 to estimate the parasite density per microliter of blood. A thick film was considered negative if no parasites were detected after examining 100 microscopic fields each containing approximately 20 white blood cells. All thick films were examined by two skilled microscopists and any discrepancies (positive *versus* negative or more than 25% difference in parasite density) were solved by repeating the readings.

Hemoglobin concentration

Hemoglobin concentration was measured using a HemoCueÒ machine (HemoCue Incorporated, Angelholm, Sweden). A sample of 10µl of blood obtained from the study participant was pipetted into a cuvette and inserted into the machine. The microcuvettes were stored at 59-86°F (15–30°C) in a dry place and the containers were closed at all times.

Human subjects

IRB approval for the study was granted by the University of North Carolina and the College of Medicine, University of Malawi. The study nurses ensured that all the study procedures were explained, before obtaining informed consent. Personal information was not included on study documents and samples to ensure confidentiality.

Blood samples were obtained by well trained nurses to ensure safety and minimize side effects.

2.1.5. Data management

Data were collected on standardized proformas and dually entered, and validated into a database (ACCESS, Microsoft Corporation, Virginia, USA). Each study proforma was entered as a separate table linked in the database by the unique identifier assigned to each study subject. Personal identifiers were maintained separately in one main file in a password protected computer file and used only as necessary to match this information with the other databases. Only the investigators of the study had access to this information. To ensure quality control, questionnaires were checked at the end of each interview. On a monthly basis, a random sample of computerized records was compared with hard-copy case report forms for confirmation of consistency. Questionable values were flagged and cross-checked with paper records. When a discrepancy was noted, the nurse who was involved in entering the data was consulted to resolve the discrepancy, and if necessary, and if necessary and possible, the study participant was also contacted to resolve the issue. Data analysis was conducted using STATA version 8 (StataCorp, College Station, USA).

2.1.6. Data analysis

A priori α-level of 0.05 was set for all statistical comparisons unless otherwise specified. Below is a description of the main study variables:

Dependent variables

Birth weight: Birth weight was analyzed as a categorical variable. Newborns were classified as having normal birth weight (≥ 2500 g) or low birth weight (< 2500 g), regardless of gestational age.

Maternal anemia: Hemoglobin concentration was also analyzed as a categorical variable. Women were classified as having anemia if they had hemoglobin concentration less than 11g/L. Anemia was further classified into mild anemia (9-10.9 g/dL), moderate anemia (7-8.9 g/dL) and severe anemia (<7g/dL) [12].

Independent variable

The main exposure variable, malaria infection during pregnancy, was defined as follows:

1. The frequency of malaria infection was categorized according to the total number of episodes malaria experienced by the women between enrolment and delivery. We were expecting that very few women would have more than 3 episodes of malaria detected during pregnancy. Therefore, the frequency of malaria infection was analyzed as a disjoint indicator variable with 3 levels: 0 (the referent group representing women who had no malaria during pregnancy; 1 (women who had one episode of malaria detected); 2 (women who had 2 or more episodes of malaria detected between enrolment and delivery).

To assess if there was a dose-response relation in the risk of low birth weight or maternal anemia with increase in the number of episodes of malaria, the frequency of infections was coded using incremental coding, and women who had no infection during pregnancy were the reference category. The coding was as follows;

Z_1= 1 if ≥ 1 episode of malaria during pregnancy, 0 otherwise

Z_2=1, if ≥ 2 episodes of malaria during pregnancy, 0 otherwise

2. The timing of malaria infection during pregnancy was classified as:

(a) Malaria infection in the antenatal period only, and at delivery only.

(b) Malaria infection in the first trimester (gestation age ≤ 12 weeks); infection in the second trimester (gestation age 13-28 weeks); and infection in the third trimester (gestation age 29 weeks-delivery). Because very few women attend antenatal clinics in the first trimester of pregnancy, we were expecting very few women to be in the first trimester category. Therefore women who had infection in the first trimester of pregnancy were excluded from this analysis.

For both definitions of timing of infection, women who had no malaria infection during pregnancy were the reference group.

Other study variables

Gravidity was included in the analysis as an effect measure modifier. The other variables; age, use of insecticide treated bed-net (ITNs), use of SP for IPT, body mass

index (BMI), use of anti-malarial drugs prior to enrolment into the study, and gestation age at enrolment into the study were treated as potential confounders.

Univariate analyses

Univariate analyses involved examination of the distribution of baseline socio-demographic characteristics of the study participants. Descriptive statistics for continuous characteristics included frequencies, means, standard deviation and range. The distribution of continuous variables was examined for presence of outliers, and to decide whether there was need for transformation, rescaling or recoding into categorical variables. Cut-off points were developed using information from previous literature, critical percentiles or based on the distribution of data. The distribution of categorical data was described using frequencies and percentages. Some of the nominal variables with more than two categories were collapsed to a smaller number of categories if there were small numbers in some categories.

Bivariate analyses

Bivariate analyses compared baseline socio-demographic characteristics across the different exposure groups. Normally distributed continuous variables were compared using analysis of variance, and non-normally distributed continuous variables were compared using the Kruskal-Wallis tests. Differences in proportions for categorical variables were compared using the Chi-square test. Tabular analyses were also used to measure the association between categorical covariates and the exposure and outcome variables. For continuous variables, the assumption of linearity was assessed by creating

categories based on previous literature, using critical percentiles or based on the distribution of the data, and assessing a plot of the log odds of the outcomes in the created categories for linearity. For ordinal variables, linearity of the log odds of the outcome was assessed while maintaining the original categories if sufficient data was available for all categories or combining categories with insufficient data. When the assumption of linearity was violated, continuous and ordinal variables were converted into indicator variables.

Assessment of effect measure modification

The presence of effect measure modification was assessed using both tabular and regression analyses. In tabular analyses, effect estimates were computed for each stratum of the covariate. The Breslow Day test for homogeneity was used to compare the strata estimates. An a priori significance level of 0.15 was chosen, because the test for homogeneity has low power. Effect measure modification was also considered present if the effects in the stratum were different in relation to the null. Further evaluation of effect measure modification using regression analysis consisted of creating an interaction term between the main exposure variable and the potential effect measure modifier. This model was compared to one without the interaction term using the Likelihood ratio test. Effect measure modification was considered present if the Wald test p-value for the interaction term was less than 0.05. If effect measure modification was present, separate estimates were reported for each strata of the effect measure modifier.

Assessment of confounding

The directed acyclic graph (DAG) was used to identify potential confounders of the association between malaria and LBW or anemia [13]. Using the DAG criteria, a variable was considered a potential confounder if it was associated with the exposure and the outcome, but the variable was not an effect of the exposure or outcome, and there was an unblocked backdoor pathway from the exposure to the outcome. A backward elimination strategy was used to make select the best model that explained the association between exposure to malaria during pregnancy and low birth weight or maternal anemia. Confounding was assessed using the change in estimate criteria. The absolute change in estimate of at least 0.10 comparing the difference in the log odds of the crude estimate (full model) and the adjusted estimate (reduced model) was considered to indicate the presence of confounding by that variable. The full model, containing all the potential confounders was compared with models without each of the covariates. Variables that caused the least change in estimate were dropped subsequently until all variables left in the model caused a change in estimate of at least 0.10. If there was effect measure modification, the change in estimate was assessed for the individual stratum specific estimates in the full model and corresponding stratum specific estimates in the reduced model. The following variables were included as potential confounders: ITNs use (yes/no); use of SP (none, 1 dose, 2 doses and 3 doses); use of anti-malarials prior to enrolment (yes/no); and gestation age at enrolment (second/third trimester). Maternal age which was continuous in the original dataset was converted into a binary variable, dichotomized at the age of 20 years, after assessing for linearity. Body mass index was entered as continuous in the model.

Multivariate analyses

1. Malaria and low birth weight

Multivariate analysis of the association between frequency and timing of *P. falciparum* infection during pregnancy and LBW or maternal anemia was performed using binomial regression [14]. The full multivariate model included the outcome, main exposure variable, and all variables that were identified as confounders. Analyses were stratified by the number of follow-up visits. Gravidity was considered an effect measure modifier based on previous studies that have consistently found a difference in the effect of malaria on LBW and maternal anemia according to parity [15] . Therefore, effect estimates were reported separately for primigravidae (PG), and women of other parities (MG). The beta coefficients for the model were estimated using the Maximum likelihood method.

The equation for the full model was as below:

$$P(Y_1=1|\mathbf{X}_1) = \exp(\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots\ldots\ldots + \beta_k X_k)$$

Where Y is the dependent variable (low birth weight, maternal anemia)

$\mathbf{X}_1$ is vector representing the independent variables (exposure to malaria and the propensity score)

β represents the parameters estimating the effect of covariates in the model

We used the Cox proportional hazards model and the Poisson regression model to confirm the estimates obtained from the binomial regression model. We assigned a constant risk period to every woman in the study so that the hazard rate ratio estimated by Cox regression and rate estimated by the Poisson regression model would equal the prevalence ratio [16,17]. A robust variance estimator was included in the Cox proportional hazards model and the Poisson model for correction of the standard errors [18]. The goodness-of-fit of the final model was also assessed using the Deviance test and examination of the distribution of standardized Pearson residuals for covariate patterns that were outliers.

2.2. Specific Aim II

To assess the efficacy of azithromycin or artesunate combined with SP compared with SP monotherapy as treatment for malaria in pregnant women

2.2.1. Rationale

Currently SP is the drug of choice for IPT and treatment of malaria in pregnant women in Malawi and other countries in sub-Saharan Africa [19]. With the emergence of resistance, the efficacy of SP is decreasing. This has raised concerns about its effectiveness in reducing malaria-related adverse events during pregnancy. There are insufficient safety data on the newer anti-malarial drugs and, therefore, a need to evaluate new anti-malarial drugs that could be used for prevention and treatment in pregnant women. One of the strategies being advocated to delay the development of drug resistance to anti-malarial drugs is the use of artemisinin-based combination therapy. Artemisinin derivatives are highly efficacious, rapidly active, and have action against a broader range of parasite developmental stages [20]. Artesunate, one of the artemisinin derivatives has been used for treatment of malaria in malaria in combination with other drugs [21-23]. However, due to the small number of women recruited into these studies, there is still need for more studies to assess its safety during pregnancy before it can be recommended for routine use. Azithromycin, a macrolide antibiotic, has anti-malarial activity [24,25]and has been used widely in pregnant women to control sexually transmitted infection (STIs) such as gonorrhea and Chlamydia with a good safety profile [26] However there have been

no studies that have assessed its efficacy in treating and preventing malaria in pregnant women.

2.2.2. Objectives

The primary objective of the study was:

1. To compare the recrudescence rates of malaria following SP monotherapy, SP-artesunate and SP-azithromycin

The secondary objectives of the study were:

1. To compare the parasite clearance times of SP monotherapy, SP-artesunate, and SP-azithromycin
2. To examine the tolerability of SP-artesunate and SP-azithromycin.
3. To examine the effect of SP-artesunate and SP-azithromycin on birth outcomes, maternal parasitemia and hemoglobin concentration.

2.2.3. Primary hypothesis

Hypothesis addressed include:

1. Treatment with SP-azithromycin and SP-artesunate results in a few recrudescence rates compared with SP monotherapy.
2. Parasite clearance time is faster for SP-azithromycin and SP-artesunate compared with SP monotherapy

3. SP-azithromycin and SP-artesunate administration during pregnancy does not cause any severe maternal and pregnancy outcomes
4. SP-azithromycin and SP-artesunate reduce the risk of low birth weight, maternal parasitemia and anemia

2.2.4. Study design and methods

Study setting

The study setting was the same as already described for Specific Aim 1

Study Population

All pregnant women presenting at the two health centers for antenatal care were considered eligible for the study if they had peripheral parasitemia, were 15 to 49 years old, the fetal gestation age was between 14 and 26 weeks, the mother had felt fetal movements and if the women were available for follow-up during the entire period of the study. Women were excluded from the study if they had a history of chronic diseases such as tuberculosis, diabetes, kidney and/or liver disease, multiple gestations, a mental disorder that could have affected their comprehension of the study or success to follow-up, known allergies to drugs containing sulfonamides, macrolides or pyrimethamine, pregnancy complications at enrollment, and received anti-malarial drugs within 28 days before enrollment. A total of 141 women were recruited over a period of 8 months from September 2003 to May 2004.

Study procedures

Informed consent was obtained prior to enrollment into the study. Each woman was assigned a unique identification number and an identifying sticker was placed on their antenatal cards. Detailed contact information was obtained to be used at a later date to trace subjects, if they did not return for follow-up visits. Demographic information, past medical and obstetric history was obtained using a standardized questionnaire through an interview conducted by trained study nurses. The women also received routine antenatal assessment which included height, weight, temperature and blood pressure measurements and abdominal examination. Study nurses also investigated for the presence of high risk factors of the pregnancy. All the women enrolled into the study had the option to receive HIV counseling and testing. The counseling was done by trained counselors who were fluent in the local language, Chichewa. A venous blood sample was obtained for measurement of maternal hemoglobin concentration, preparation of thick films, and syphilis testing.

Subjects were randomly assigned to 3 treatment arms using computer generated random numbers that were grouped into blocks of 4-10. The three treatment arms consisted of: (1) SP (3 tablets) only; (2) SP (3 tablets) plus azithromycin 1g/day for 2 days and (3) SP (3 tablets) plus artesunate 200 mg/day for 3 days. Administration of the study drug was under direct observation by the study nurse. If the medication was vomited within 30 minutes of ingestion, another similar dose was administered. Women were asked to remain at the health center for at least an hour after the administration of drugs. Women who lived far away were asked to stay at the health center for 2 nights or were

visited by the study nurse in their home village. The women were given 200 mg of ferrous sulfate and 0.25 mg of folic acid for daily administration throughout pregnancy as part of the Malawi government policy. They were also given insecticide-treated bed-nets (ITNs) with instructions on how to use them.

Follow-up

Each woman received two doses of the assigned treatment and these were given at least 4 weeks apart. Thick films were examined on days 1,2,3,7 and 14 after receiving the assigned treatment. Body temperature was recorded by the study nurses every 6 hours until it became normal (<37.5 ^{0}C). Women with peripheral parasitemia between day 7 and 28 after administration of the first or the second dose of study drug were treated with quinine 600 mg, 3 times a day for 5 days as per Malawi government policy. At all the visits, blood samples (50 μl spots, 4 per sample) were spotted onto filter paper, air dried and stored in sealed plastic bags with dessicant for molecular analysis. The women were followed until delivery and were asked not to self-administer anti-malarial drugs. Instead they were asked to return to the health centers for evaluation any time they experienced symptoms of malaria, were febrile and for any other reason between scheduled visits. At each of the subsequent visits, they received routine antenatal care, and a standardized interview was conducted to obtain information on malarial illnesses, use of anti-malarial drugs and potential side effects of the study drugs. Thick blood films were also prepared.

Delivery

The community TBAs and nurses at the QECH labor and delivery wards were enlisted to cooperate with this study. They were trained to obtain the requisite samples and information. All HIV-positive women were given nevirapine (Roxane Laboratories, Columbus, Ohio, USA) at the onset of active labor according to the HIVNET 012 protocol [27] which was adopted by the Government of Malawi to prevent mother-to-child transmission (MTCT) of HIV. Newborns delivered at the health centers or QECH were weighed using a digital scale, and gestational age was assessed using the Ballard score within 24 hours of delivery by trained study nurses [28]. Head and arm circumference and body length were measured using a tape measure. The newborns were also examined for the presence of congenital anomalies.

Full thickness biopsies from a healthy paracentric area of the placenta were collected in 10% neutral buffered formalin for preparation of histology slides. A blood sample was obtained from a peripheral vein, the placenta and umbilical cord to prepare thick films, blood spots on a filter paper and to estimate maternal hemoglobin concentration. The filter papers were air-dried and stored in plastic Ziploc bags with dessicant for molecular assays. If a TBA was present for women delivering at home, birth weight was measured using color-coded scales that indicated green if the baby weighed more than 2500 grams and red if the baby weighed less than 2500 grams. Peripheral, placental and cord thick blood films were prepared and forwarded to the health centers through community health workers.

Infant follow-up

The women and their babies returned to the health centers at 1 and 4 weeks after delivery and their general health status was assessed. An attempt was made to determine the cause of any neonatal death by questioning the mother about premortal symptoms using an unstructured questionnaire. Additional follow up visits were at 1, 3, 6 and 12 months after delivery when the children were seen at the under five clinics. Health Surveillance Assistants (HSAs) tracked women and children who did not return and encouraged them to attend the clinics.

Monitoring of adverse events

All adverse events were followed closely by the study staff. Symptoms present at baseline were considered to be attributable to malaria. All patients requiring hospitalization were monitored at the health centers or were referred to QECH and visited daily in order to document progression/resolution of the adverse event and to provide any clinical services deemed necessary. Some of the clinical events considered serious were seizures, coma, diabetic ketoacidosis, diffuse petechiae and disseminated intravascular coagulation. Less serious adverse events were monitored at the health centers with outpatient management as deemed appropriate by the study physician. Treatment was discontinued if there were serious adverse events and these were reported to the institutional review boards (IRBs) of the University of Malawi, College of Medicine and the University of North Carolina within 48 hours. A Data Safety Management Committee (DSMB) was put in place to review data if there was an occurrence of serious adverse events from azithromycin or artesunate patients.

Laboratory Procedures

Malaria diagnosis

The diagnosis of malaria was the same as that described for specific Aim 1

Placental biopsies

Fixed placental biopsies were wax embedded and 4μm thick sections were cut into slides. Histological slides were prepared by staining with Gurr's modified Giemsa and/or Hematoxylin and Eosin [8]. The slides were examined for the presence of *P. falciparum* infected erythrocytes, and hemozoin deposition in fibrin and monocytes. Using a systematic method, 500 intervillous blood cells were counted under oil immersion to determine the level of parasitemia. The slides were classified into: (1) parasites, no pigment in monocytes or fibrin, (2) parasites, pigment in monocytes ± fibrin, (3) parasites, pigment in fibrin, (4) no parasites, pigment only (past infection), and (5) no parasites or pigment (no infection) [8]. Placental histology was examined by an experienced pathologist without prior knowledge of the blood film microscopy results, treatment group assignment, maternal characteristics or pregnancy outcomes.

Distinguishing reinfection from recrudescence

Molecular genotyping of the *msp-1* gene using a heteroduplex tracking assay (HTA) was performed on paired samples of women who had more than one episode of malaria during follow-up to distinguish between recrudescence and reinfection. The *msp-1* gene is highly variable, and therefore allows the identification of different strains of

infection [29]. The HTA used a radioactive labeled oligonucleotide, which when bound to similar sequences, migrated down a polyacrylamide gel electrophoresis at a speed determined by the shape of the DNA duplexes [30,31]. A strain with a unique sequence was identified in samples based on the location of the band on a gel. Investigators were blinded of the treatment allocation when deciding the outcome of a subsequent episode of malaria.

HIV diagnosis

HIV test was performed simultaneously using two enzyme immunoassays (EIA), Determine HIV-1/2 Rapid Test (Abbott Laboratories, Illinois, USA) and Unigold Test (Trinity Biotech plc, Dublin, Ireland) according to the manufacturers' instructions. If these tests produced discordant results, a third assay, the Hemastrip Rapid Test (ChemBio Diagnostic Systems) was used to resolve the discrepancy.

Measurement of Hemoglobin Levels

Measurement of hemoglobin concentration was the same as that described for specific Aim 1

Human Subjects

IRB approval for the proposed study was granted by the University of North Carolina and the College of Medicine, University of Malawi. To ensure privacy and confidentially, HIV pre- and post-test counseling was conducted in private settings by trained counselors. The administered dose of SP was according to the Malawi government

national guidelines, and the doses of azithromycin and artesunate were similar to those used in previous studies which reported no adverse events [26,32,33]. All drugs were purchased in one batch and stored at a dry cool place, to maintain quality.

2.2.5. Data Management

Procedures for data management were the same as described for specific aim I.

2.2.6. Data analysis

A priori α-level of 0.05 was set for all statistical comparisons unless otherwise specified. Below is a description of main study variables.

Dependent Variables

***Recrudescence*:** The primary outcome of interest was time to recrudescence after treatment with the assigned study drug. Recurrent infections were classified as recrudescence if parasitemia was detected from day 7 after drug administration [34]. Recurrent episodes detected between days 7 and 14 were classified as recrudescence without genotyping because they were assumed very unlikely to be a new infection. For recurrent episodes that were detected after day 14, we genotyped the *msp-1* gene to distinguish between recrudescence and new infections. Recurrent episodes that were genotyped were classified as recrudescence if the recurrent episode contained all or a

subset of variants (at least one identical uncommon band with prevalence <10% or at least 2 common bands with a prevalence >10%) that were present in the initial infection.

Parasite clearance time: This was defined as the time, in days, from the beginning of anti-malarial treatment until parasites were no longer detectable in a peripheral thick blood film.

Birth outcomes: Abortion was defined as delivery of a non-viable fetus before 28 weeks gestation. Stillbirth was defined as delivery of a dead fetus after 28 weeks of gestation. Death occurring within 28 days of delivery was classified as neonatal death. Low birth weight was defined as weight less than 2500 g at delivery.

Maternal anemia: Women were classified as being anemic if they had hemoglobin concentration less than 11g/dL [12].

Independent variable

Treatment groups: The main exposure of interest was the treatment group. Data analysis was performed using the intention to treat principle. None of the women crossed over from the original assigned group to another treatment group during the follow-up period.

Other study variables

Variables included in the analysis as potential effect measure modifiers or confounders were maternal age, gravidity, education and socio-economic status. Socio-

economic status was evaluated using a number of questions which enquired about the type of job, roofing and wall material of the house, sanitary facilities, source of drinking water and possession of a car, radio, mattress or bicycle. Clinical characteristics included baseline parasite density, HIV-infection status and use of quinine after recrudescence.

Univariate analysis

Univariate analyses involved the examination of the distribution of baseline demographic and clinical characteristics of the women by treatment group. Descriptive statistics for continuous characteristics included frequencies, means, standard deviation and range. The distribution of continuous variables was examined to identify possible cut-off points for multivariate analysis. The presence of outliers was noted and cross-checked with raw data for inconsistencies. The distribution of categorical and ordinal data was described using frequencies and percentages. Some categorical variables with more than two levels were either dichotomized, or changed to indicator variables with more than two levels, if the assumption of linearity was violated.

Survival analysis was used to determine the efficacy of SP-artesunate and SP-azithromycin compared with SP monotherapy. Individuals had an event if they had an episode of malaria that was classified as recrudescence during the follow-up period. Individuals were censored if they were lost to follow-up or had no event at the end of follow-up (delivery). Individuals contributed to the time at risk from the time they had an episode of malaria until they had an event, or when censored. There was no difference in

the distribution of failure times in censored and non-censored individuals when compared conditional on outcome. Therefore it was concluded that censoring was non-informative.

The mechanism of missingness of data was also investigated. Except for HIV status which had 37% of the data missing, none of the other baseline characteristics in the dataset had missing data. There was no difference in the failure times and recrudescence rates between women who had an HIV test and those who did not have an HIV test. Data was missing on outcomes [maternal parasitemia (28%), hemoglobin concentration (29%) and birth weight (16%)] because women had moved out of the study areas, were lost to follow-up, delivered outside the study area or blood samples were not collected during a home delivery. There was no significant difference in the baseline characteristic between women who were lost to follow-up and those not lost to follow-up. Therefore, we assumed that missingness was completely at random, and complete case analysis was performed.

Bivariate analysis

Kaplan-Meier survival curves were used to calculate the conditional survival probability for the independent predictors of treatment outcome. Comparison of the survival curves was performed using the Log-rank or the Wilcoxon test. The assumption of a constant proportion hazard over time for the independent predictors was assessed graphically using log (-log (S (t))) curves, and the Cox test by adding an interaction term between a predictor and time in a Cox proportional hazards model. If the time dependent covariates were significant, it indicated that the hazards were not proportional and the

assumption was relaxed by either stratifying by that variable or including a time interaction term in the model.

Analysis of the secondary outcomes (the cumulative incidence proportion of abortions, still births, low birth weight, maternal anemia and adverse events) was performed using tabular analysis. Differences in the proportions of the outcomes across the treatment groups were compared using the Fischer exact methods.

Multivariate analysis

Multivariate analysis of the efficacy of SP-azithromycin and SP-Artesunate compared with SP monotherapy was performed using a conditional risk-set Cox proportional hazards model, an extension of the Cox model that was stratified by the number of treatment doses received [35]. Each individual had two data lines representing the two doses of the drug they received. The risk intervals for the model were defined using gap time. Follow-up time for the first data line for each subject was time from the first treatment to time of event, censoring or just before the second treatment. Follow-up period for the second data line represented the time from second treatment to the event, or censoring (delivery or loss to follow-up). The model assumed a different baseline hazard for each stratum, and therefore, was stratified by the number of treatment doses received. A robust variance estimator was included in the model to account for the correlation of data in each woman [18]. The Efron estimation of the partial likelihood was used to account for ties.

A backward elimination strategy was used to select the best model that assessed the efficacy of SP-azithromycin and SP-artesunate compared with SP monotherapy. The multivariate model included the main exposure variable, potential confounders and effect measure modifiers. The presence of effect measure modification was assessed using the Likelihood-ratio test. This was done by comparing the full model containing all variables including an interaction term with a reduced model that did not contain the interaction term. The remaining covariates were assessed for confounding using the change in estimate criteria. Although treatment allocation was randomized, we still adjusted for confounding to control for residual confounding. The absolute change in estimate of at least 0.10 comparing the difference in the log hazard ratio of the crude estimate (full model) and the adjusted estimate (reduced model) was considered to indicate the presence of confounding by a variable and it was retained in the full model. Variables with more than two levels were tested together as a chunk. The variable which caused the least change in estimate was the first to be removed from the full model followed consecutively by variables which caused the least estimate. Only variables that caused an absolute change in estimate of at least 0.10 were left in the final model.

Goodness of fit of the model

We examined the correlation between the Schoenfeld partial residuals of the final model and the rank order of failure time for goodness of fit of the model [36].

Sensitivity analysis

Malawi is a high-transmission area with a diverse *P. falciparum* parasite population. We identified three *msp*-1 variants that were common, with prevalence rates between 11% and 16%. These common variants would have been classified as recrudescence if they were detected in a recurrent episode, even if it were a new infection. We conducted sensitivity analysis to assess the impact that misclassification of recurrent infections that shared only a single common variant (prevalence >10%) with the initial infection would have had on the efficacy of the combination therapies. We used the prevalence of the common bands and the total number of variants in the recurrent episode to calculate the probability the recurrent episode was a recrudescence. For a recurrent episode with x variants and sharing a single variant of prevalence y with the initial infection, the binomial probability that this variant was found by chance in a recurrent infection was equal to $1-(1-y)^x$ (and therefore the probability that it represented a failure $=(1-y)^x$). Using the calculated probabilities, we recalculated the hazard ratios by varying the probability of recrudescence from 0.10, when all infections that shared a single common band were classified as recrudescent to 0.8 were none of the infections that shared a single common band were considered recrudescence.

2.3. References

(1) Brabin BJ. An analysis of malaria in pregnancy in Africa. Bull.World Health Organ. 1983;61(6):1005-1016.

(2) McGregor IA. Epidemiology, malaria and pregnancy. Am.J.Trop.Med.Hyg. 1984 Jul;33(4):517-525.

(3) Steketee RW, Wirima JJ, Hightower AW, Slutsker L, Heymann DL, Breman JG. The effect of malaria and malaria prevention in pregnancy on offspring birthweight, prematurity, and intrauterine growth retardation in rural Malawi. Am.J.Trop.Med.Hyg. 1996;55(1 Suppl):33-41.

(4) Matteelli A, Donato F, Shein A, Muchi JA, Leopardi O, Astori L, et al. Malaria and anaemia in pregnant women in urban Zanzibar, Tanzania. Ann.Trop.Med.Parasitol. 1994 Oct;88(5):475-483.

(5) Shulman CE, Graham WJ, Jilo H, Lowe BS, New L, Obiero J, et al. Malaria is an important cause of anaemia in primigravidae: evidence from a district hospital in coastal Kenya. Trans.R.Soc.Trop.Med.Hyg. 1996 Sep-Oct;90(5):535-539.

(6) Shulman CE, Marshall T, Dorman EK, Bulmer JN, Cutts F, Peshu N, et al. Malaria in pregnancy: adverse effects on haemoglobin levels and birthweight in primigravidae and multigravidae. Trop.Med.Int.Health 2001 Oct;6(10):770-778.

(7) Matteelli A, Caligaris S, Castelli F, Carosi G. The placenta and malaria. Ann.Trop.Med.Parasitol. 1997 Oct;91(7):803-810.

(8) Rogerson SJ, Pollina E, Getachew A, Tadesse E, Lema VM, Molyneux ME. Placental monocyte infiltrates in response to *Plasmodium falciparum* malaria infection and their association with adverse pregnancy outcomes. Am.J.Trop.Med.Hyg. 2003 Jan;68(1):115-119.

(9) Sullivan AD, Nyirenda T, Cullinan T, Taylor T, Harlow SD, James SA, et al. Malaria infection during pregnancy: intrauterine growth retardation and preterm delivery in Malawi. J.Infect.Dis. 1999 Jun;179(6):1580-1583.

(10) National Statistical Office (NSO). Malawi population and housing census. Summary of final results, volume 1, Zomba, Malawi 1998.

(11) Warhurst DC, Williams JE. ACP Broadsheet no 148. July 1996. Laboratory diagnosis of malaria. J.Clin.Pathol. 1996 Jul;49(7):533-538.

(12) World Health Organization. Prevention and Management of Severe Anaemia in Pregnancy. Geneva: WHO; 1993.

(13) Greenland S, Pearl J, Robins JM. Causal diagrams for epidemiologic research. Epidemiology 1999 Jan;10(1):37-48.

(14) Wacholder S. Binomial regression in GLIM: estimating risk ratios and risk differences. Am.J.Epidemiol. 1986 Jan;123(1):174-184.

(15) Brabin BJ, Romagosa C, Abdelgalil S, Menendez C, Verhoeff FH, McGready R, et al. The sick placenta-the role of malaria. Placenta 2004 May;25(5):359-378.

(16) Lee J. Odds ratio or relative risk for cross-sectional data? Int.J.Epidemiol. 1994 Feb;23(1):201-203.

(17) Clayton, David.; Hills, Michael. Statistical Models in Epidemiology. New York, Oxford University Press Inc.;; 1996. p. 367.

(18) D. Lin and L. Wei. The Robust Inference for the Cox Proportional Hazards Model, J. American Statistical Association 1989;84:1074-1078.

(19) WHO 2002. Strategic Framework for Malaria Control During Pregnancy in the WHO Africa Region.

(20) Bloland P2. Drug resistance in malaria. WHO monograph WHO/CDS/CSR/DRS/2001.4. World Health Organization, Geneva, Switzerland.

(21) McGready R, Ashley EA, Moo E, Cho T, Barends M, Hutagalung R, et al. A randomized comparison of artesunate-atovaquone-proguanil versus quinine in treatment for uncomplicated *falciparum* malaria during pregnancy. J.Infect.Dis. 2005 Sep 1;192(5):846-853.

(22) McGready R, Cho T, Keo NK, Thwai KL, Villegas L, Looareesuwan S, et al. Artemisinin antimalarials in pregnancy: a prospective treatment study of 539 episodes of multidrug-resistant Plasmodium falciparum. Clin.Infect.Dis. 2001 Dec 15;33(12):2009-2016.

(23) McGready R, Cho T, Samuel, Villegas L, Brockman A, van Vugt M, et al. Randomized comparison of quinine-clindamycin versus artesunate in the treatment of *falciparum* malaria in pregnancy. Trans.R.Soc.Trop.Med.Hyg. 2001 Nov-Dec;95(6):651-656.

(24) Andersen SL, Ager A, McGreevy P, Schuster BG, Wesche D, Kuschner R, et al. Activity of azithromycin as a blood schizonticide against rodent and human *Plasmodia in vivo*. Am.J.Trop.Med.Hyg. 1995 Feb;52(2):159-161.

(25) Andersen SL, Oloo AJ, Gordon DM, Ragama OB, Aleman GM, Berman JD, et al. Successful double-blinded, randomized, placebo-controlled field trial of azithromycin and

doxycycline as prophylaxis for malaria in western Kenya. Clin.Infect.Dis. 1998 Jan;26(1):146-150.

(26) Gray RH, Wabwire-Mangen F, Kigozi G, Sewankambo NK, Serwadda D, Moulton LH, et al. Randomized trial of presumptive sexually transmitted disease therapy during pregnancy in Rakai, Uganda. Am.J.Obstet.Gynecol. 2001 Nov;185(5):1209-1217.

(27) Guay LA, Musoke P, Fleming T, Bagenda D, Allen M, Nakabiito C, et al. Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: HIVNET 012 randomised trial. Lancet 1999 Sep 4;354(9181):795-802.

(28) Ballard JL, Khoury JC, Wedig K, Wang L, Eilers-Walsman BL, Lipp R. New Ballard Score, expanded to include extremely premature infants. J.Pediatr. 1991 Sep;119(3):417-423.

(29) Snounou G, Beck HP. The use of PCR genotyping in the assessment of recrudescence or re-infection after antimalarial drug treatment. Parasitol Today 1998(14):462-467.

(30) Ngrenngarmlert W, Kwiek JJ, Kamwendo DD, Ritola K, Swanstrom R, Wongsrichanalai C, et al. Measuring allelic heterogeneity in *Plasmodium falciparum* by a heteroduplex tracking assay. Am.J.Trop.Med.Hyg. 2005 Jun;72(6):694-701.

(31) Yeramian P, Meshnick SR, Krudsood S, Chalermrut K, Silachamroon U, Tangpukdee N, et al. Efficacy of DB289 in Thai patients with *Plasmodium vivax* or acute, uncomplicated *Plasmodium falciparum* infections. J.Infect.Dis. 2005 Jul 15;192(2):319-322.

(32) Adam I, Ali DM, Abdalla MA. Artesunate plus sulfadoxine-pyrimethamine in the treatment of uncomplicated *Plasmodium falciparum* malaria during pregnancy in eastern Sudan. Trans.R.Soc.Trop.Med.Hyg. 2006 Jan 24.

(33) Deen JL, von Seidlein L, Pinder M, Walraven GE, Greenwood BM. The safety of the combination artesunate and pyrimethamine-sulfadoxine given during pregnancy. Trans.R.Soc.Trop.Med.Hyg. 2001 Jul-Aug;95(4):424-428.

(34) WHO 2005. Susceptibility of *Plasmodium Falciparum* to Antimalarial Drugs. Report on global monitoring 1996-2004. WHO/HTM/MAL/2005.1103. Accessed at http://www.who.int/malaria/includes_en/whomalariapublications19982004.htm.

(35) T. Therneau and P. Grambsch. Modeling Survival Data: Extending the Cox Model. New York: Springer-Verlag, 2000.

(36) D. Schoenfeld. Partial Residuals for the Proportional Hazards Regression Model. Biometrika 1982;69:239-241.

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CHAPTER 3

The effect of timing and frequency of *Plasmodium falciparum* infection during pregnancy on low birth weight and maternal anemia

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Abstract

Background: In areas of endemic transmission, *Plasmodium falciparum* infection during pregnancy causes maternal anemia and low birth weight. However the effect frequency and timing of infection on the severity of these adverse effects is not known. This information would be important for developing prevention policies in pregnant women.

Methods: We conducted a prospective observational study recruiting 2,462 pregnant women in Blantyre, Malawi. The presence of malaria was assessed by microscopy at scheduled visits during follow-up and delivery. Birth weight and maternal hemoglobin concentration were measured at delivery. The association between timing and frequency of malaria infection on the risk of low birth weight and maternal anemia was analyzed using a binomial regression model..

Results: The prevalence of low birth weight increased with the number of malaria episodes: [Primigravidae: 1 episode (prevalence ratio [PR] =2.72; 95% C.I. 1.06-7.00), and 2 or more episodes (PR=3.16; 95% C.I. 1.11-9.04); Multigravidae: 1 episode (PR =1.26; 95% C.I. 0.51-3.11) and 2 or more episodes (PR=3.00; 95% C.I. 0.79-11.41)]. The prevalence of anemia also increased with the number of episodes of malaria: [Primigravidae: 1 episode (PR= 1.07; 95% C.I. 0.52-2.19) and 2 or more episodes (PR= 1.57; 95% C.I. 0.70-3.54); Multigravidae: 1 episode (PR= 1.09; 95% C.I. 0.62-1.91) and 2 or more episodes (PR= 1.53; 95% C.I. 0.54-4.30]. The prevalence of low birth weight was higher with infection in the second trimester (PR=2.97; 95% CI 1.60-5.53) than in the third trimester (PR=1.42; 95% CI 0.63-3.22). The prevalence of maternal anemia was

higher with infection in the third trimester (PR=1.44; 95% CI 0.90-2.29) compared with infection in the second trimester (PR=0.94; 95% CI 0.51-1.71).

Conclusion: Timing and frequency of *Plasmodium falciparum* infection during pregnancy affect the risk of low birth weight and maternal anemia. There is need to increase coverage of prevention measures against malaria throughout pregnancy to reduce the risk of these complications.

Introduction

Each year more than 50 million pregnant women become pregnant in malaria endemic areas [1]. The complications of *P. falciparum* infection during pregnancy are dependent on transmission rates and the level of acquired immunity. Women in low transmission areas are at risk for severe disease with a potential for mortality [2,3]. They are also at risk for severe pregnancy complications such as spontaneous abortions, still births and low birth weight (LBW). In high transmission areas, women have acquired partial immunity by the time they reach reproductive age [4,5]. Consequently they are not at risk for severe disease, but are at an increased risk for developing anemia and delivering babies with LBW.

The actual mechanisms on how *P. falciparum* infection during pregnancy causes LBW still remain unclear. Some factors that have been cited include maternal anemia [6], placental insufficiency caused by the thickening of the trophoblastic membrane[7,8], elevated proinflammatory cytokine levels[9,10], poor oxygen and glucose transfer from the parasitized erythrocytes and use of nutrients by macrophages sequestered in the placenta[10]. The etiology of maternal anemia caused by *P. falciparum* infection during pregnancy is also multi-factorial. It is mediated through hemolysis of both infected and uninfected erythrocytes [11-13], phagocytosis of erythrocytes by macrophages, and poor bone marrow response[14]. However, the contribution of each of these factors to the development of maternal anemia has not been clearly elucidated.

Most studies have examined the association between *P. falciparum* infection and the adverse outcomes using placental histology to describe the evolution of infection during pregnancy[4,7,15-21]. However, the assessment of malaria at a single time point may result in misclassification. Other studies have limited recruitment to primigravidae [22]. Although *P. falciparum* infection in multigravidae is not infrequent, it still poses a significant risk for severe anemia and LBW. To our knowledge, no studies have investigated the effect of frequency of *P. falciparum* infection during pregnancy on the risk of low birth weight and maternal anemia. We therefore conducted a prospective study following women during pregnancy to investigate the impact of timing and frequency of *P. falciparum* malaria on the risk of LBW and maternal anemia. This information would aid policy makers to identify the crucial time point when intermittent presumptive therapy (IPT) would be most effective in preventing malaria during pregnancy and its complications.

Methods

Study site

The study was conducted at Mpemba and Madziabango health centers in Blantyre District, in the southern region of Malawi. Blantyre district covers 2012 km^2 with a population of approximately 950,000 people [23]. Malaria transmission is perennial but peaks during the rainy season (November to March). *P. falciparum* causes over 90% of all malaria infections. The two health-centers have maternity beds for delivery of low risk pregnancies, a malaria microscopy laboratory with a trained medical technologist, 4 full-time and 9 part-time nurses. There are 18 traditional birth attendants (TBAs) that take care of births that occur in the community. Women with high risk pregnancies are referred to

Queen Elizabeth Central Hospital (QECH), a tertiary hospital located approximately 10 km away from the health centers for management and delivery.

Study population and recruitment

All pregnant women attending routine antenatal care at the two health facilities were invited to participate in the study. A total of 2,462 women were recruited over a period of 15 months, between April 2002 and July 2003. Signed or witnessed verbal consent was sought for participation in the study before enrollment. A standardized questionnaire was used to obtain information on demographics, past obstetric history, malaria symptoms and factors associated with malaria prevention measures such as use of insecticide-treated bed nets (ITNs) and anti-malarial drugs in pregnancy. Study participants were given study numbers to retain until delivery for easy identification. Detailed contact information was obtained to aid in follow-up. The women received routine antenatal assessment which included height, weight, temperature and blood pressure measurements, and abdominal examination. A sample of venous blood was collected for hemoglobin concentration estimation and examination of thick blood films. The women were given a dose of sulfadoxine-pyrimethamine (SP) as intermittent preventive therapy (IPT) under direct observation by the antenatal clinic personnel. Antenatal records were checked to ensure that SP was not given at intervals less than one month.

Follow-up

The follow-up visits were scheduled according to the normal antenatal routine care. Depending on the gestation age at enrollment, women were seen at the following gestational ages: 12 weeks, 26weeks, 32 weeks and 36-38 weeks age and at delivery. At these visits, women received routine antenatal care and a standardized interview was conducted to obtain information on malarial illnesses and use of anti-malarial drugs. A peripheral blood sample was obtained for examination of thick films and the women were given a second dose of SP according to the Malawi national policy schedule. The women were followed until delivery and were encouraged to deliver at the health centers.

Delivery

Blood samples were obtained soon after delivery for measurement of maternal hemoglobin concentration and examination of peripheral, placental and cord thick films. Newborns were weighed within 24 hours of delivery using a digital scale to the nearest gram. A system was set up such that community nurses and TBAs were able to identify women enrolled in the study, who delivered at home. TBAs were trained to prepare peripheral, placental and cord thick films from women who delivered in the community and forward them to the clinics through community health workers. Newborns delivered in the community were weighed using color coded scales that indicated green if the newborn weighed more than 2,500 grams and red if the newborn weighed less than 2,500 grams.

Laboratory methods

Malaria diagnosis was performed using microscopy of Giemsa stained thick blood films. Slides were examined under a microscope using a magnification of 100x oil immersion objective to detect and quantify parasitemia. Parasite densities were estimated using an assumed leukocyte count of 6,000 leukocytes/μl of blood. A thick film was considered negative if no parasites were detected after examining 100 microscopic fields each containing approximately 20 white blood cells. All thick films were read by two skilled microscopists and any discrepancies (positive *versus* negative or more than 25% difference in parasite density) were solved by repeating the readings. Hemoglobin concentration was determined using a HemoCueÒ machine (HemoCue Incorporated, Angelholm, Sweden).

Outcome definitions

Newborns were classified as having normal birth weight (≥ 2,500 g) or LBW (< 2,500 g), regardless of gestational age. Women were defined as having anemia if they had hemoglobin concentration less than 11g/dL [24].

Ethical approval and consent

This study was approved by the College of Medicine Research Committee (University of Malawi, Blantyre, Malawi) and the Institutional Review Boards of the University of North Carolina (Chapel Hill, NC).

Statistical Analysis

Data analysis was performed using STATA version 8.0 software (StataCorp, College Station, Texas, USA). Comparison of the baseline demographic characteristics was performed using the chi-square test for categorical data, analysis of variance for normally distributed, and the Kruskal-Wallis tests for non-normally distributed continuous variables. The frequency of malaria infection was classified according to the number of episodes detected during pregnancy. The timing of malaria infection during pregnancy was defined in two ways. Firstly, timing of infection during pregnancy was defined as: (1) parasitemia in the antenatal period only and (2) parasitemia at delivery only. Secondly, timing of infection was defined as: (1) parasitemia in the second trimester (gestation age 13 to 28 weeks), and (2) parasitemia in the third trimester (gestation age 29 weeks to term). Multivariate analyses were performed using a binomial regression model to estimate the association between timing and frequency of *P. falciparum* infection during pregnancy and LBW or maternal anemia. We stratified the analysis by the total number of visits during follow-up. Gravidity was considered an effect measure, and estimates were reported separately for primigravidae (PG) and multigravidae (MG). Variables were considered confounders if there was an absolute change in estimate of 0.10 after comparing the full model and the reduced model without the variable. A backward elimination strategy was used to identify the best model. Covariates that were considered as potential confounders included maternal age, body mass index (BMI), use of ITNs, SP use during pregnancy, gestation age at enrolment, taking anti-malarial drugs and prior to enrolment into the study. All p-values are two-sided, and confidence intervals (CIs) were calculated at the 95% level. Statistical significance was set at $p \leq 0.05$.

Results

Study Population

Of the 2,462 women enrolled into the study, birth weight was recorded for 1,548 newborns and information on hemoglobin concentration at delivery was available for 1,253 women. Data was missing for birth weight and maternal hemoglobin concentration because women had moved or delivered outside the study area, or failed to return for follow-up and the study team were unable to locate their houses. There were no significant differences in most of the baseline characteristics in the women who were included in the analysis and those lost to follow, except for reported ITNs ownership and use of IPT SP (Table 3.1). Analysis was restricted to women who were enrolled at their first antenatal visit with information on outcome [birth weight (*n*=1,162), and maternal hemoglobin concentration at delivery (*n*=973)]. The total number of visits by the women enrolled into the study was as follows: 1 visit [9.6%, (*n*=190)], 2 visits [44.0%, (*n*=870)], 3 visits [40.3% (*n*=798)] and 4 visits [6.1% (*n*=121)]. The characteristics of women enrolled into the study are shown in Table 3.2. The mean maternal age was 24 years, and the mean gestational age at enrollment was 22 weeks, and 12.7% of the women reported owning ITNs.

Malaria

The prevalence of parasitemia at enrolment was 18.6%, higher in PG compared with MG, (38.2% *vs.* 12.2%; prevalence ratio [PR] = 3.13; 95% confidence interval (CI), 2.60-3.75). The prevalence of parasitemia throughout pregnancy was also higher in PG compared with MG (49.6% versus 20.6%; PR= 2.40, 95% CI 2.09-2.76). More women

had parasitemia in the antenatal period than at delivery irrespective of gravidity (20.5% versus 4.6%, p<0.0001). The frequency and timing of infection during pregnancy according to gravidity are shown in Table 3.3. After adjusting for other baseline characteristics, parasitemia was more common among PG compared with MG (PR=2.35, 95% CI 1.93-2.86). Women who were enrolled in the third trimester of pregnancy had less parasitemia compared with women enrolled in the second trimester (PR=0.77, 95% CI 0.62-0.96). The association between *P. falciparum* infection during pregnancy and other baseline characteristics of the study population are shown in Table 3.4.

Low birth weight

The mean birth weight was 3018.2 g (standard deviation (SD), 482.3 g), and the prevalence of LBW was 9.3%. Gravidity, maternal age and bed net use were significantly associated with low birth weight in bivariate analysis, p<0.05. After adjustment for other baseline characteristics, only gravidity was still significantly associated with the risk of LBW. The proportion of LBW deliveries was more common among PG than MG (17.5% *vs.* 6.8%; PR=2.24; 95% CI, 1.34 -3.75) (Table 3.4).

Maternal hemoglobin

The prevalence of mild and moderate-to-severe anemia at enrollment was 53.1% and 14.8%, respectively. The prevalence of mild and moderate-to-severe anemia at delivery was 19.4% and 3.2% respectively. After adjusting for other baseline characteristics, the prevalence of anemia at delivery was higher in women who had malaria during pregnancy compared with those who did not have infection (PR=1.28;

95% CI 0.98-1.66). Women who did not own ITNs were more likely to have anemia at delivery than women who owned ITNs (PR=1.24; 95% CI 0.87-1.77). Other factors associated with anemia during pregnancy are shown in Table 3.5.

Effect of frequency of malaria infection on low birth weight and maternal anemia

The prevalence of LBW increased with the number of episodes experienced during pregnancy. Compared with PG who had no malaria during pregnancy, the prevalence of LBW for PG with one episode of malaria was (PR=2.72; 95% 1.06-7.00), and 2 or more episodes was (PR=3.16; 95% 1.11-9.04). Similarly in MG, the prevalence of LBW increased with the number of episodes of malaria [1 episode: (PR 1.26, 95% CI 0.51-3.11), and 2 or more episodes: (PR 3.00, 95% CI 0.79-11.41)]. The prevalence of maternal anemia also increased with the number of episodes of infection both in PG and MG. Compared with PG who had no malaria during pregnancy, the prevalence of anemia with 1 episode of malaria was (PR=1.07, 95% CI 0.52-2.19), and 2 or more episodes was (PR=1.57, 95% CI 0.70-3.54). The prevalence of maternal anemia comparing MG who had no infection with MG who had 1 episode of malaria was (PR=1.09, 95% CI 0.62-1.91), and 2 or more episodes was (PR=1.53, 95% CI 0.54-4.30), (Table 3.6).

The effect of timing of malaria infection on low birth weight and maternal anemia

The prevalence of LBW and maternal anemia varied with the time of infection during pregnancy. Because of small numbers, estimates were combined for PG and MG. Compared with women who had no infection during pregnancy, the prevalence of LBW was higher in women who had infection in the antenatal period (PR=2.24; 95% CI 1.21-4.13) than in women who had infection at delivery (PR=2.13; 95% CI 0.90-5.01). In the

antenatal period, the prevalence of LBW was higher for women who had infection in the second trimester (PR=2.97; 95% CI 1.60-5.53) compared with infection in the third trimester (PR=1.42; 95% CI 0.63-3.22). The prevalence of maternal anemia was higher in women who had infection at delivery (PR=1.52; 95% CI 0.83-2.78) compared with infection in the antenatal period (PR=1.03; 95% CI 0.63-1.68). When timing was classified using trimester of pregnancy, the prevalence of maternal anemia was also higher in women with infection in the third trimester (PR=1.44; 95% CI 0.90-2.29) compared with infection in the second trimester (PR=0.94; 95% CI 0.51-1.71), (Table 3.7).

Discussion

We investigated the association between the frequency and timing of *P. falciparum* infection during pregnancy on the risk of low birth weight and maternal anemia in a large sample of pregnant women in Malawi. We found that the prevalence of low birth weight and maternal anemia increased with the number of episodes of infection experienced during pregnancy, irrespective of gravidity. Timing of infection also affected the risk of LBW and maternal anemia. Infection detected early in pregnancy (antenatal period or second trimester) increased the prevalence of LBW more than infection detected in late in pregnancy (third trimester or at delivery). By contrast, the prevalence of maternal anemia was higher with infection late in pregnancy compared with infection early in pregnancy.

P. falciparum infection during pregnancy is characterized by the sequestration of infected erythrocytes in the maternal placental vascular space [25]. Studies have shown that this sequestration causes an infiltration of inflammatory cells and

elevation of cytokine levels in the infected placentae [9,10,26]. It has been suggested that this inflammatory response reduces the materno-fetal exchange of nutrients through poor oxygen and glucose transfer by parasitized erythrocytes, use of nutrients by the growing parasites and placenta mechanical blockage due to thickening of the basement membrane [7-10], leading to growth restriction in the fetus. We found that the risk of low birth weight increased with the number of episodes experienced during pregnancy. This suggests that the damage caused in the placenta by the sequestration of malaria parasites is reversible. If the damage had been permanent, the risk for low birth weight would not have changed irrespective of the number of episodes experienced during pregnancy. Previous studies have found that women with infections early in pregnancy did not show histological differences seen in infected placentae at delivery[8,27,28], suggesting that the placenta is able to recover from acute infections. We also found that the timing of infection during pregnancy affected the risk of low birth weight. The prevalence of low birth weight was higher in women who had infection early in pregnancy than infection detected late in pregnancy. Maximum fetal growth rate occurs between 20 and 28 week gestation, this would explain why infection in the second trimester increased the risk of low birth weight more than infection in the third trimester.

We also found that the risk of maternal anemia increased with the number of episodes of infection experienced during pregnancy both in primigravidae and multigravidae. Compared with women who had no infection during pregnancy, the mean hemoglobin concentration was 0.2 g/dL lower for women who had 1 episode, and 0.8 g/dL lower for women with 2 or more episodes of malaria during pregnancy. Women who had infection in the third trimester of pregnancy or at delivery had a higher risk of

maternal anemia than women who had infection in the second trimester or in the antenatal period. Malaria infection during pregnancy causes anemia through hemolysis of infected and uninfected erythrocytes, and poor bone marrow response [11,12,14]. The women in our study were given SP for IPT and ferrous sulphate supplementation during follow-up, which could have raised the hemoglobin concentration after infection early in pregnancy, reducing the risk of anemia at delivery.

Our findings are important for developing preventive policies in pregnant women. Most malaria-endemic areas have limited resources and most women do not receive the recommended two doses of SP during pregnancy. Access is limited by many factors including low providers' awareness of the regimen, poor health-seeking behavior of pregnant women who tend not to receive antenatal care and poor availability of drugs. Additionally, most women in malaria-endemic areas present late in pregnancy for antenatal care [29,30]. Administration of sulfadoxine-pyrimethamine at this time could still reduce the prevalence of low birth weight. Although the prevalence of low birth weight and maternal anemia was higher in PG, the adverse effects were also seen in multigravidae, indicating the need to include MG in future studies and policies on prevention of malaria during pregnancy.

Our study had several limitations. We did not obtain information on HIV which is associated with malaria and its complications and this could have biased our results. There were very few women who presented during their first trimester of pregnancy. Other previous studies have also noted that women in rural areas of Malawi tend to seek health

care in the middle to late second trimester [29,30]. Therefore, we were unable to investigate the impact of malaria infection during the first trimester of pregnancy. We had expected that many women would deliver at home and, therefore, recruited traditional birth attendants to help us collect data for home deliveries. Despite extensive attempts, loss to follow-up was a concern in this investigation and occurred in part because of the highly mobile nature of this peri-urban study population. There were no significant differences in the baseline characteristics of the women who were lost to follow-up and women included in the analysis. Most of the women who were lost to follow up had moved out of the study area and therefore it is impossible to determine whether the outcomes in these women would have been different from the women who remained and delivered in the study area. Despite these limitations, our study sample was community based, recruiting women who were using the government health centers, which are primarily used by the majority of population living in the study area. A recent survey conducted in Malawi found that more than 90% of pregnant women visit antenatal clinics at least once during pregnancy [31]. Therefore, the study sample was most likely representative of the pregnant women population living in the rural areas of Malawi.

In conclusion, the timing and frequency of *P. falciparum* infection during pregnancy have an effect on the risk of low birth weight and maternal anemia. The prevalence of both maternal anemia and LBW increased with the number of episodes of malaria experienced during pregnancy. The prevalence of LBW was higher with infection early in pregnancy whereas the risk of maternal anemia was higher with infection late in

pregnancy. There is need to improve access to intermittent preventive therapy and other preventive measures throughout pregnancy to reduce the risk of complications of malaria.

Acknowledgements

We thank the entire staff of the Mpemba and Madziabango health centers, Mrs Ebby Chaluluka and all the midwives for cooperation and support in this research project. We are especially grateful to the mothers who volunteered to participate in this study.

References

(1) WHO. World Malaria Report, 2005. Accessed at http://rbm.who.int/wmr2005/.

(2) Nosten F, Rogerson SJ, Beeson JG, McGready R, Mutabingwa TK, Brabin B. Malaria in pregnancy and the endemicity spectrum: what can we learn? Trends Parasitol. 2004 Sep;20(9):425-432.

(3) Luxemburger C, McGready R, Kham A, Morison L, Cho T, Chongsuphajaisiddhi T, et al. Effects of malaria during pregnancy on infant mortality in an area of low malaria transmission. Am.J.Epidemiol. 2001 Sep 1;154(5):459-465.

(4) McGregor IA. Epidemiology, malaria and pregnancy. Am.J.Trop.Med.Hyg. 1984 Jul;33(4):517-525.

(5) Brabin BJ, Romagosa C, Abdelgalil S, Menendez C, Verhoeff FH, McGready R, et al. The sick placenta-the role of malaria. Placenta 2004 May;25(5):359-378.

(6) Verhoeff FH, Brabin BJ, van Buuren S, Chimsuku L, Kazembe P, Wit JM, et al. An analysis of intra-uterine growth retardation in rural Malawi. Eur.J.Clin.Nutr. 2001 Aug;55(8):682-689.

(7) Steketee RW, Wirima JJ, Hightower AW, Slutsker L, Heymann DL, Breman JG. The effect of malaria and malaria prevention in pregnancy on offspring birthweight, prematurity, and intrauterine growth retardation in rural Malawi. Am.J.Trop.Med.Hyg. 1996;55(1 Suppl):33-41.

(8) Ismail MR, Ordi J, Menendez C, Ventura PJ, Aponte JJ, Kahigwa E, et al. Placental pathology in malaria: a histological, immunohistochemical, and quantitative study. Hum.Pathol. 2000 Jan;31(1):85-93.

(9) Fried M, Muga RO, Misore AO, Duffy PE. Malaria elicits type 1 cytokines in the human placenta: IFN-gamma and TNF-alpha associated with pregnancy outcomes. J.Immunol. 1998 Mar 1;160(5):2523-2530.

(10) Moormann AM, Sullivan AD, Rochford RA, Chensue SW, Bock PJ, Nyirenda T, et al. Malaria and pregnancy: placental cytokine expression and its relationship to intrauterine growth retardation. J.Infect.Dis. 1999 Dec;180(6):1987-1993.

(11) Omodeo-Sale F, Motti A, Basilico N, Parapini S, Olliaro P, Taramelli D. Accelerated senescence of human erythrocytes cultured with *Plasmodium falciparum*. Blood 2003 Jul 15;102(2):705-711.

(12) Greve B, Lehman LG, Lell B, Luckner D, Schmidt-Ott R, Kremsner PG. High oxygen radical production is associated with fast parasite clearance in children with *Plasmodium falciparum* malaria. J.Infect.Dis. 1999 Jun;179(6):1584-1586.

(13) Ekvall H. Malaria and anemia. Curr.Opin.Hematol. 2003 Mar;10(2):108-114.

(14) Verhoeff FH, Brabin BJ, Chimsuku L, Kazembe P, Broadhead RL. An analysis of the determinants of anaemia in pregnant women in rural Malawi--a basis for action. Ann.Trop.Med.Parasitol. 1999 Mar;93(2):119-133.

(15) Brabin BJ. An analysis of malaria in pregnancy in Africa. Bull.World Health Organ. 1983;61(6):1005-1016.

(16) Matteelli A, Donato F, Shein A, Muchi JA, Leopardi O, Astori L, et al. Malaria and anaemia in pregnant women in urban Zanzibar, Tanzania. Ann.Trop.Med.Parasitol. 1994 Oct;88(5):475-483.

(17) Shulman CE, Graham WJ, Jilo H, Lowe BS, New L, Obiero J, et al. Malaria is an important cause of anaemia in primigravidae: evidence from a district hospital in coastal Kenya. Trans.R.Soc.Trop.Med.Hyg. 1996 Sep-Oct;90(5):535-539.

(18) Shulman CE, Marshall T, Dorman EK, Bulmer JN, Cutts F, Peshu N, et al. Malaria in pregnancy: adverse effects on haemoglobin levels and birthweight in primigravidae and multigravidae. Trop.Med.Int.Health 2001 Oct;6(10):770-778.

(19) Matteelli A, Caligaris S, Castelli F, Carosi G. The placenta and malaria. Ann.Trop.Med.Parasitol. 1997 Oct;91(7):803-810.

(20) Menendez C, Ordi J, Ismail MR, Ventura PJ, Aponte JJ, Kahigwa E, et al. The impact of placental malaria on gestational age and birth weight. J.Infect.Dis. 2000 May;181(5):1740-1745.

(21) Rogerson SJ, Mkundika P, Kanjala MK. Diagnosis of *Plasmodium falciparum* malaria at delivery: comparison of blood film preparation methods and of blood films with histology. J.Clin.Microbiol. 2003 Apr;41(4):1370-1374.

(22) Sullivan AD, Nyirenda T, Cullinan T, Taylor T, Harlow SD, James SA, et al. Malaria infection during pregnancy: intrauterine growth retardation and preterm delivery in Malawi. J.Infect.Dis. 1999 Jun;179(6):1580-1583.

(23) National Statistical Office (NSO). Malawi population and housing census. Summary of final results, volume 1, Zomba, Malawi 1998.

(24) World Health Organization. Prevention and Management of Severe Anaemia in Pregnancy. Geneva: WHO; 1993.

(25) Walter PR, Garin Y, Blot P. Placental pathologic changes in malaria. A histologic and ultrastructural study. Am.J.Pathol. 1982 Dec;109(3):330-342.

(26) Abrams ET, Brown H, Chensue SW, Turner GD, Tadesse E, Lema VM, et al. Host response to malaria during pregnancy: placental monocyte recruitment is associated with elevated beta chemokine expression. J.Immunol. 2003 Mar 1;170(5):2759-2764.

(27) Sullivan AD, Nyirenda T, Cullinan T, Taylor T, Lau A, Meshnick SR. Placental haemozoin and malaria in pregnancy. Placenta 2000 May;21(4):417-421.

(28) Cottrell G, Deloron P, Fievet N, Sow S, Gaye O, Le Hesran JY. Prediction of *Plasmodium falciparum* placental infection according to the time of infection during pregnancy. Acta Trop. 2006 Jul;98(3):255-260.

(29) Rogerson SJ, van den Broek NR, Chaluluka E, Qongwane C, Mhango CG, Molyneux ME. Malaria and anemia in antenatal women in Blantyre, Malawi: a twelve-month survey. Am.J.Trop.Med.Hyg. 2000 Mar;62(3):335-340.

(30) Steketee RW, Wirima JJ, Slutsker L, Breman JG, Heymann DL. Comparability of treatment groups and risk factors for parasitemia at the first antenatal clinic visit in a study of malaria treatment and prevention in pregnancy in rural Malawi. Am.J.Trop.Med.Hyg. 1996;55(1 Suppl):17-23.
(31) Malawi Demographic and Health Survey, 2004.

Table 3.1. Comparison of the baseline characteristics of women who were lost and not lost to follow-up

Characteristic	Women not lost to follow-up (*n*=1759)	Women lost to follow-up (*n*=703)	P-value
Age [median, (IQR)], years	22 (20-27)	23 (20-27)	0.74
Gravidity (%)			
Primigravidae	76.9	74.4	0.18
Multigravidae	23.4	25.7	
Bed-net usage (%)	14.6	7.5	0.001
Gestation age [median, (IQR)], weeks	24 (22-28)	24 (22-27)	0.54
Body mass index [mean, (SD)], kg/m^2	21.6 (2.2)	21.6 (2.5)	0.99
Hemoglobin [mean, (SD)],g/dl	10.2 (1.5)	10.3 (1.4)	0.19
Maternal anemia† (%)	66.8	64.8	0.35
Routine SP treatment ≥ 2 doses (%)	67.8	70.1	<0.0001

†- Anemia defined as as hemoglobin concentration less than 11g/dL ; IQR- interquartile range ; SD- standard deviation ; SP-sulfadoxine-pyrimethamine

Table 3. 2. The baseline characteristics of the women enrolled into the study with and without malaria during pregnancy

Characteristic	All women (n=1869)	Malaria positive§ (*n*=515)	Malaria negative¶ (*n*=1354)	P-value
Age [median, (IQR)], years	23 (20-27)	21 (19-25)	23 (20-28)	<0.0001
Gravidity (%)				
Primigravidae	23.9	43.0	16.7	<0.0001
Multigravidae	76.1	57.0	83.4	
Bed-net usage (%)	12.7	11.3	13.2	NS
Gestation age [median, (IQR)], weeks	24 (22-26)	24 (20-26)	24 (22-27)	NS
Body mass index [mean, (SD)], kg/m^2	21.6 (2.4)	21.3 (2.4)	21.7 (2.3)	0.01
Hemoglobin [mean, (SD)],g/dl	10.1 (1.4)	9.9 (1.4)	10.2 (1.3)	<0.0001
Maternal anemia† (%)	67.9	73.6	65.7	<0.00001
Routine SP treatment ≥ 2 doses (%)	63.6	70.5	60.8	<0.0001

IQR- Interquartile range; SD-standard deviation; SP-sulfadoxine-pyrimethamine
§ Women who had at least one episode of *P. falciparum* infection detected during pregnancy
¶ Women who had no *P. falciparum* infection detected during pregnancy
† Anemia was defined as hemoglobin concentration less than 11g/dL

Table 3.3. The frequency and timing of *P. falciparum* infection during pregnancy by gravidity

	Primigravidae (*n*=475)	Multigravidae (*n*=1482)	P-value
Frequency of infection			
No episodes	223 (50.5)	1116 (79.4)	
1 Episode	170 (38.5)	255 (18.1)	<0.0001
2 or more episodes	49 (11.1)	35 (2.5)	
Time of infection§			
Antenatal period only	149 (37.0)	215 (15.5)	<0.0001
Delivery only	31 (7.7)	53 (3.8)	
Time of infection†			
Second trimester	120 (30.0)	133 (9.7)	<0.0001
Third trimester	57 (14.3)	128 (9.3)	

§Time of infection during pregnancy was classified as having parasitemia in the antenatal period only, or at delivery only

† Time of infection during pregnancy was classified according to trimester of pregnancy: second trimester (gestation age 13 to 28 weeks), and third trimester (gestation age 28 weeks to term)

Table 3.4. Characteristics associated with *P. falciparum* infection during pregnancy

Characteristic	N	(%)†	Crude PR (95% CI)	Adjusted PR (95% CI)
Age				
≥ 20 years	1424	23.2	1.00	1.00
Less than 20 years	425	42.1	1.92 (1.63-2,26)	0.97 (0.79-1.18)
Gravidity				
Multigravidae	1406	20.6	1.00	1.00
Primigravidae	442	49.6	2.58 (2.21-3.02)	2.35 (1.93-2.86)
Bed-net use				
Yes	233	24.4	1.00	1.00
No	1604	28.0	1.02 (0.98-1.06)	1.03 (0.80-1.33)
Use of SP as IPT				
1 dose	681	22.3	1.00	1.00
2 doses	975	28.9	1.30 (1.09-1.54)	0.84 (0.65-1.08)
3 doses	213	38.0	1.70 (1.37-2.13)	0.90 (0.58-1.4133)
Body mass index (kg/m2)				
<18.5	130	36.1	1.00	1.00
18.5-24.9	1544	26.8	0.74 (0.58-0.94)	0.84 (0.65-1.08)
≥ 25	136	27.2	0.75 (0.53-1.08)	0.84 (0.58-1.21)
Prior use of antimalarial drugs				
Yes	94	26.6	1.00	1.00
No	1770	27.6	1.04 (0.73-1.47)	1.00 (0.71-1.42)
Gestation at enrollment				
Second trimester	1412	29.5	1.00	1.00
Third trimester	412	20.2	0.68 (0.55-0.84)	0.77 (0.62-0.96)
Total number of visits during follow-up				
2 visits	801	20.5	1.00	1.00
3 visits	767	33.3	1.62 (1.37-1.92)	1.78 (1.36-2.32)
4 visits	121	46.3	2.26 (1.79-2.86)	2.37 (1.51-3.71)

N- Total number of women with information in the specified category; PR- Prevalence ratio; CI- Confidence interval; SP-sulfadoxine-pyrimethamine ; IPT-intermittent preventive therapy
† Percentage of people with malaria; *Use of antimalarial drugs prior to enrolment into the study

Table 3.5. Characteristics associated with low birth weight

Characteristic	N	(%)†	Crude PR (95% CI)	Adjusted PR (95% CI)
Age				
≥ 20 years	870	7.5	1.00	1.00
Less than 20 years	283	14.8	1.70 (1.31-2.21)	1.05 (0.63-1.77)
Gravidity				
Multigravidae	851	6.8	1.00	1.00
Primigravidae	303	16.5	1.91 (1.52-2.41)	2.24 (1.34-3.75)
Maternal anemia				
No	269	7.1	1.00	1.00
Yes	827	10.0	1.09 (0.98-1.20)	1.11 (0.72-1.72)
Bed-net use				
Yes	174	5.2	1.00	1.00
No	969	10.1	1.09 (1.02-1.16)	1.93 (0.96-3.92)
Use of SP as IPT				
1 dose	449	10.2	1.00	1.00
2 doses	602	8.9	0.88 (0.60-1.27)	0.85 (0.58-1.25)
3 doses	111	7.2	0.70 (0.34-1.45)	0.70 (0.33-1.50)
Body mass index (kg/m2)				
<18.5	85	11.8	1.00	1.00
18.5-24.9	945	9.4	0.80 (0.43-1.48)	0.89 (0.47-1.68)
≥ 25	90	5.6	0.47 (0.17-1.33)	0.55 (0.19-1.57)
Gestation at enrolment				
Second trimester	904	9.8	1.00	1.00
Third trimester	235	6.8	0.69 (0.41-1.15)	0.75 (0.43-1.30)
Prior use of antimalarial drugs*				
Yes	61	9.8	1.00	1.00
No	1097	9.3	0.95 (0.43-2.07)	0.87 (0.41-1.82)

N- Total number of babies with birth weight information in the specified category; PR-Prevalence ratio; CI-Confidence interval; SP-sulfadoxine-pyrimethamine ; IPT-intermittent preventive therapy
†-percentage of babies with low birth weight; ‡ women who had anemia at delivery
*-Use of antimalarial drugs prior to enrolment into the study

Table 3.6. Characteristics associated with maternal anemia* at delivery

Characteristic	N	(%)†	Crude PR (95% CI)	Adjusted PR (95% CI)
Age				
≥ 20 years	729	21.8	1.00	1.00
Less than 20 years	239	25.5	1.17 (0.91-1.49)	1.04 (0.74-1.45)
Parity				
Multigravidae	713	21.3	1.00	1.00
Primigravidae	255	25.9	1.20 (0.94-1.52)	1.09 (0.78-1.53)
Malaria during pregnancy				
No	661	21.0	1.00	1.00
Yes	312	26.0	1.20 (0.98-1.47)	1.28 (0.98-1.66)
SP use for IPT				
1 dose	362	28.2	1.00	1.00
2 doses	518	18.2	0.64 (0.50-0.82)	0.62 (0.48-0.80)
3 doses	93	25.8	0.92 (0.63-1.34)	0.76 (0.49-1.16)
Bed-net use				
Yes	150	19.3	1.00	1.00
No	808	23.3	1.04 (0.97-1.10)	1.24 (0.87-1.77)
Body mass index (kg/m2)				
<18.5	70	19.0	1.00	1.00
18.5-24.9	795	23.0	1.16 (0.71-1.88)	1.14 (0.70-1.86)
≥ 25	72	22.2	1.11 (0.59-2.10)	1.05 (0.83-2.37)
Gestation at enrolment				
Second trimester	761	22.3	1.00	1.00
Third trimester	193	23.8	0.96(0.74-1.25)	1.07 (0.79-1.44)
Prior use of antimalarial drugs				
Yes	49	34.7	1.00	1.00
No	920	22.1	0.64 (0.43-0.95)	0.77 (0.48-1.23)

*- Anemia (hemoglobin <11g/dL) present at enrolment or at delivery
N- Total number of women with information in the specified category
†-percentage of women with anemia at either at enrolment or at delivery
PR- Prevalence ratio; CI- Confidence interval; SP-sulfadoxine-pyrimethamine ; IPT-intermittent preventive therapy

Table 3.7. The effect of frequency of *P. falciparum* infection during pregnancy on low birth weight and maternal anemia

Malaria Exposure		Primigravidae		Multigravidae	
	N (%)	Crude PR (95% CI)	Adjusted PR† (95% CI)	Crude PR (95% CI_)	Adjusted †PR (95% CI)
Low birth weight					
No malaria	55 (6.95)	1.00	1.00	1.00	1.00
1 episode	38 (12.7)	2.71 (1.04-7.05)	2.72 (1.06-7.00)	1.13 (0.46-2.74)	1.26 (0.51-3.11)
2 or more episodes†	15 (20.8)	3.19 (1.10-9.21)	3.16 (1.11-9.04)	2.89 (0.76-11.00)	3.00 (0.79 -11.41)
Maternal anemia					
No malaria	139 (21.0)	1.00	1.00	1.00	1.00
1 episode	56 (23.0)	1.10 (0.54-2.21)	1.07 (0.52-2.19)	1.03 (0.58-1.81)	1.09 (0.62-1.91)
2 episodes	25 (36.8)	1.43 (0.63-3.24)	1.57 (0.70-3.54)	1.56 (0.57-4.31)	1.53 (0.54-4.30)

PR- Prevalence ratio; CI- Confidence interval, N- number of children with low birth weight
† -Adjusted for gestation at enrolment, number of visits, and body mass index and net usage

Table 3.8. The effect of timing of *P. falciparum* infection during pregnancy on low birth weight and maternal anemia

Malaria Exposure	N (%)	Crude PR (95% CI)	Adjusted* PR† (95% CI)
Low Birth Weight			
No malaria	55 (7.0)	1.00	1.00
Antenatal only	26 (11.5)	2.12 (1.17-3.87.)	2.24 (1.21-4.13)
Delivery only	14 (16.7)	2.06 (0.89-4.78)	2.13 (0.90-5.01)
Second Trimester‡	24 (14.6)	2.90 (1.59-5.30)	2.97 (1.60-5.53)
Third Trimester‡‡	15 (10.3)	1.29 (0.58-2.91)	1.42 (0.63-3.22)
Maternal Anemia			
No malaria	139 (21.0)	1.00	1.00
Antenatal only	39 (22.2)	0.99 (0.60-1.63)	1.03 (0.63-1.68)
Delivery only	19 (24.7)	1.47 (0.83-2.59)	1.52 (0.83-2.78)
Second Trimester‡	29 (22.8)	0.89 (0.40-1.62)	0.94 (0.51-1.71)
Third Trimester‡‡	28 (21.9)	1.39 (0.87-2.22)	1.44 (0.90-2.29)

* Adjusted for bed-net use, gestation at enrolment, body mass and number of visits

‡- 13 to 28 weeks gestation age
‡‡- 29 weeks to term

CHAPTER 4

A randomized controlled pilot trial of azithromycin or artesunate combined with sulfadoxine-pyrimethamine as treatment for *Plasmodium falciparum* infection in pregnant women

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Abstract

Context: In areas of endemic transmission, malaria in pregnancy is associated with severe maternal anemia and low-birth weight deliveries. New anti-malarial treatment regimens are urgently needed in sub-Saharan Africa because of the increase in drug resistance.

Objective: We investigated the efficacy and safety of anti-malarial treatment regimens in pregnant women at two rural health centers in Blantyre, Malawi.

Design, Setting and Patients: We conducted a randomized open-label clinical trial, recruiting 141 pregnant women. They were randomly allocated to 3 treatment groups: sulfadoxine-pyrimethamine (SP; 3 tablets, 500mg sulfadoxine and 25mg pyrimethamine per tablet); SP plus azithromycin (1g/day x 2 days); or SP plus artesunate (200mg/day x 3 days). Women received two doses of the pre-assigned treatment, administered 4 weeks apart. Heteroduplex tracking assays were performed to distinguish recrudescence from new infections. The number of recrudescent episodes in the three treatment groups was analyzed using the conditional risk set Cox-regression model.

Main Outcome Measures: Recrudescence rates and adverse outcomes.

Results: All treatment regimens were well tolerated. Two women vomited soon after ingesting azithromycin. Recrudescent episodes of malaria were less frequent with SP-azithromycin [Hazard Ratio 0.14 (95% confidence interval 0.02 to 0.78)] and SP-artesunate

[Hazard Ratio 0.13 (95% confidence interval 0.03 to 0.53)] compared with SP monotherapy. There were more abortions in the SP-azithromycin group, and more stillbirths in the SP-artesunate groups, but they were probably unrelated to treatment. Because of the small sample size, the effect on birth outcomes, maternal malaria or maternal anemia could not be evaluated.

Conclusion: Both SP-artesunate and SP-azithromycin appeared to be safe, well tolerated and efficacious for the treatment of malaria during pregnancy. A larger study is needed to determine its safety and efficacy in preventing poor birth outcomes**.**

Introduction

More than 30 million women become pregnant in malaria-endemic areas in Africa each year[1]. Infection with *Plasmodium falciparum* during pregnancy can cause maternal anemia, abortions, stillbirths, preterm deliveries and low birth weight[2, 3]. In areas of high transmission, most of the infections in pregnant women are asymptomatic and consequently, malaria infection may go undiagnosed[4]. All pregnant women in these areas should be provided with intermittent preventive therapy (IPT) at antenatal visits to prevent infection[5]. Sulfadoxine-pyrimethamine (SP), first introduced in Malawi in 1993, has now been adopted by many countries in sub-Saharan Africa as the drug of choice for IPT. Studies have shown that IPT significantly reduces the prevalence of placental malaria and low birth weight (LBW)[6-11].

The increase in the resistance to SP in sub-Saharan Africa is recognized as a growing public health problem[12]. There are currently few chemotherapeutic options available for treatment and prevention of malaria during pregnancy. In addition, limited data exist on the safety of anti-malarial drugs as pregnant women have been frequently excluded from most malaria treatment trials for fear of toxicity to the fetus[13]. Research is urgently needed to identify alternative anti-malarial drugs that are safe, acceptable and efficacious in treating and preventing malaria infection in pregnant women.

The use of combination anti-malarial drugs that target different pathways is being strongly advocated as a strategy for improving efficacy and delaying the emergence of drug-resistant parasites[14]. The combination of conventional anti-malarial drugs with artemisinin

derivatives has been proposed as the next best option for the treatment of resistant *P. falciparum*. Artesunate, one of the artemisinin derivatives, has been used widely in South-East Asia in non-pregnant individuals. There have been no reports of severe adverse effects in studies when it was given as treatment in the second and third trimesters of pregnancy[15, 16]. However, due to the small number of women in these studies, there is still need for more information on its safety in pregnant women. Azithromycin, a macrolide antibiotic, which has been found to have anti-malarial effect [17, 18], has been used in pregnant women to treat sexually transmitted infection (STIs), and other infections with a good safety profile[19, 20]. However, there have been no studies that have investigated its efficacy in treating malaria in pregnant women. The objective of this pilot study was to compare the efficacy and safety of SP combined with azithromycin or artesunate with SP monotherapy.

Methods

Study population

The study was conducted at Mpemba and Madziabango health-centers in Blantyre District, Malawi from September 2003 to September 2004. Blantyre district has a population of approximately 950,000 people[21]. Malaria transmission is perennial, but peaks during the rainy season (November–March). *P. falciparum* causes over 90% of all malaria infections. The two health-centers have maternity beds for delivery of low risk pregnancies. Women with high risk pregnancies are referred to Queen Elizabeth Central Hospital (QECH), a tertiary hospital located about 10 km from the health centers for management. Women were eligible if they had peripheral parasitemia, were 15-49 years old, fetal gestation age 14-26

weeks, and were available for follow-up until delivery. Women were excluded if they had multiple gestation, a history of chronic diseases such as tuberculosis and diabetes, a mental disorder, known allergies to drugs containing sulfonamides, macrolides or pyrimethamine, pregnancy complications, or if they had taken anti-malarial drugs within 28 days before enrollment.

Study procedures

Written or witnessed verbal informed consent was obtained prior to enrollment. A standardized questionnaire was administered on demographic information, history of malaria illnesses, and past medical and obstetric history. The women received routine antenatal assessment and had the option to be tested for human immunodeficiency virus (HIV) infection, with pre- and post-test counseling. The HIV test was only done in women who accepted to have the test. A venous blood sample was obtained to measure hemoglobin concentration, prepare thick blood films, and HIV testing. Subjects were randomly assigned using computer generated random numbers to three treatment arms: (1) SP (3 tablets; 500mg sulfadoxine and 25mg pyrimethamine per tablet); (2) SP (3 tablets) plus azithromycin (1g/day for 2 days) and (3) SP (3 tablets) plus artesunate (200mg/day for 3 days). Administration of the drug was under direct observation. A full dose of the drug was re-administered if the medication was vomited within 30 minutes of ingestion. Women stayed at the health-center for at least an hour after taking the drug. Women who lived far away spent 2 nights at the health-center or were visited at home. The women were given 200mg of ferrous sulfate and 0.25mg of folic acid for daily administration, and insecticide treated bed-nets (ITNs).

Follow-up

Women received two doses of the assigned treatment, given on average 4 weeks apart. Thick films were examined for parasitemia on days 1,2,3,7 and 14 after each treatment, at subsequent antenatal visits, and at unscheduled visits. Body temperature was recorded every 6 hours until it became normal (<37.5 ^{0}C). Symptoms present at baseline were considered to be attributable to malaria. Mild or moderate adverse events were monitored at the health-centers with outpatient management. All patients requiring hospitalization were monitored at the health-centers or referred to QECH. Women with parasitemia between days 7 and 28 after taking the assigned treatment were given quinine (600mg, 3 times/day for 5 days). The women were followed until delivery, and at each subsequent visit women received routine antenatal care, information was obtained on malarial illnesses, use of anti-malarial drugs, and potential side effects of the study drugs. The women were asked not to self-administer anti-malarial drugs, but to return to the health-centers for evaluation any time they experienced symptoms of malaria.

Delivery

All HIV-positive women were given nevirapine (Roxane Laboratories, Columbus, Ohio, USA) at the onset of active labor according to the HIVNET 012 protocol[22]. Newborns delivered at the health-centers or QECH were weighed using a digital scale (to the nearest gram) and gestational age was estimated using the Ballard score within 24 hours of delivery [23]. A blood sample was obtained from a maternal peripheral vein, the placenta and umbilical cord for preparation of thick films and estimation of maternal hemoglobin concentration. Full thickness placental biopsies were collected to prepare histology slides. If a traditional birth

attendant was present during home deliveries, birth weight was measured using color-coded scales that indicated red for birth weight less than 2,500g and green for birth weight at least 2,500g. Peripheral, placental and cord blood thick blood films were prepared and forwarded to the health-centers through community health workers.

Outcome measures

Treatment failure

Recurrent episodes detected between days 7 and 14 were classified as recrudescence without genotyping, because they were assumed very unlikely to be new infections[24]. For recurrent episodes detected after day 14, we genotyped the *msp-1* gene using a Heteroduplex Tracking Assay (HTA)[25, 26] to distinguish between recrudescence and new infections. Genotyped recurrent episodes were classified as recrudescence if all the variants or a subset of the alleles (at least one identical uncommon band with prevalence <10% or at least 2 common bands with a prevalence >10%) detected in a recurrent episode, were identical to those present in the initial infection.

Maternal and birth outcomes

Newborns were classified as having normal (≥ 2,500g) or low (< 2,500g) birth weight, regardless of gestational age. Prematurity was defined as delivery before 37 estimated weeks of gestation. Abortion was defined as delivery of a non-viable fetus before 28 weeks of gestation. Stillbirth was defined as delivery of a dead fetus after 28 weeks of gestation. Neonatal death was defined as death occurring during the first 27 completed day of

life. Women were defined as having anemia if they had hemoglobin concentration less than 11g/dL.

Laboratory procedures

Malaria diagnosis was performed by microscopy of Giemsa stained thick blood films. Parasite density was estimated using an assumed leukocyte count of 6,000 leukocytes/μl of blood. A thick film was considered negative if no parasites were detected after examining 100 microscopic fields each containing approximately 20 leukocytes. All thick films were read by two skilled microscopists. Discrepancies were solved by repeating the readings. An assay using real-time PCR and sequence-specific probes[27] was used to detect point mutations in the genes, encoding dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*), which have been associated with SP resistance[28]. Fixed placental biopsies were wax embedded, and 4μm thick sections were cut onto slides. Histological slides were prepared by staining with Gurr's modified Giemsa and/or Hematoxylin and Eosin. The slides were examined for presence of *P. falciparum* infected erythrocytes and hemozoin deposition in fibrin or monocytes[29]. Hemoglobin concentration was measured using a HemoCueÒ hemoglobinometer (HemoCue Incorporated, Angelholm, Sweden). HIV testing was performed using two enzyme immuno-assays, Determine HIV-1/2 Rapid Test (Abbott Laboratories, Illinois, USA) and Unigold Test (Trinity Biotech plc, Dublin, Ireland) according to the manufacturers' instructions. Discordant results were resolved using a third assay, Hemastrip Rapid Test (ChemBio Diagnostic Systems).

Ethical approval

This study was approved by the College of Medicine Research Committee (University of Malawi) and the Institutional Review Boards of the University of North Carolina (Chapel Hill, NC).

Statistical Analysis

Data analysis was conducted using STATA version 8 (Stata Corporation, TX, USA). Baseline characteristics and birth outcomes across the treatment groups were compared using the Chi-square test for categorical variables, analysis of variance for normally distributed, and the Kruskal-Wallis tests for non-normally distributed continuous variables. The main efficacy end point was HTA-adjusted recrudescence rates determined by survival analysis. Follow-up time was calculated in days from initial infection to occurrence of an event (recrudescence), or censoring (delivery or loss to follow-up). Kaplan-Meier survival curves assessed the independent predictors of treatment outcome. Comparison of the survival curves was performed using the Log-rank test. A conditional risk-set Cox proportional hazards regression model compared the efficacy of the three treatment regimens[30]. The baseline hazard was stratified by the number of treatment doses received. A robust variance estimator was included in the model to account for the correlation in each individual[31]. Other covariates included in the analysis were baseline parasite density, gravidity, HIV status, and history of receiving quinine during the follow-up period. The baseline parasite density was log-transformed to obtain a normal distribution. Since the number of women with unknown HIV status was large, regression models were derived with and without this variable. Safety end points were the incidence of adverse events.

Results

A total of 141 pregnant women with uncomplicated *P. falciparum* infection were recruited into the study, 47 women in each treatment group. Data on treatment allocation and follow-up losses are shown in Figure 1. Twenty-three women were lost to follow-up. Reasons included permanent movement from the study area (*n*=5), withdrawal from the study (*n*=16), and delivery outside the study area (*n*=2). The median follow-up period was 102 days (range 2-178 days). All women received at least one treatment course of the assigned drug and 121 women received a second course. Of the samples obtained at enrollment, 138 (98%) were genotyped. Mutations were present in 132 (97%) of the samples at *dhfr*-59 and 118 (90%) of the samples at *dhps*-540. The genotyping results imply a moderate to high level of SP resistance.

Baseline characteristics

There were no significant differences in most of the baseline demographic and clinical characteristics across the treatment groups (Table 1). Only 89 (63.1%) women accepted to have an HIV test result, and of these, 26 (29.2%) were HIV-positive. The proportions of HIV-infected women, although not significantly different, were higher in the SP [33.3%, (9/27)] and SP-azithromycin groups [34.5%, (10/29)] than in the SP-artesunate group [21.2%, (7/33)].

Treatment efficacy

Microscopic re-appearance of *P. falciparum* parasites after the first treatment was detected in 30 (24.8%) of the 121 evaluable women. HTA-genotyping indicated that 21

(70%) of these episodes were recrudescence. The overall median time to recrudescence was 34 days (range 7-133 days). The highest recrudescence rates was 35% (14/40) in women receiving SP, compared with 9.5% (4/42) in women given SP-azithromycin, and 7.7% (3/39) for the SP-artesunate. Of the 9 new infections identified at the time of receiving the second treatment, 2 resulted in recrudescence (HTA-corrected), both in the SP-artesunate group, detected on days 34 and 104, respectively. Overall, recrudescent episodes were significantly less frequent in the SP-azithromycin [Hazard ratio (HR) 0.23, (95% confidence interval (CI) 0.08-0.71)] and SP-artesunate treatment groups [HR 0.25 (95% CI 0.10-0.61)] compared with SP monotherapy (Figure 2).

After adjusting for gravidity and baseline parasite density, recrudescent episodes of malaria were still less frequent in the SP-azithromycin [HR 0.19 (95% CI 0.06-0.63)] and the SP-artesunate groups [HR 0.25 (95% CI 0.10-0.65)] than with SP monotherapy. There was no significant difference in efficacy between the SP-azithromycin and SP-artesunate groups. Recrudescent episodes remained less frequent in the SP-azithromycin [HR 0.14 (95% CI 0.02 to 0.78)] and the SP-artesunate groups [HR 0.13 (95% CI 0.03 to 0.53)] after including HIV in the model. Sensitivity analysis confirmed that there were lower recrudescence rates in women who received the two combination therapies compared with those given SP monotherapy, after taking into account possible misclassification of recrudescence [32].

Parasite clearance

SP-artesunate significantly accelerated the clearance of parasites compared with SP-azithromycin or SP (Figure 3). By day 2, the parasite clearance rates were significantly

higher for women allocated to SP-artesunate [39/47, (83.0%)] than those given SP [12/47, (25.5%)] and SP-azithromycin [11/47, (23.4%)]; p< 0.001. By day 3, the parasite clearance rates for SP-artesunate were (46/47, 97.8%) compared with [33/47, (70.2%)] and [35/47, (74.5%)] in the SP and SP-azithromycin groups respectively. None of the women in the SP-azithromycin and SP-artesunate groups had parasitemia on day 7. However, 4 (8.5%) women in the SP group still had parasitemia on day 7 and 2 (4.3%) were parasitemic on day 14.

Maternal parasitological and hematological responses at delivery

The prevalence of peripheral parasitemia at delivery was similar across the treatment groups (Table 2). There were more women with placental malaria diagnosed via microscopy for SP-azithromycin [10, (30.3%)] than SP [5, (16.1%)] and SP-artesunate [6, (17.1%)] groups, p< 0.05. However, histologically there was no significant difference in malaria prevalence across the treatment groups, SP [11, (47.8%)] *vs.* SP-azithromycin [9, (50.0%)] *vs.* SP-artesunate [13, (44.8%)), p=0.23. The maternal hemoglobin concentration was higher at delivery than at enrolment for all treatment groups [SP (12.6g/dL *vs*. 10.2g/dL); SP-azithromycin (12.6g/dL *vs*. 10.0g/dL) and SP-artesunate (13.0g/dL *vs*. 10.5g/dL)], but there was no significant difference between the groups (p=0.48).

Pregnancy outcomes

Of the 118 deliveries with known outcomes, there were 109 live births, 4 spontaneous abortions and 5 still births (Table 2). All 4 abortions occurred in the SP-azithromycin group. Three of the abortions occurred 25-29 days after treatment. Two of these women had malaria at the time of abortion, of which one had HIV-coinfection; the other woman was HIV-

positive. There was 1 stillbirth in the SP, none in the SP-azithromycin and 4 in the SP-artesunate group. Two stillbirths occurred after prolonged labor, 1 occurred after the mother had taken traditional medicine to induce labor, 1 had the cord around the neck at delivery, and in 1 stillbirth, the mother had a positive syphilis test at delivery. There were more neonatal deaths in the SP (*n*=4) compared with the SP-azithromycin (*n*=1) and the SP-artesunate group (*n*=3). Four of the deaths occurred soon after delivery, secondary to premature delivery at 29-31weeks. Two neonatal deaths occurred a few hours after delivery due to complications of labor (obstructed labor). Thus, the abortions, still births and neonatal deaths all potentially had other proximal causes and were probably unrelated to treatment.

Kernicterus was detected in one infant, born at 36 weeks gestation, weighing 2,150g at delivery, whose mother was treated with SP. Table 3 contains further details related to the pregnancy outcomes.

Birth weight

One hundred and six (89.8%) of the 118 babies were weighed and gestational ages assessed within 24 hours of delivery. There was no difference in the gestation age between the groups, and no physical abnormalities were detected in any of the newborn babies. Although not significantly different, the mean birth weights of infants born to mothers in the SP (2,868.6g, SD 625.2g) and SP-artesunate (2,836.2g, SD 482.0g) was higher than the SP-azithromycin group (2,784.7g, SD 536.6g) (Table 2).

Maternal adverse effects

Artesunate and azithromycin were well tolerated. There were no serious or clinically significant treatment-associated adverse reactions reported. Early drug-induced vomiting occurred in 2 women given SP-azithromycin, who vomited the drug soon after ingestion. Mild adverse reactions frequently reported included general body pains 5.7% (8/141), headache 3.5% (5/141), vomiting 2.1% (3/141), anorexia 1.4% (2/141), nausea 1.4% (2/141), and diarrhea 1.4% (2/141). However, these symptoms were indistinguishable from malaria symptoms. There was no significant difference in the reporting of these symptoms across the treatment groups.

Discussion

Our results show that SP plus azithromycin or artesunate were more effective in treating malaria in pregnant women than SP monotherapy. In addition, the artesunate-combination shortened the clearance time of parasites. Both regimens were well tolerated. No treatment-related severe adverse events were detected, but larger studies will be needed to rule out associations with abortions, still births and neonatal deaths. We did not find a significant difference in the birth weight, maternal hemoglobin concentration, peripheral and placental parasitemia across the treatment groups. This may have been due to the small sample size in this study.

Recent studies have shown that the combination SP-artesunate is effective against uncomplicated *P. falciparum* infection in African children[33, 34]. However, very few studies have investigated the safety and efficacy of SP-artesunate in treating malaria during

pregnancy. A study conducted in Sudan found that SP-artesunate was efficacious in treating uncomplicated *P. falciparum* malaria in 32 pregnant women with no adverse effects[35]. Women who were exposed to SP-artesunate in Gambia during mass drug administration did not experience adverse effects and delivered babies with higher birth weight compared with women who did not receive treatment[36]. Other studies have also found that artesunate alone or in combination with mefloquine, atovaquone, proguanil or quinine was efficacious and safe in all trimesters of pregnancy[15, 16, 37-39].

However, SP-azithromycin may have several advantages. First, although the parasite clearance rate was slow compared with the SP-artesunate group, the rate of recrudescence was similar to SP-artesunate. Secondly, azithromycin has been used to treat STIs in pregnant women with a very good safety profile, and reduced the prevalence of low birth weight, and neonatal deaths[19]. Therefore, using SP-azithromycin to treat pregnant women with malaria may also protect mothers against other infectious causes of poor birth outcomes. Thirdly, azithromycin has a relatively long half-life compared with artesunate. The combination of artesunate with a long half-life partner drug could promote resistance to the partner [40].

The two combination regimens were relatively well tolerated. The minor side-effects reported by the women, were difficult to distinguish from symptoms of uncomplicated malaria. There were 4 abortions in the SP-azithromycin group which were probably not treatment-related. However, a larger study is needed in order to rule out the possibility of an association.

Malawi introduced SP as first-line treatment for uncomplicated *P. falciparum* infection and for IPT in 1993. However, the prevalence of clinical failure and mutations in parasite enzymes associated with resistance to SP have risen in recent years [41]. We also found high prevalence levels of mutations (90-97%) associated with SP resistance, which may explain why SP monotherapy was inferior to SP combination therapies. Our results indicate that there will be need to change to other anti-malarial drugs in the near future. Choosing the optimal drug combination will depend on many factors such as transmission dynamics, cost, safety, dosing requirements and acceptability. There is also a need to consider interactions with anti-retroviral drugs in areas where the HIV prevalence is high, as is the case for Malawi [42].

In conclusion, this pilot trial found that SP combined with azithromycin or artesunate was efficacious in treating and preventing malaria in pregnant women. These preliminary results are promising and should encourage further and larger treatment studies to confirm the efficacy and safety of the combination of SP with artesunate or azithromycin in pregnant women. This information will be very important for the formulation of anti-malarial drug policy.

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Financial Disclosures: None reported.

Funding/Support: This study was funded by CDC grant number CDC/ASPH/ASTDR S1935-21/21. Dr Steve Rogerson is supported by a Wellcome Trust Senior Research Fellowship.

Role of the Sponsor: The CDC had no role in the design and conduct of the study, data collection, analysis and interpretation of the data, in the preparation, review, or approval of the manuscript.

Acknowledgements

We would like to thank Mrs Chaluluka, the drivers, study nurses, technicians and traditional birth attendants in Mpemba and Madziabango areas, the nurses at QECH labor ward for their co-operation and all the women who agreed to participate in this study We also thank Ella Nkhoma for the laboratory work in genotyping the blood samples, contributions to the study design from Dr Victor Mwapasa, Professor Cameron Bowie and statistical analysis suggestions from Dr William Miller..

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References

1. WHO. World Malaria Report, 2005. Accessed at http://rbm.who.int/wmr2005/ on October 20,2006.

2. Brabin BJ. An analysis of malaria in pregnancy in Africa. *Bull World Health Organ*. 1983;61:1005-1016.

3. Guyatt HL, Snow RW. The epidemiology and burden of *Plasmodium falciparum*-related anemia among pregnant women in sub-Saharan Africa. *Am J Trop Med Hyg*. 2001;64:36-44.

4. Shulman CE. Malaria in pregnancy: Its relevance to safe-motherhood programmes. *Ann Trop Med Parasitol*. 1999;93 Suppl 1:S59-66.

5. WHO 2002. Strategic framework for malaria prevention and control during pregnancy in the African region. Brazzaville, World Health Organization; 2004,AFR/MAL/04/01.

6. Shulman CE, Dorman EK, Cutts F, et al. Intermittent sulphadoxine-pyrimethamine to prevent severe anaemia secondary to malaria in pregnancy: A randomised placebo-controlled trial. *Lancet*. 1999;353:632-636.

7. van Eijk AM, Ayisi JG, ter Kuile FO, et al. Effectiveness of intermittent preventive treatment with sulphadoxine-pyrimethamine for control of malaria in pregnancy in western Kenya: A hospital-based study. *Trop Med Int Health*. 2004;9:351-360.

8. Steketee RW, Wirima JJ, Hightower AW, Slutsker L, Heymann DL, Breman JG. The effect of malaria and malaria prevention in pregnancy on offspring birth weight, prematurity, and intrauterine growth retardation in rural Malawi. *Am J Trop Med Hyg*. 1996;55:33-41.

9. Parise ME, Ayisi JG, Nahlen BL, et al. Efficacy of sulfadoxine-pyrimethamine for prevention of placental malaria in an area of Kenya with a high prevalence of malaria and human immunodeficiency virus infection. *Am J Trop Med Hyg*. 1998;59:813-822.

10. Rogerson SJ, Chaluluka E, Kanjala M, Mkundika P, Mhango C, Molyneux ME. Intermittent sulfadoxine-pyrimethamine in pregnancy: Effectiveness against malaria morbidity in Blantyre, Malawi, in 1997-99. *Trans R Soc Trop Med Hyg*. 2000;94:549-553.

11. Schultz LJ, Steketee RW, Macheso A, Kazembe P, Chitsulo L, Wirima JJ. The efficacy of antimalarial regimens containing sulfadoxine-pyrimethamine and/or chloroquine in preventing peripheral and placental *Plasmodium falciparum* infection among pregnant women in Malawi. *Am J Trop Med Hyg*. 1994;51:515-522.

12. Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR. Epidemiology of drug-resistant malaria. *Lancet Infect Dis*. 2002;2:209-218.

13. Phillips-Howard PA, Wood D. The safety of antimalarial drugs in pregnancy. *Drug Saf*. 1996;14:131-145.

14. White NJ, Nosten F, Looareesuwan S, et al. Averting a malaria disaster. *Lancet*. 1999;353:1965-1967.

15. McGready R, Ashley EA, Moo E, et al. A randomized comparison of artesunate-atovaquone-proguanil versus quinine in treatment for uncomplicated *falciparum* malaria during pregnancy. *J Infect Dis*. 2005;192:846-853.

16. McGready R, Cho T, Samuel, et al. Randomized comparison of quinine-clindamycin versus artesunate in the treatment of falciparum malaria in pregnancy. *Trans R Soc Trop Med Hyg*. 2001;95:651-656.

17. Andersen SL, Oloo AJ, Gordon DM, et al. Successful double-blinded, randomized, placebo-controlled field trial of azithromycin and doxycycline as prophylaxis for malaria in western Kenya. *Clin Infect Dis*. 1998;26:146-150.

18. Dunne MW, Singh N, Shukla M, et al. A multi-center study of azithromycin, alone and in combination with chloroquine, for the treatment of acute uncomplicated plasmodium falciparum malaria in India. *J Infect Dis*. 2005;191:1582-1588.

19. Gray RH, Wabwire-Mangen F, Kigozi G, et al. Randomized trial of presumptive sexually transmitted disease therapy during pregnancy in Rakai, Uganda. *Am J Obstet Gynecol*. 2001;185:1209-1217.

20. Sarkar M, Woodland CC, Koren G, Einarson AR. Pregnancy outcome following gestational exposure to azithromycin. *BMC Pregnancy Childbirth*. 2006;6:18.

21. National Statistical Office (NSO). Malawi Population and Housing Census. Summary of final results, Volume 1, Zomba, Malawi 1998.

22. Guay LA, Musoke P, Fleming T, et al. Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: HIVNET 012 randomised trial. *Lancet*. 1999;354:795-802.

23. Ballard JL, Khoury JC, Wedig K, Wang L, Eilers-Walsman BL, Lipp R. New ballard score, expanded to include extremely premature infants. *J Pediatr*. 1991;119:417-423.

24. WHO 2005. Susceptibility of *Plasmodium falciparum* to antimalarial drugs. report on global monitoring 1996-2004. WHO/HTM/MAL/2005.1103.

25. Ngrenngarmlert W, Kwiek JJ, Kamwendo DD, et al. Measuring allelic heterogeneity in plasmodium falciparum by a Heteroduplex tracking assay. *Am J Trop Med Hyg*. 2005;72:694-701.

26. Yeramian P, Meshnick SR, Krudsood S, et al. Efficacy of DB289 in thai patients with *Plasmodium vivax* or acute, uncomplicated *Plasmodium falciparum* infections. *J Infect Dis*. 2005;192:319-322.

27. Alker AP, Mwapasa V, Meshnick SR. Rapid real-time PCR genotyping of mutations associated with sulfadoxine-pyrimethamine resistance in *Plasmodium falciparum*. *Antimicrob Agents Chemother*. 2004;48:2924-2929.

28. Kublin JG, Dzinjalamala FK, Kamwendo DD, et al. Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. *J Infect Dis*. 2002;185:380-388.

29. Rogerson SJ, Pollina E, Getachew A, Tadesse E, Lema VM, Molyneux ME. Placental monocyte infiltrates in response to *Plasmodium falciparum* malaria infection and their association with adverse pregnancy outcomes. *Am J Trop Med Hyg*. 2003;68:115-119.

30. *T. Therneau and P. Grambsch. Modeling Survival Data: Extending the Cox Model. New York: Springer-Verlag*, 2000.

31. D. Lin and L. Wei. The robust inference for the Cox proportional hazards model, *J. American Statistical Association*. 1989;84:1074-1078.

32. Kwiek J.J., AlkerA. P.,Wenink E.C., et al. Estimating true antimalarial efficacy by Heteroduplex tracking assay in patients with complex *Plasmodium falciparum* infections. *Antimicrobial agents and chemotherapy*.

33. Priotto G, Kabakyenga J, Pinoges L, et al. Artesunate and sulfadoxine-pyrimethamine combinations for the treatment of uncomplicated *Plasmodium falciparum* malaria in uganda: A randomized, double-blind, placebo-controlled trial. *Trans R Soc Trop Med Hyg*. 2003;97:325-330.

34. Dorsey G, Njama D, Kamya MR, et al. Sulfadoxine/pyrimethamine alone or with amodiaquine or artesunate for treatment of uncomplicated malaria: A longitudinal randomised trial. *Lancet*. 2002;360:2031-2038.

35. Adam I, Ali DM, Abdalla MA. Artesunate plus sulfadoxine-pyrimethamine in the treatment of uncomplicated *Plasmodium falciparum* malaria during pregnancy in eastern Sudan. *Trans R Soc Trop Med Hyg*. 2006.

36. Deen JL, von Seidlein L, Pinder M, Walraven GE, Greenwood BM. The safety of the combination artesunate and pyrimethamine-sulfadoxine given during pregnancy. *Trans R Soc Trop Med Hyg*. 2001;95:424-428.

37. McGready R, Cho T, Cho JJ, et al. Artemisinin derivatives in the treatment of *falciparum* malaria in pregnancy. *Trans R Soc Trop Med Hyg*. 1998;92:430-433.

38. McGready R, Cho T, Keo NK, et al. Artemisinin antimalarials in pregnancy: A prospective treatment study of 539 episodes of multidrug-resistant *Plasmodium falciparum*. *Clin Infect Dis*. 2001;33:2009-2016.

39. Piola P, Fogg C, Bajunirwe F, et al. Supervised versus unsupervised intake of six-dose artemether-lumefantrine for treatment of acute, uncomplicated *Plasmodium falciparum* malaria in Mbarara, Uganda: A randomised trial. *Lancet*. 2005;365:1467-1473.

40. Hastings IM, Ward SA. Coartem (artemether-lumefantrine) in Africa: The beginning of the end? *J Infect Dis*. 2005;192:1303-4; author reply 1304-5.

41. Plowe CV, Kublin JG, Dzinjalamala FK, et al. Sustained clinical efficacy of sulfadoxine-pyrimethamine for uncomplicated *falciparum* malaria in Malawi after 10 years as first line treatment: Five year prospective study. *BMJ*. 2004;328:545.

42. Brentlinger PE, Behrens CB, Micek MA. Challenges in the concurrent management of malaria and HIV in pregnancy in sub-Saharan Africa. *Lancet Infect Dis*. 2006;6:100-111.

Table 4.1. The baseline characteristics of pregnant women enrolled into the clinical trial

Characteristic	Treatment Group			P-value
	SP Only	SP & Azithromycin	SP & Artesunate	
Age in years, mean (SD)	21.6 (4.9)	21.2 (4.8)	20.9 (4.6)	0.72
Body weight in kg, mean (SD)	53.3 (5.9)	52.2 (6.1)	52.9 (5.9)	0.62
Married (%)	44 (93.6)	38 (80.0)	43 (91.5)	0.18
Education (%)				
None	3 (6.4)	9 (19.2)	8 (17.0)	
Primary	42 (89.4)	31 (65.9)	32 (68.1)	0.06
Secondary/Tertiary	2 (4.26)	7 (14.9)	7 (14.9)	
Gravidity, median [range]	1 (1-8)	1 (1-9)	1 (1-7)	
Primigravidae (%)	27 (57.5)	25 (53.1)	28 (59.6)	0.91
Secundigravidae (%)	5 (10.60	7 (14.89)	4 (8.5)	
Gestation weeks at 1st consultation, mean (SD)	21.9 (3.6)	22.4 (3.1)	22..0 (3.2)	0.81
Parasitemia/µl, geometric mean [range]	964.5 (180-22500)	1184.2 (150-22500)	686.6 (120-4260)	0.05
Hemoglobin concentration (g/dL)	10.2 (1.8)	10.0 (1.4)	10.5(1.6)	0.27
HIV Positive† (%)	9 (33.3)	10 (34.5)	7 (21.2)	0.45

†Only 89 women accepted to have an HIV test; SP group (*n*=27), SP-azithromycin group (*n*=29), SP-artesunate group (*n*=33)

Table 4.2. Maternal and fetal outcomes according to treatment group

Outcome	Treatment Group			P-value
	SP	SP & Azithromycin	SP & Artesunate	
Peripheral parasitemia at delivery (%)§	10 (30.3)	10 (30.3)	8 (22.9)	0.15
Placental parasitemia by microscopy (%)¶	5 (16.1)	10 (30.3)	6 (17.1)	0.04
Placental parasitemia by histology #	11 (47.8)	9 (50.0)	13 (44.8)	0.23
Cord blood parasitemia	0	2 (4.3)	1 (2.1)	0.09
Hemoglobin concentration††, g/dL (mean, SD)	12.6 (2.3)	12.6 (2.3)	13.0 (2.0)	0.59
Maternal anemia (%)	8 (24.2)	8 (25.8)	5 (14.2)	0.48
Spontaneous abortions (%)	0	4 (8.5)	0	0.02
Still birth (%)	1 (2.1)	0	4 (8.5)	0.03
Neonatal deaths (%)	4 (8.5)	1 (2.1)	3 (6.4)	0.34
Gestational age at delivery†, weeks (median, IQR)	38 (29-42)	36 (33-42)	37 (34-42)	0.21
Birth weight , grams‡, (mean, SD)	2868.6 (625.2)	2784.7 (536.6)	2836.2 (482.0)	0.87
Low birth weight (%)	8 (22.2)	6 (20.0)	6(17.7)	0.45

§ 101 women had peripheral parasitemia results; SP only (*n*=33), SP-azithromycin (*n*=33), SP-artesunate (*n*=35)
¶99 women had placental parasitemia results; SP only (*n*=31), SP-azithromycin (*n*=33), SP-artesunate (*n*=35)
#70 women had placental histology results; SP only (*n*=23), SP-azithromycin (*n*=18), SP-artesunate (*n*=29)
††99 women had hemoglobin results; SP only (*n*=33), SP-azithromycin (*n*=31), SP-artesunate (*n*=35)
† Estimated using the Ballard score
‡Includes only live born singletons with birth weight

Table 4.3. Details of women who had adverse pregnancy outcomes

Patient No	Treatment Group	Outcome	Details related to death	Days after last dose
1	SP	Stillbirth	Prolonged labor	92 days
2	SP	NND after 5 days	Birth weight 2150g, had Kernicterus diagnosed by clinical symptoms	78 days
3	SP	NND after 9 days	NA	142 days
4	SP	NND	Preterm delivery at 29 weeks	33 days
5	SP	NND	Preterm delivery at 29 weeks, birth weight 930g, MPs+	30 days
6	SP-Artesunate	NND	Preterm delivery at 28 weeks	29 days
7	SP-Artesunate	Stillbirth	Full term delivery, weight 2.7kg, cord around the neck, MPs+	11 days
8	SP-Artesunate	NND	Preterm delivery at 28 weeks, birth weight 1.3 kg	31 days
9	SP-Artesunate	Stillbirth	Full term, took traditional medicines to enhance labor, MPs+	99 days
10	SP-Artesunate	Stillbirth	Preterm delivery at 31 weeks, birth weight 2 kg, VDRL 1:64 positive and TPHA positive	29 days
11	SP-Artesunate	Stillbirth	Prolonged labor, birth weight 2600g	127 days
12	SP-Artesunate	NND	Obstructed labor, birth weight 2600g	79 days
13	SP-Azithromycin	NND	Obstructed labor, birth weight 2800g, MPS+	54 days
14	SP-Azithromycin	Abortion at 26 weeks	NA	4 days
15	SP-Azithromycin	Abortion at 25 weeks	MPs+	25 days
16	SP-Azithromycin	Abortion at 27 weeks	HIV Positive, MPs+	28 days
17	SP-Azithromycin	Abortion at 27 weeks	HIV positive	29 days

SP- sulfadoxine-pyrimethamine; NND- neonatal death; MPs-malaria parasites detected in the maternal peripheral or placental blood; NA-no information available related to the abortion or neonatal death; VDRL-Venereal Disease Research Laboratory test; TPHA- *Treponema pallidum* hemagglutination assay; HIV-human immunodeficiency virus

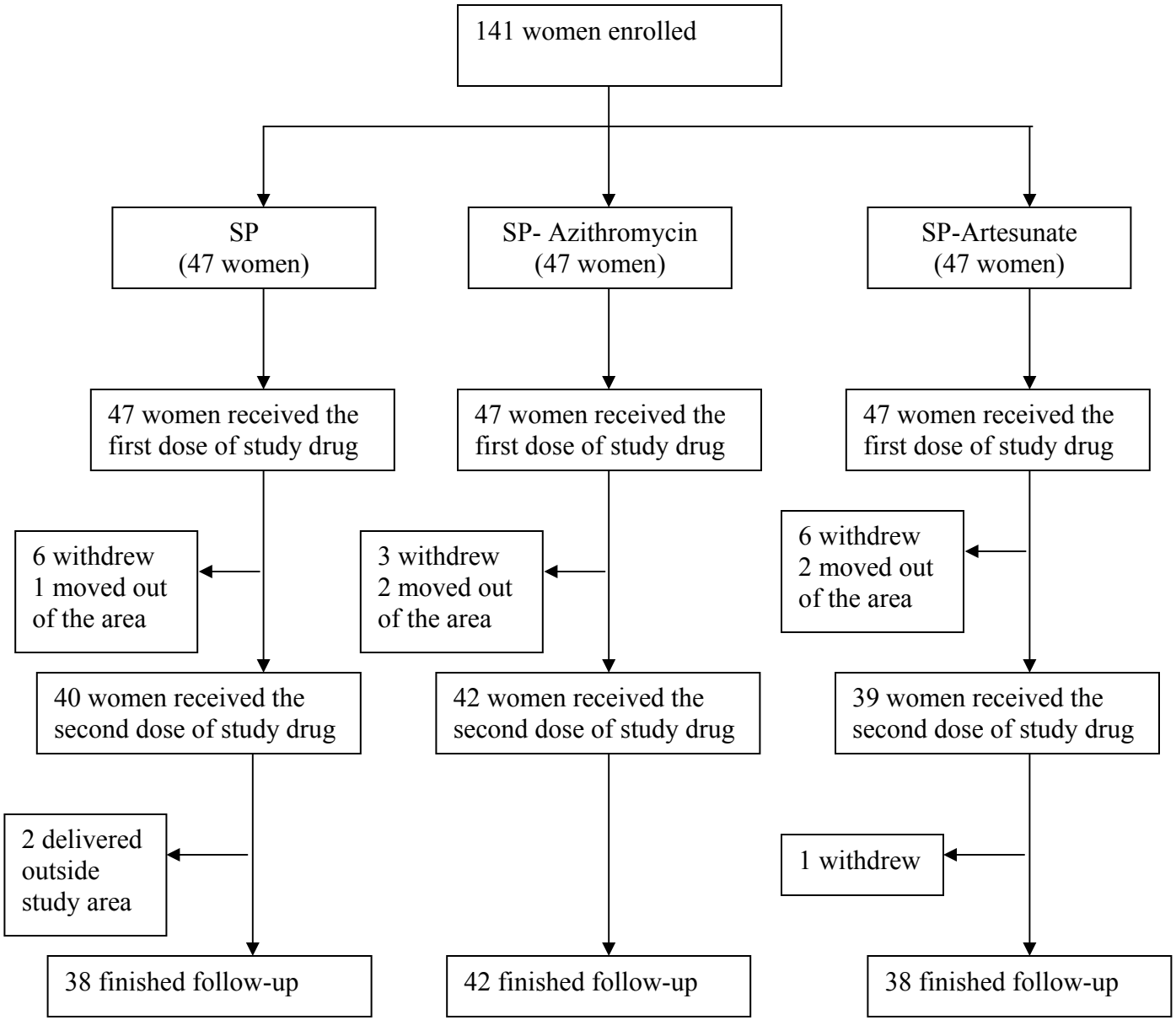

Figure 4.1. Flow diagram of the study participants

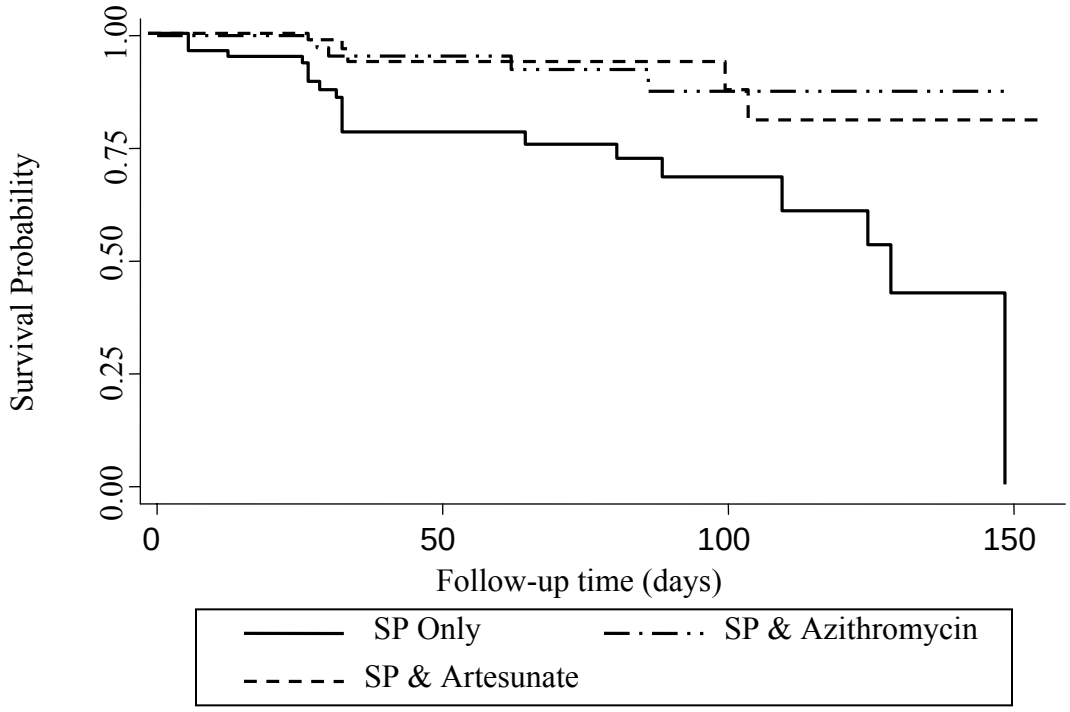

Figure 4.2. The rate of recrudescence according to treatment group

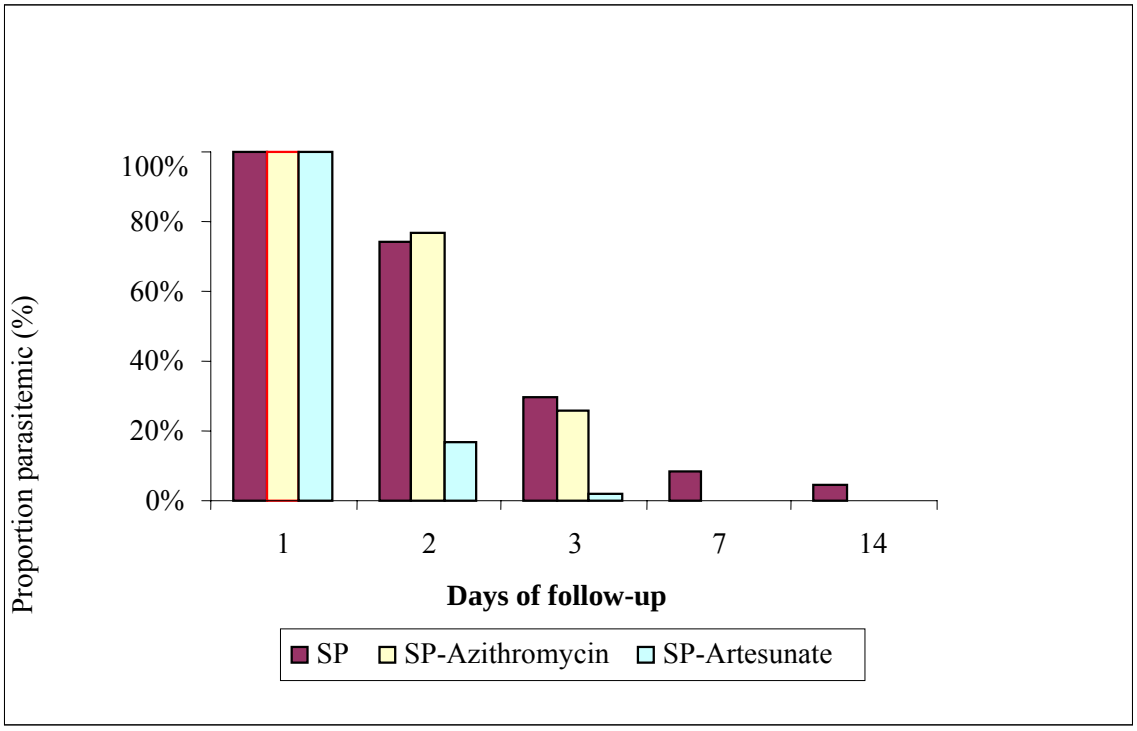

Figure 4.3. Parasite clearance time after treatment

CHAPTER 5

DISCUSSION

5.1. Specific AIM I

We found that the risk of low birth weight and maternal anemia increased with the number of episodes of malaria experienced during pregnancy. Furthermore, the time of infection also had an effect on the risk of low birth weight and maternal anemia. The prevalence of low birth weight was higher when parasitemia was detected in the antenatal period, especially in the second trimester of pregnancy. In contrast, the prevalence of maternal anemia was higher in women who had infection late in pregnancy detected in the third trimester of pregnancy or at delivery compared with women who had parasitemia in the antenatal period or in the second trimester.

These results present to date, a study of a large sample of women of all parities followed longitudinally during pregnancy to estimate the effect of timing and frequency of infection during pregnancy on low birth weight and maternal anemia. Most previous studies have examined the association between malaria and its adverse effects by measuring parasitemia at one time point during pregnancy, or examining placental histology at delivery [1-8] . However, there is a strong potential for misclassification when information on malaria infection is collected at one point in time during the entire pregnancy period because studies have shown that peripheral parasitemia is not a very sensitive indicator of the presence of *P. falciparum* infection during pregnancy [9,10]. Although placental histology is the most sensitive indicator of infection with malaria during pregnancy [10], it has some limitations. The placenta can only be examined after delivery and some infections occurring early in pregnancy may completely resolve by the time of delivery, leaving no residual pathology [9,11,12]. Therefore to increase the sensitivity

of detecting parasitemia, and to reduce the probability of misclassification, we obtained blood samples from the pregnant women during pregnancy, and placental parasitemia at delivery. We did not examine placental biopsies and therefore could still have missed some of the women who had malaria during pregnancy.

Our study population was community based. We recruited women who were presenting at the two health centers for antenatal care. Surveys have shown that more than 90% of pregnant women in Malawi visit antenatal clinics at least once during pregnancy [13]. Therefore the study sample was more likely representative of pregnant women living in rural areas in Malawi. Traditional birth attendants and community health workers were recruited to collect data for home deliveries to minimize loss to follow-up. Despite these measures, loss to follow-up was a concern in this investigation and occurred in part because of the highly mobile nature of the study population.

We only recruited a very small sample of women in the first trimester of pregnancy. Previous studies have shown that women in Malawi tend to seek health care in the middle to- late second trimester [14,15]. Therefore we were unable to investigate the impact of malaria infection during the first trimester of pregnancy. We also did not obtain information on HIV-serostatus which is associated with increased risk for malaria infection and its complications during pregnancy and therefore our estimates could be biased by lack of this information.

Our findings have policy implications in preventing malaria during pregnancy. Currently, it is recommended that pregnant women in malaria endemic areas should receive at least two doses of sulfadoxine-pyrimethamine as intermittent preventive therapy (IPT) during pregnancy, one dose soon after quickening and the other dose in the third trimester of pregnancy [16]. We found that the number of episodes of malaria experienced during pregnancy increased the risk of low birth weight and maternal anemia. This is especially important in HIV-infected women who are at risk for multiple infections during pregnancy[17]. In most malaria endemic areas women do not receive the recommended two doses of SP during pregnancy as IPT. A recent study conducted in Malawi found low compliance to IPT among pregnant women, especially multigravidae, despite widespread awareness on its benefits and recommended improved delivery mechanisms of IPT [18]. Furthermore, most women in Malawi tend to present for antenatal care late in pregnancy [14,15]. It is therefore important to ensure that women receive the required doses of IPT during pregnancy to reduce the number of infections. Pregnant women should be given SP even when they present for antenatal care late in pregnancy as this could still reduce the risk of low birth weight, because we found that infection late in pregnancy increased the risk of low birth weight more than early in pregnancy.

Improved antenatal care services would be one way of improving compliance and coverage. In order to maximize the benefits of preventive measures such as use of insecticide-treated bed nets and intermittent preventive therapy, it is also important that women attend ANC at an earlier stage of pregnancy than is currently the case. However, this demands allocation of more resources to the health services in terms of manpower, training, stable drug supply and quantitative as well as qualitative improvements of health

facilities. Health systems and services research is needed to improve effectiveness and efficiency of existing services and for a better prioritization of resource allocations so that more women will be motivated to use them. Since the majority of pregnant women do not have access to malaria preventive measures and other services delivered through health units, ways of administering anti-malarial drugs to this high risk group at community level, through accessible, acceptable and safe means need to be explored. Although the risk of malaria and its adverse effects was higher in primigravidae, multigravidae still represented a big proportion of women who had malaria who were also at risk for low birth weight and maternal anemia. Therefore multigravidae should also be targeted in prevention policies that are established to prevent malaria in pregnant women.

We have identified the impact of timing and frequency of infection during pregnancy on low birth weight and maternal anemia. Few prospective studies have studies the evolution of infection during pregnancy and it effect on low birth weight and maternal anemia, recruiting women of all parities. A prospective study building on the research in this dissertation could offer more contribution to the knowledge of the pathophysiology of malaria during pregnancy. Future research studies could improve on the identification of malaria during pregnancy by including the examination of placental histology in addition to examining parasitemia at different points during pregnancy. Furthermore, recruiting women in the first trimester of pregnancy would be important to understand the impact of malaria at this point when embryogenesis takes place. Follow-up of women during pregnancy while monitoring the growth of the fetus using more sensitive methods such as ultrasound sonography would provide more information on how malaria causes low birth weight during pregnancy.

5.2. Specific AIM II

For our second aim, we investigated the safety and efficacy of sulfadoxine-pyrimethamine (SP) combined with azithromycin or artesunate compared with SP monotherapy in treating uncomplicated *Plasmodium falciparum* infection in pregnant women. We found high recrudescence rates when we used SP monotherapy compared with SP combined with azithromycin or artesunate to treat malaria in pregnant women. We also found high prevalence rates of mutations (90-97%) that are associated with SP resistance in our study population. Therefore, there will be need to change from SP monotherapy to a more efficacious regimen in the near future. Apart from two women who had drug induced vomiting n the SP-azithromycin treatment group, none of the other women reported severe adverse events that were directly related to treatment. Additionally, SP-artesunate significantly shortened the parasite clearance.

This was the first study to investigate the use of SP-azithromycin and SP-artesunate for treatment and prevention of malaria in pregnant women. The two combination regimens were relatively well tolerated. The minor side-effects reported by the women, were indistinguishable from symptoms of uncomplicated malaria. There were more abortions in the SP-azithromycin group which were probably not treatment-related. However, a larger study is needed in order to determine if the abortions were treatment related. There were more stillbirths in the SP-artesunate group compared with the other two groups. However, they were directly related to obstetric complications. Most of the neonatal deaths were secondary to premature delivery. The prevalence of neonatal deaths

and stillbirths found in our study are similar to reports from other studies in Blantyre (personal communication Dr Metaferia).

The median follow-up period was 80 days. Therefore we were able to detect most of the recrudescent infections. We performed molecular genotyping using a Heteroduplex tracking assay [19,20], to differentiate between recrudescent and new infections because of the long follow-up time. However, it is possible that a patient might have had a new parasite infection that had the same multilocus genotype as the pretreatment infection. We conducted a sensitivity analysis to assess the impact of such misclassification on our results and found that the combination therapies were still significantly more efficacious compared with SP monotherapy.

One of the limitations of our study was that information on the use of anti-malarials outside the study setting was self-reported and was not confirmed by measuring drug levels in urine or blood. This could have underestimated or overestimated our results, depending on the efficacy of the drugs obtained outside the study settings. In addition, the overall sample size was small and therefore we had low power to detect significant association of the some of the outcomes if present.

There is a big overlap in the geographical distribution of malaria and HIV. In most malaria-endemic areas, the prevalence of HIV in women in the reproductive age is high [21]. Studies have suggested that the immune response against malaria in women co-infected with HIV is impaired [17]. A number of studies from Malawi and Kenya have

shown that *P. falciparum* parasitemia occurs more frequently in HIV-infected pregnant women [22]. These women required more doses of SP to prevent malaria [23]. The optimum approach to IPT in women who are HIV-positive is still not known. Most of the women in our study refused to have an HIV test. Therefore we were unable to conduct further analysis to assess the effect of HIV on efficacy on the combination therapies. More research is therefore needed to assess if the efficacy of the combination therapies would be affected by coinfection with HIV.

Currently, there is a big drive to roll-out antiretroviral medication on a large scale in areas where HIV prevalence is high. However, there is limited information on the interaction between anti-malarial and antiretroviral drugs. One study found that HIV-1 protease inhibitors, ritonavir and saquinavir decrease CD36-mediated adhesion of parasitized erythrocytes, and non-opsonic phagocytosis of parasitized erythrocytes by human macrophages, which may modify malaria sequestration *in vivo* [24]. This might lead to decreased phagocytosis of parasitized erythrocytes, higher parasite densities and possibly more severe infection. As numerous trials are being conducted to identify new alternatives to SP for the treatment and prevention of malaria in pregnant women, there is also need for research on how these new drugs would interact with antiretroviral drugs as this could present a substantial problem in most malaria endemic areas.

While there is urgency to offer more effective treatment whilst minimizing the risks of developing further drug resistance, there are still many operational constraints such as affordability, acceptability, and adherence by patients and access through public and private sectors. In order to obtain the benefits of combination therapy and protect the

component drugs, complete adherence to the full recommended dosing regimen is necessary. Long-duration regimens, complex dosing schemes, high cost, poor understanding of how or why to adhere to recommended regimens, and adverse reactions to treatment contribute to non-adherence. A more comprehensive operational research, information gathering and consultation strategy needs conducted before recommended wide use of the combination therapies in pregnant women.

5.3. References

(1) Brabin BJ. An analysis of malaria in pregnancy in Africa. Bull.World Health Organ. 1983;61(6):1005-1016.

(2) McGregor IA. Epidemiology, malaria and pregnancy. Am.J.Trop.Med.Hyg. 1984 Jul;33(4):517-525.

(3) Steketee RW, Wirima JJ, Hightower AW, Slutsker L, Heymann DL, Breman JG. The effect of malaria and malaria prevention in pregnancy on offspring birthweight, prematurity, and intrauterine growth retardation in rural Malawi. Am.J.Trop.Med.Hyg. 1996;55(1 Suppl):33-41.

(4) Matteelli A, Donato F, Shein A, Muchi JA, Leopardi O, Astori L, et al. Malaria and anaemia in pregnant women in urban Zanzibar, Tanzania. Ann.Trop.Med.Parasitol. 1994 Oct;88(5):475-483.

(5) Shulman CE, Graham WJ, Jilo H, Lowe BS, New L, Obiero J, et al. Malaria is an important cause of anaemia in primigravidae: evidence from a district hospital in coastal Kenya. Trans.R.Soc.Trop.Med.Hyg. 1996 Sep-Oct;90(5):535-539.

(6) Shulman CE, Marshall T, Dorman EK, Bulmer JN, Cutts F, Peshu N, et al. Malaria in pregnancy: adverse effects on haemoglobin levels and birthweight in primigravidae and multigravidae. Trop.Med.Int.Health 2001 Oct;6(10):770-778.

(7) Matteelli A, Caligaris S, Castelli F, Carosi G. The placenta and malaria. Ann.Trop.Med.Parasitol. 1997 Oct;91(7):803-810.

(8) Menendez C, Ordi J, Ismail MR, Ventura PJ, Aponte JJ, Kahigwa E, et al. The impact of placental malaria on gestational age and birth weight. J.Infect.Dis. 2000 May;181(5):1740-1745.

(9) Ismail MR, Ordi J, Menendez C, Ventura PJ, Aponte JJ, Kahigwa E, et al. Placental pathology in malaria: a histological, immunohistochemical, and quantitative study. Hum.Pathol. 2000 Jan;31(1):85-93.

(10) Rogerson SJ, Mkundika P, Kanjala MK. Diagnosis of *Plasmodium falciparum* malaria at delivery: comparison of blood film preparation methods and of blood films with histology. J.Clin.Microbiol. 2003 Apr;41(4):1370-1374.

(11) Sullivan AD, Nyirenda T, Cullinan T, Taylor T, Lau A, Meshnick SR. Placental haemozoin and malaria in pregnancy. Placenta 2000 May;21(4):417-421.

(12) Cottrell G, Deloron P, Fievet N, Sow S, Gaye O, Le Hesran JY. Prediction of *Plasmodium falciparum* placental infection according to the time of infection during pregnancy. Acta Trop. 2006 Jul;98(3):255-260.

(13) Malawi Demographic and Health Survey, 2004.

(14) Rogerson SJ, van den Broek NR, Chaluluka E, Qongwane C, Mhango CG, Molyneux ME. Malaria and anemia in antenatal women in Blantyre, Malawi: a twelve-month survey. Am.J.Trop.Med.Hyg. 2000 Mar;62(3):335-340.

(15) Steketee RW, Wirima JJ, Slutsker L, Breman JG, Heymann DL. Comparability of treatment groups and risk factors for parasitemia at the first antenatal clinic visit in a study of malaria treatment and prevention in pregnancy in rural Malawi. Am.J.Trop.Med.Hyg. 1996;55(1 Suppl):17-23.

(16) WHO 1993. A Global Strategy for Malaria Control. Geneva, World Health Organisation, 1993.

(17) ter Kuile FO, Parise ME, Verhoeff FH, Udhayakumar V, Newman RD, van Eijk AM, et al. The burden of co-infection with human immunodeficiency virus type 1 and malaria in pregnant women in sub-saharan Africa. Am.J.Trop.Med.Hyg. 2004 Aug;71(2 Suppl):41-54.

(18) Holtz TH, Kachur SP, Roberts JM, Marum LH, Mkandala C, Chizani N, et al. Use of antenatal care services and intermittent preventive treatment for malaria among pregnant women in Blantyre District, Malawi. Trop.Med.Int.Health 2004 Jan;9(1):77-82.

(19) Ngrenngarmlert W, Kwiek JJ, Kamwendo DD, Ritola K, Swanstrom R, Wongsrichanalai C, et al. Measuring allelic heterogeneity in Plasmodium falciparum by a heteroduplex tracking assay. Am.J.Trop.Med.Hyg. 2005 Jun;72(6):694-701.

(20) Yeramian P, Meshnick SR, Krudsood S, Chalermrut K, Silachamroon U, Tangpukdee N, et al. Efficacy of DB289 in Thai patients with Plasmodium vivax or acute, uncomplicated Plasmodium falciparum infections. J.Infect.Dis. 2005 Jul 15;192(2):319-322.

(21) Meshnick SR, Mwapasa V, Rogerson SJ. Protecting Pregnant Women from Malaria in Areas of High HIV Infection Prevalence. J.Infect.Dis. 2006 Aug 1;194(3):273-275.

(22) Verhoeff FH, Brabin BJ, Hart CA, Chimsuku L, Kazembe P, Broadhead RL. Increased prevalence of malaria in HIV-infected pregnant women and its implications for malaria control. Trop.Med.Int.Health 1999 Jan;4(1):5-12.

(23) Filler SJ, Kazembe P, Thigpen M, Macheso A, Parise ME, Newman RD, et al. Randomized Trial of 2-Dose versus Monthly Sulfadoxine-Pyrimethamine Intermittent Preventive Treatment for Malaria in HIV-Positive and HIV-Negative Pregnant Women in Malawi. J.Infect.Dis. 2006 Aug 1;194(3):286-293.

(24) Nathoo S, Serghides L, Kain KC. Effect of HIV-1 antiretroviral drugs on cytoadherence and phagocytic clearance of Plasmodium falciparum-parasitised erythrocytes. Lancet 2003 Sep 27;362(9389):1039-1041.