CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

An estimated 25-30 million women become pregnant annually in malaria-endemic areas of Africa; most of them live in areas of stable malaria transmission (WHO, 2004). The immunosuppression associated with pregnancy and the absence of specific immunity to the unique subset of *P. falciparum* that sequester in the placenta, especially in primigravidae, have been adduced as reasons for the increased susceptibility of pregnant women to malaria infection (Fried and Duffy, 1996; Hviid and Salanti, 2007). The anti-disease immunity acquired prior to pregnancy keeps the infection asymptomatic in presentation. However, the subclinical infection still poses a great danger to both the mother and the foetus. The presence of parasites in the placenta can lead to maternal anaemia (potentially responsible for maternal death when severe), low birth weight (LBW), intrauterine growth retardation, congenital malaria, premature delivery, abortion and stillbirth (Steketee *et al.,* 1996; Newman *et al.,* 2003; Rogerson and Boeuf, 2007).

Reports on the burden of MIP in Nigeria vary widely, ranging from 8.4% to 72% (Okwa, 2003; Adefioye *et al.,* 2007; Falade *et al.,* 2008), thus exposing the obvious challenge in methodology and competency of microscopists used in these assessments. With the increased level of malaria interventions currently sponsored by the managers of the Global Fund (for HIV/AIDS, tuberculosis and malaria), it becomes difficult to assess their impact since the baseline data for such comparisons are questionable and unrealistic. It is therefore necessary to determine acceptable prevalence data on MIP using standard protocol. The paucity of data on MIP in Nigeria was shown in two consecutive World Malaria Reports of 2008 and 2009 (WHO, 2008; 2009).

To prevent the adverse effects of MIP, antimalarial chemoprophylaxis is generally recommended. For a long time, prophylaxis with weekly pyrimethamine (common brand

Daraprim® also known as "*Sunday Sunday medicine*") or chloroquine (CQ) was widely adopted in many African countries as the mainstay in malaria prevention in pregnancy (WHO, 1986). However, as the resistance to CQ and pyrimethamine increased to unacceptable levels, coupled with issues of compliance to frequency of dosing with CQ and pyrimethamine, there was a shift in national policy on the control of MIP (FMOH, 2005a). The current strategies employed to control MIP are: intermittent preventive treatment during pregnancy with sulphadoxine-pyrimethamine (IPTp-SP); use of insecticide-treated bed nets; and case management of malaria illness and anemia (WHO, 2004; FMOH, 2005a). The evidence on the effectiveness of IPTp-SP on which the Federal Ministry of Health adopted the strategy was obtained from studies conducted in Malawi and Kenya because there was none from Nigeria (FMOH, 2005a). There have also been concerns about the appropriate dosing regimen in relation to the level of malaria endemicity, suggesting that in hyper- and holoendemic areas the two dose regimen may not be adequate.

IPTp-SP is the administration of two or more therapeutic doses of SP regardless of the presence of malarial infection, at an interval of at least four weeks, starting in the second trimester of pregnancy (after quickening). IPTp-SP strategy ensures that the placenta is cleared of parasites at the time of rapid foetal growth (White, 2005). In Nigeria, there is no data on the protective efficacy of IPTp-SP. Similarly, no parasitologic monitoring of pregnant women on IPTp-SP has been done considering the vigorous promotion of SP for the prevention of MIP.

Molecular surveillance of specific mutations in some genes of *P. falciparum* is useful in monitoring the level of resistance to antimalarial drugs in *P. falciparum* isolates in the general circulation. Specific mutations in *P. falciparum chloroquine-resistance transporter (Pfcrt)* and *P. falciparum multi-drug resistance 1 (Pfmdr1)* genes determine the level of resistance to

quinolones such as chloroquine, amodiaquine and mefloquine; while mutations in specific loci of *dihydrofolate reductase (dhfr)* and *dihydropteroate synthase (dhps)* genes determine the level of resistance to pyrimethamine and sulphadoxine respectively.

1.2 STATEMENT OF THE PROBLEM

The prevalence of MIP as reported by various authors varies widely even within a particular geographic region, 8.4-72% in southwest Nigeria (Okwa, 2003; Adefioye *et al.*, 2007; Falade *et al.*, 2008), raising concerns on the adequacy of methods employed, competency of the microscopists used in these assessments and reliability of available data. In Tanzania, Mwanziva *et al.*, (2008) reported that trained research scientists confirmed <1% of the slides read as malaria positive by clinic microscopists. In most of the local reports on MIP, standard protocol for malaria microscopy and quality assurance were not followed. Thus, obtaining reproducible results was difficult.

Anaemia has been identified as a risk factor for maternal mortality mostly in developing countries. The average estimate for all-cause anaemia-attributable mortality for Africa is 6.37% (WHO, 1991a). Few studies have reported on anaemia in Nigeria using the international definition of anaemia for pregnant women (haematocrit <33%) (WHO, 1989).

The efficacy of antimalarial drugs used for the prevention and/or treatment of malaria infection is determined by the level of resistance of malaria parasites to these drugs. The level of *P. falciparum* resistance to CQ and pyrimethamine has been reported to be very high both by molecular and clinical studies in children (FMOH, 2005; Happi *et al.*, 2006). However, these results cannot be extrapolated to the pregnant population. The level of resistance to SP among *P. falciparum* isolates circulating in the general human population is also on the rise, thus posing a

threat to the IPTp-SP strategy. The dearth of studies on monitoring the protective efficacy of SP and pregnancy outcome in women on IPTp-SP made the longitudinal study on IPTp-SP imperative among pregnant women attending antenatal clinics in Lagos.

Molecular surveillance of antimalarial resistance markers has been used to determine the level of resistance in *P. falciparum* isolates from non-pregnant population, mostly in children (Happi *et al.*, 2005; Ojurongbe *et al.*, 2007). It is known that the dynamics of selection of dominant clones of *P. falciparum* is influenced by the pre-existing immunity and drug use (Bloland, 2001). Considering that the immune status of pregnant women and children are not similar, extrapolating results of studies carried out with children to pregnant women may not be appropriate. However, there is paucity of data on the molecular surveillance of antimalaria resistance markers in isolates of *P. falciparum* from pregnant women in Nigeria.

1.3 OBJECTIVES OF STUDY

The overall objective of the study was to assess the effectiveness of some antimalarial drugs used for the control of malaria in pregnancy in Lagos.

The specific objectives of the study were to:

1. determine the prevalence of malaria and anaemia in pregnancy in Lagos.
2. assess the protective efficacy of IPTp-SP and equivalence of the standard 2-dose regimen of IPTp-SP to the experimental monthly IPTp-SP against *P. falciparum* infection.
3. describe the haplotypes of *pfcrt*, *pfmdr1* and *dhfr* genes of *P. falciparum* isolates from pregnant women.

1.4 SIGNIFICANCE OF THE STUDY

- The prevalence (7.7%) of malaria in asymptomatic pregnant women in Lagos was determined using standard protocol and quality assurance procedures.
- Anaemia prevalence in pregnancy was determined based on the internationally accepted definition. The study also provides information on the association between anaemia and asymptomatic malaria in pregnancy.
- This study provides information on the protective efficacy of IPTp-SP (98.4% in the first month) in Lagos. The equivalence of experimental monthly SP dosing to the standard 2-dose regimen of IPTp-SP in Lagos was established.
- This study reported high resistant haplotypes of *Pfcrt* CVIET (75.9%), and *dhfr* ACIRNVI (66.7%) among *P. falciparum* isolates from pregnant women, indicating high level of resistance to chloroquine and pyrimethamine respectively; while the absence of *dhfr* 16V, 108T, 164L and the relatively low level of *Pfmdr1 86Y* (25%) indicate absence of resistance to proguanil and low resistance to amodiaquine respectively.

1.5 LIMITATION OF THE STUDY

Challenges with amplification of some parasite DNA due to low parasiataemia limited this study. However their impact on the overall goal of the study was not significant.

1.6 OPERATIONAL DEFINITION OF TERMS

Chemoprophylaxis: Prevention of disease or infection by the use of a chemotherapeutic agent.

Gravida: A pregnant woman

Haplotype: A contraction of the phrase "haploid genotype", is a set of closely linked genetic markers present on one chromosome which tend to be inherited together (not easily separable by recombination).

Intermittent preventive treatment: The administration of therapeutic doses of a drug at specified intervals with an aim to both treat and prevent a disease.

Low birth weight: Birth weight less than 2.5 kg.

Multigravida: A woman pregnant for 3 or more times

Mutation: A change in the sequence of base pairs in the chromosomal molecule.

Parity: The condition of having given birth to an infant or infants, alive or dead; a multiple birth is considered as a single parous experience.

Prevalence: the number of cases of a disease existing in a given population at a given time

Primigravida: A woman pregnant for the first time

Protective efficacy: The ability of a drug to prevent the infection in over a period of time.

Quickening: Signs of life felt by the mother as a result of the fetal movements, usually noted from 16 to 20 weeks of pregnancy.

Secundigravida: A woman pregnant for the second time

Trimester: A period of 3 months; one-third of the length of a pregnancy.

1.7 LIST OF ABBREVIATIONS

C.I.	Confidence interval
CSA:	Chondroitin sulphate A
DHFR:	dihydrofolate reductase
DHPS:	dihydropteroate synthase
DNA:	Deoxynucleic acid
FMOH:	Federal Ministry of Health
GLUT1:	Glucose transporter-1
HA:	Hyaluronic acid
HIV	Human Immunodeficiency Virus
IPTp-SP	Intermittent Preventive Treatment with Sulphadoxine-Pyrimethamine
ICAM1:	Intercellular adhesion molecule 1
IE:	Infected erythrocyte
IFN-γ:	Interferon- γ
IgG:	Immunoglobin G
IgM:	Immunoglobin M
ITN	Insecticide-treated nets
IUGR:	Intrauterine growth retardation
LBW:	Low Birth Weight
MAbs :	Monoclonal antibodies
M0:	Month 0 (recruitment month)
M1:	Month 1 (one month after recruitment)
M2:	Month 2 (two months after recruitment)
M3:	Month 3 (Three months after recruitment)
M4:	Month 4 (Four months after recruitment)
MIP:	Malaria in pregnancy

NBW: Normal Birthweight

PCV: Packed cell volume

PfEMP1: *Plasmodium falciparum* erythrocyte membrane-binding protein 1

SP: Sulphadoxine-Pyrimethamine

TNF: Tissue necrosis factor

VCAM1: vascular cellular adhesion molecule-1

VSA_{PAM}: Pregnancy-Associated malaria variant surface antigen

WHO: World Health Organization

χ^2 Chi square

CHAPTER TWO

LITERATURE REVIEW

2.1 MALARIA IN PREGNANCY

Malaria is a protozoal infection transmitted to man by the female anopheline mosquito infected with human malaria parasites. Five *Plasmodium* species have been reported to infect man: *P. falciparum, P. malariae, P. ovale, P. vivax and P. knowlesi* (Singh *et al.,* 2004). *Plasmodium falciparum* is the most virulent (Aleyamma *et al.,* 2006) as well as the most prevalent, accounting for about 98% of the malaria cases in the country (FMOH, 2005).

In Nigeria, malaria transmission is all year round. The main vectors belong to *Anopheles gambiae* complex (*An. gambiae ss* and *An. arabiensis*) though *An. funestus* is also present in Nigeria (FMOH, 2005). Morbidity and mortality are high in early childhood (especially in children < 5 yrs of age), but with constant exposure to malaria parasites older children and adults develop effective anti-disease immunity to malaria, which though not total, prevents life-threatening parasite burdens and suppresses the pro-inflammatory responses which cause illness. During pregnancy, the acquired immunity is not able to prevent sequestration and multiplication of *P. falciparum* in the placenta but is able to keep the infection at asymptomatic level in majority of the cases (Mockenhaupt *et al.,* 2002). Depending on the endemicity of malaria in an area, it can be expected that 1-50 % of pregnant women may carry malaria parasitaemia especially in the placenta, without noticing it (Brabin, 1983; Steketee *et al.,* 2001).

The life cycle of malaria parasites and the potential drug targets are shown Figure 2.1.

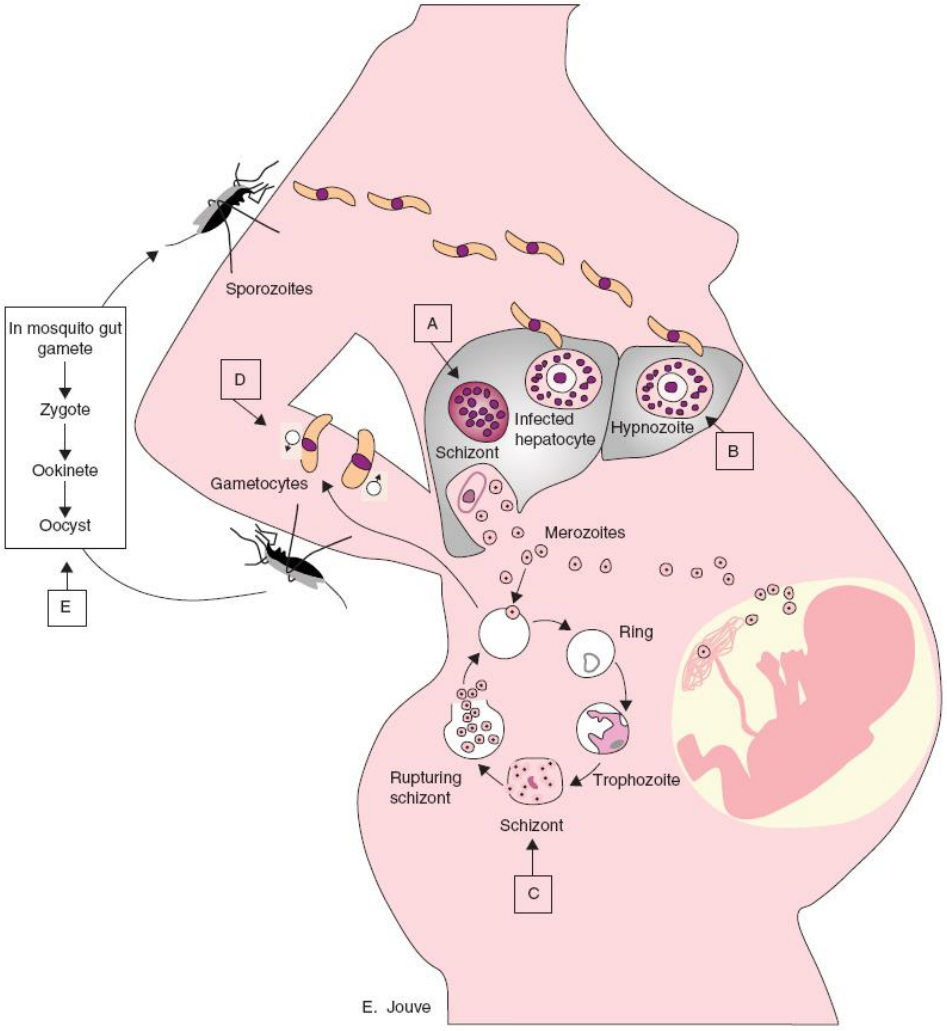


Figure 2.1: Malaria life cycle and antimalarial drug targets (Sevene *et al.*, 2010)

Plasmodium sporozoites travel from the salivary glands of the anopheline mosquito through the bloodstream of the human host to the liver, where they invade hepatocytes and divide to form multinucleated schizonts. Hypnozoites can be found in P. vivax and P. ovale infections as a quiescent stage in the liver. Liver schizonts rupture and release merozoites into the circulation, where they invade red blood cells. Within the red cells, merozoites mature from ring forms to trophozoites to multinucleated schizonts. Some merozoites differentiate into male or female gametocytes that can then be ingested by the anopheline mosquito. The Plasmodium cycle is completed in the mosquito gut. Capital letters indicate the Plasmodium-stage targets of antimalarial drugs.

A: Tissue schizonticides; B: Hypnozoiticides; C: Blood schizonricides; D: Gametocytocides; E: Sporontocides.

In areas of stable malaria transmission, the main consequences of MIP are maternal anaemia, which when severe could cause maternal death; and low birth weight, which is a significant contributor to infant mortality.

In pregnancy, the infected erythrocytes (IEs) express parasite-derived immunologically and functionally unique subset of variant surface antigens (VSA) not found in non-pregnant individuals (Hviid and Salanti, 2007), though they belong to the *P falciparum* erythrocyte membrane protein1 (PfEMP1) family encoded by *var* genes. The var genes are highly polymorphic and thus are responsible for the transcription of different types of PfEMP1 with diverse antigenic and binding properties. This distinct sub-population of parasites, which bind principally to chondroitin sulfate A (CSA), is responsible for cytoadhesion in the placenta (Fried and Duffy, 1996; Fried *et al.*, 2006).

Natural protective immunity against MIP is acquired during the course of pregnancies, with the development of anti-PfEMP1 antibodies recognizing placental IEs from different geographical regions. These antibodies are female-specific and parity-dependent (Beeson *et al.*, 2002). In malaria-endemic regions, a first pregnancy allows a woman to develop an immune response against the receptors used by IEs for binding to the intervillous spaces of the placenta (Beeson *et al.*, 2002).

2.2 SEQUESTRATION OF *PLASMODIUM FALCIPARUM*-INFECTED ERYTHROCYTES IN THE PLACENTA

Malaria in pregnancy is characterized by the sequestration of *Plasmodium falciparum*-infected erythrocytes in placental intervillous spaces. Sequestration is the localization of *P.falciparum* infected erythrocytes in the microvasculature of organs such as the kidney, brain and the

placenta. This localization is mediated by the expression of parasite-induced variant surface antigens on the surface of IEs, which in non-pregnant individuals, bind mostly to the ubiquitous CD36 receptors and to other common receptors present in most endothelial cells of microcapillaries such as vascular cellular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin (Fried and Duffy, 1996; Beeson *et al.,* 2000; Maubert *et al.,* 2000).

Majority of placental blood parasite samples collected from pregnant women adhere to CSA, while a minority of peripheral samples (upto 25%) adhere to CD36, either exclusively or in addition to CSA (Fried and Duffy, 1996; Fried *et al.,* 2006). Placental parasites appear to express a specific phenotype defined by the adherence of *P. falciparum*-infected erythrocytes to the glycosaminoglycans: chondroitin sulfate A (CSA) and hyaluronic acid (HA), expressed by the syncytiotrophoblast (the outermost line of the placenta in contact with maternal blood) or in the intervillous spaces of the placenta (Lucchi *et al.,* 2006a). Syncytiotrophoblast expresses a large amount of CSA (Maubert *et al.,* 2000). The PfEMP1 expressed on IEs of Placental isolates of *P. falciparum* do not bind to the primary microvasculature receptors as seen in IEs from non-pregnant individuals. The complications of MIP are caused by the massive sequestration of infected erythrocytes (IEs) in the placenta.

Sequestration prevents the IEs from getting to the spleen and subsequent removal by resident macrophages. These cell-cell interactions increase the probability of erythrocyte invasion by merozoites emerging from a lysed cell.

Major differences exist between pregnancy-associated malaria VSA (VSA_{PAM}) and previously characterized VSA. Apart from being recognized only by female sera in a parity-dependent manner, VSA_{PAM} show other distinct characteristics such as:

- Adhesion to multiple receptors (IgG/IgM/HA/CSA) rather than the exclusive binding to CSA is a characteristic placental isolate (Beeson and Brown, 2004; Rasti *et al.*, 2006).
- Adhesion to HA and CSA was greatest among pigmented trophozoite-infected erythrocytes and at physiologic pH (Beeson and Brown, 2004)
- Formation of infected erythrocyte rosettes is rare when compared to CD36-binding VSA (Rogerson *et al.*, 2000; Beeson and Brown, 2004),
- Adhesion to HA is inhibited by enzymatic (hyaluronidase and trypsin) cleavage and by dodecamer or larger oligosaccharide fragments or polysaccharides (Beeson *et al.*, 2000).
- Trypsin-resistant and trypsin-sensitive CSA adhesion have been observed (Sharling *et al.*, 2004).
- Soluble HA, but not CSA, could cause aggregation or clumping of IEs.
- Different HA types varied in adhesion-inhibitory activity, which was altered by physical treatment, suggesting that structural features of HA influence IE interactions.
- High levels of HA adhesion occurred at low wall shear stress, conditions thought to prevail in the placenta.
- with the exception of rosetting isolates, non-immune IgM binding is a phenomenon only seen with CSA-binding clones (Creasey *et al.*, 2003; Rasti *et al.*, 2006),
- VSA_{PAM} do not generally mediate adhesion to CD36 (Fried and Duffy, 1996; Beeson and Brown, 2004)
- VSA_{PAM} mediated adhesion to the placenta and CSA can be resistant to concentrations of trypsin known to remove most PfEMPl molecules from the infected cell surface.

Plasmodium falciparum-IEs that infect the human placenta were found to bind IgG. A strain of *P. falciparum* cloned for IgG binding adhered massively to placental syncytiotrophoblasts in a

pattern similar to that of natural infections. Adherence was inhibited by IgG-binding proteins, but not by glycosaminoglycans or enzymatic digestion of CSA or HA acid. Normal, non-immune IgG that is bound to a duffy binding-like domain beta of the PfEMP1 might at the IE surface act as a bridge to neonatal Fc receptors of the placenta (Flick *et al.*, 2001).

Placental parasites collected in Tanzania uniformly adhered to the molecule CSA, and soluble CSA completely inhibited adhesion of most samples to placental cryosections. Three of 46 placental parasite samples also adhered to immobilized HA, but HA failed to inhibit adhesion of any placental parasites to placental cryosections. Similarly, non-immune IgG and protein A failed to inhibit adhesion of parasite samples to placental cryosection. *P. falciparum* adhesion in the placenta appears to be a non-redundant process that requires CSA as a receptor (Fried *et al.*, 2006).

Although PfEMPl-mediated CSA adhesion appears to play a role in placental malaria the molecular interactions triggering this syndrome is complex. Apart from the additional receptors such as non-immune IgM and IgG, and HA, CSA-binding laboratory clones and placental CSA binding isolates also appear to express some parasite encoded surface antigens other than PfEMPl, such as ring surface proteins 1 and 2 (RSP 1 and 2) (Pouvelle *et al.*, 2000). Interestingly, a gene 'knock-out' of the CSA binding *var* (FCR3*var*CSA) in parasite clone FCR3 abolishes CSA binding, but the 'knock-out' parasites still bind the syncytiotrophoblast of *ex vivo* placental cryosections (Andrews and Lanzer, 2002).

2.2.1 Characteristics of PFEMP-1

PfEMP-1 molecules are situated in the knob-like protrusions associated with the parasitized erythrocyte surface. PfEMP1 expression is determined and ordered mainly by host physiology and immunity. Thus infections are dominated by VSA variants that afford parasites optimal sequestration and high effective parasite multiplication rates (Lavstsen *et al.,* 2005). PfEMP-1 molecules are highly antigenic and are encoded by a diverse multigene family, the *var* genes and expression on the surface of erythrocytes can switch during the course of a clonal infection (Su *et al.*, 1995). Each haploid parasite genome contains 50-60 *var* genes (Gardner *et al.,* 2002; Lavstsen *et al.,* 2003). The hypothesis (Fried and Duffy, 1996) that the strains of *P. falciparum* implicated in placental malaria are representative of a unique sub-population, was supported by the demonstration that the expressed PfEMP-1 in infected placental parasite samples are closely related to each other (Khattab *et al.,* 2001).

Members of the PfEMP-1 family function as adhesion molecules binding to various host endothelial receptors. Receptors potentially bound include CD36, intercellular adhesion molecule 1 (ICAM-1), thrombospondin, E selectin, vascular cell adhesion molecule 1 (VCAM-1), platelet endothelial cell adhesion molecule 1 (PECAM1) and chondroitin sulfate A (CSA) (Baruch *et al.,* 1996, Khattab *et al.,* 2001).

Duffy binding like (DBL)-gamma domains expressed by placental *P. falciparum* isolates have an affinity to CSA. All parasite populations accumulating in infected placentas express only one variant of PfEMP-1, each of which contains a DBL-gamma domain with CSA binding capacities. Furthermore, sequence analysis data provide evidence for antigenic conservation among the DBL-gamma sequences expressed by different placental parasites (Khattab *et al.,*

2001).

2.3 PREVALENCE OF MALARIA IN PREGNANCY

About 25 million women become pregnant in malarious areas of Africa each year, most of them live in areas of stable malaria transmission (WHO, 2004). Among pregnant women, primi- and secunigravidae are at greatest risk of the effects of malaria. It has been reported that in malaria-endemic areas 19% of cases of infant low birth weight are due to malaria (Mutabingwa *et al.*, 2005), and that 6% of infant deaths are due to low birth weight caused by malaria (Guyatt and Snow, 2004). Also, between 200,000 and 500,000 pregnant women develop severe anaemia as a result of malaria in sub-Saharan Africa (i.e. 3-5% of maternal anaemia) (Steketee *et al.*, 2001).

Malaria is endemic in Nigeria and accounts for 60% of all clinic visits in the country (FMOH, 2000). The country's estimated 140 million people are at risk of having malaria. Each year, 70.5% of pregnant women have been reported to present with symptoms suggestive of malaria while 11% of them have been reported to die of malaria each year (FMOH, 2007). The reported prevalence rates in different parts of Nigeria are shown in Table 2.1

Table 2.1: Prevalence Rate of Malaria In Pregnancy In Nigeria.

Location	Prevalence	Reference
Yola (Adamawa State)	22.1%	Kagu *et al.*, 2007
Warri (Edo State)	32.9%	Isibor *et al.*, 2003
Benin (Edo State)	13.0%	Enato *et al.*, 2007
Lagos (Lagos State)	34.0%	Anorlu *et al.*, 2001
	60.0%	Okwa, 2003
	34.0%	Tayo *et al.*, 2009
Ibadan(Oyo State)	8.4%	Falade *et al.*, 2008
Abeokuta (Ogun State)	62.4%	Idowu *et al.*, 2008
Oshogbo (Osun State)	72%	Adefioye *et al.*, 2007
Ebonyi State	19.7%	Uneke, 2008

2.4 EFFECTS OF MALARIA IN PREGNANCY

The effects of malaria during pregnancy vary greatly from negligible to severe, depending on factors such as malaria transmission rates, use of chemoprophylaxis, maternal age, gravidity of the women, host genetics and nutrition, and concurrent infection with human immunodeficiency virus (HIV).

In areas of low transmission or in epidemics, pregnant women are at high risk of severe and cerebral malaria and even death (Shulman *et al.,* 2002; Nosten *et al.,* 2004). Whereas, in areas where malaria transmission is stable, most adult women would have developed sufficient immunity such that, even during pregnancy, *P. falciparum* infection does not usually result in fever or other clinical symptoms. In these areas, the principal impact of malaria infection is due to the presence of parasites in the placenta causing maternal anaemia (potentially responsible for maternal death when severe) and low birth weight (LBW) (Newman *et al.,* 2003; Rogerson and Boeuf, 2007). Other effects of MIP include: maternal hypoglycaemia, congenital malaria, premature delivery, abortion and stillbirth (Steketee *et al.,* 1996).

The effects of MIP have been noted to be significantly lower in multigravids as a result of acquisition of specific immunity to placental malaria due to previous exposure. In primigravids, such specific immunity to placenta-specific parasites is non-existent and so they are more affected by *P.falciparum* infection during pregnancy (Beeson and Duffy, 2005). Several studies have observed that primigravidae are at greatest risk of the effects of MIP (Beeson and Duffy, 2005; Duffy, 2007). It has been observed in some studies that secundigravidae are almost as susceptible to malarial infections as primigravidae (Brabin, 1983; Khattab *et al.,* 2004).

In general, chemoprophylaxis has been an effective way of preventing MIP. Thus, pregnant women on antimalarial chemoprophylaxis are at reduced risk of the harmful effects of malaria (Kalanda *et al.*, 2006).

Some studies have identified young maternal age (< 24 yrs), poor nutrition and host genetics as risk factors significantly associated with the adverse effects of MIP (Bouyou-Akotet *et al.*, 2003; Tako *et al.*, 2005).

Concurrent infection of a pregnant woman with HIV and malaria has been associated with more peripheral and placental parasitemia, higher parasite densities, increased frequency of clinical malaria, severe anemia and increased risk of adverse birth outcomes. (Kamya *et al.*, 2006). The impaired ability to control *P. falciparum* infection has been attributed to the impaired opsonic phagocytic clearance of malaria parasites (Keen *et al.*, 2007), and reduced levels of specific antibodies associated with protection against MIP (Mount *et al.*, 2004).

2.4.1 Intrauterine Growth Retardation

The intrauterine growth retardation (IUGR) effect is related to nutrient transport to the fetus. The intense infiltration of immune cells into the placenta as a result of sequestration of infected erythrocytes leads to a cascade of events that results in reduction in both amino acid (Thongsong *et al.* 2005) and glucose transportation to the foetus (Rogerson and Boeuf, 2007). A high density of parasites and chronic parasite infection in the placental blood and the associated cellular immune response may result in consumption of glucose and oxygen that would have gone to the fetus (Beeson and Duffy, 2005). Also, histological abnormalities described in parasitized placenta show pathological changes such as the thickening of the cytotrophoblastic membranes, which may impair materno-foetal exchanges and thus interfere with nutrient transport (Bulmer *et al.*, 1993; Verhoeff *et al.*, 2001). Malaria-associated maternal anemia may also contribute

independently to IUGR most likely through a reduction in oxygen transport to the fetus (Verhoeff *et al.*, 2001).

2.4.2 Low Birth Weight

Low birth weight (< 2.5 kg) is an important risk factor for neonatal and infant mortality (McCormick, 1985; Steketee *et al.*, 2001; Guyatt and Snow, 2004). LBW is a high risk factor for perinatal death and it is also correlated with morbidity and mortality during infancy (McCornick, 1985; Bloland *et al.*, 1996). The causes of LBW are multifactorial, 19% of which is attributed to MIP. Other causes of LBW include preterm delivery and intrauterine growth retardation.

2.4.3 Anaemia

In malaria endemic regions, anaemia is a common feature in pregnancy. The average estimate for all-cause anaemia-attributable mortality for Africa is 6.37% (WHO, 1991a). For pregnant women, anaemia is defined as haemoglobin levels of less than 11.0 (WHO, 1989). In a study in Northeastern Nigeria, the overall prevalence of anaemia among pregnant women was 72.0% where mild, moderate and severe anaemia constituted 31.8%, 39.4% and 0.9%, respectively (Kagu *et al.*, 2007).

Malnutrition and helminthiasis are believed to be the commonest causes of anaemia. However, in a study carried out in Uganda helminthiasis was not significantly associated with anaemia in pregnant women, rather malaria and HIV infection were significantly associated with anaemia in pregnancy (Muhangia *et al.*, 2007). Malaria can also aggravate an existing anaemia during pregnancy. Anaemia due to malaria is more common and severe between 16-29 weeks of gestation. This could be due to haemolysis of parasitized erythrocytes, increased demands of pregnancy and pre-existing folate deficiency (Malaria site, 2006). Anaemia, when severe,

increases the risk of perinatal mortality, and maternal morbidity and mortality (Steketee *et al.*, 2001).

2.4.4 Hypoglycaemia

Hypoglycaemia is a complication of malaria that is more common in pregnancy. The major cause is the high energy demands of the infecting parasites as well as the hypercatabolic state associated with pregnancy. Increased response of pancreatic islets to secretory stimuli (like quinine) leads to hyper-insulinaemia and hypoglycaemia. However, hypoglycaemia is usually undetected because its symptoms such as tachycardia, sweating and giddiness are also associated with malaria. Some patients may have abnormal behaviour, convulsions, altered sensorium, and sudden loss of consciousness. These symptoms may easily be confused with cerebral malaria (Malaria Site, 2006).

Therefore, in all pregnant women with falciparum malaria, particularly those receiving quinine, blood sugar should be monitored. In some cases, hypoglycaemia can be associated with lactic acidosis and in such cases mortality is very high. Maternal hypoglycaemia can also cause fetal distress without any signs (Malaria Site, 2006).

2.4.5 Congenital Malaria

Congenital malaria is rare and occurs in less than 5% of affected pregnancies. Placental barrier and maternal IgG antibodies which cross the placenta may protect the foetus to some extent. However, it is thought that the structural damage caused by binding of infected erythrocytes to the placental surface may impair the transplacental transfer of protective antibodies and permit the transfer of infected erythrocytes to the foetus (Okoko *et al.*, 2001). Congenital malaria is more common in areas where malaria transmission is unstable (Onyenekwea *et al.*, 2004).

2.5 PATHOGENESIS OF PLACENTAL MALARIA

Severity of malaria is related to the capacity of the *P. falciparum* IEs to cytoadhere and to sequester in the microvascular capillaries of vital organs (Kyes *et al.,* 2001). Cytoadherence could influence the invasion of erythrocytes by merozoites emerging from mature rupturing schizont (Ginsburg and Hoshen, 2002) thus effectively increasing the population of infected erythrocytes in the placenta. In cases of low peripheral parasitemia, women can be asymptomatic, even if there is a large accumulation of parasites in the placenta. In the chronic stage an inflammatory reaction occurs, with accumulation of leukocytes and necrosis of the neighboring placental tissue (Bulmer *et al.,* 1992).

An intense infiltration of immune cells, including macrophages, into the placental intervillous spaces, and the production of pro-inflammatory cytokines often occur in response to infection. Expression of alpha and beta chemokines may initiate or facilitate this cellular infiltration during placental malaria. Specific immunity against placenta-binding parasites may prevent infection or facilitate clearance of parasites prior to the influx of inflammatory cells, thereby avoiding a cascade of events leading to disease and death (Beeson and Duffy, 2005).

Amino acids and glucose act as regulators of placental and foetal development (Baumann *et al.,* 2002; Regnault *et al.* 2005) and intrauterine growth retardation (IUGR) is associated with impairment of the active transport mechanisms operating at the level of the syncytiotrophoblast. Interleukin 1b, which is increased in MIP (Moormann *et al.* 1999), inhibits amino acid uptake of the trophoblast-like cell line (BeWo) in a dose-dependent manner (Thongsong *et al.* 2005), while tissue necrosis factor (TNF) has been shown to down-regulate amino acid transport across the placenta *in vivo* (Carbo *et al.*, 1995). Local inflammation, triggered by malaria infection and

supported by maternal monocyte infiltrates, could thus cause a decrease in amino acid transfer across the placenta, impairing foetal growth.

The basal membrane expression of glucose transporter-1 (GLUT1) is positively regulated by insulin-like growth factor I, low levels of which have been reported in human malaria (Mizushima *et al.* 1994). GLUT1 is the main glucose transporter in the placenta and sits in the microvillous and basal membranes of the syncytiotrophoblast. Since the microvillous membrane contains more transporter than the basal membrane, alteration in the density of transporters in the basal membrane is the main factor regulating glucose transplacental transport (Rogerson and Boeuf, 2007). This could lead to a decrease of glucose flux across the placenta, possibly leading to IUGR.

Placental hypoxia is a well-defined cause of LBW whether it is normobaric hypoxia in animal models of IUGR (Regnault *et al.* 2007) or hypobaric hypoxia in high-altitude pregnancies (Zamudio, 2003). From peak oxygen saturation of 60% in mid pregnancy, foetal blood oxygen saturation falls to around 40% by term, due to increasing foetal demand (Soleymanlou *et al.* 2005). Any fall in oxygen saturation in uteroplacental blood, or significant decrease in blood supply, will result in decreased oxygen availability to the foetus. Placental hypoxia could be caused by sub-optimal placental blood flow due to inadequate placentation, or by the massive monocyte and IE infiltrates in the intervillous spaces. Moreover, by adhering to the syncytiotrophoblast, IEs and monocytes could physically decrease the surface of exchange between maternal and foetal blood leading to a further decrease in nutrient and oxygen transport across the placenta. In term placental tissue, hypoxia leads to apoptosis (Levy *et al.* 2000), increased production of pro-inflammatory cytokines (Benyo *et al.*, 1997) and impaired amino acid and glucose transport (Zamudio *et al.* 2006).

Monocyte infiltrates can develop in the intervillous space in response to malaria and have been associated with intrauterine growth restriction (IUGR) and anaemia (Menendez *et al.* 2000; Rogerson *et al.* 2003). IE sequestered in the placenta induce the secretion of b-chemokines by maternal mononuclear cells (Abrams *et al.* 2003; Chaisavaneeyakorn *et al.* 2003; Suguitan *et al.* 2003) and by foetal syncytiotrophoblast (Abrams *et al.* 2003; Lucchi *et al.* 2006a), attracting monocytes to the placenta. Macrophage migration inhibitory factor is found in increased levels in women with placental malaria (Chaisavaneeyakorn *et al.* 2005), and it may help to retain the recruited monocytes and to activate them to secrete b-chemokines, setting up a positive feedback loop.

Placental monocytes are relatively more activated than circulating monocytes (Diouf *et al.,* 2004). Several factors present in the intervillous space of malaria-infected placentae could activate monocytes. In particular, IEs, malaria pigment (haemozoin), glycosylphosphatidylinositol and fibrinogen have all been shown to induce TNF production by monocytes (Bate *et al.,* 1988; Pichyangkul *et al.,* 1994; Smiley *et al.,* 2001; Krishnegowda *et al.* 2005).

Increase in placental levels of TNF, interleukin 6 (which come mainly from monocytes) and of interferon c (a major activator of monocytes) have been associated with LBW (Fried *et al.,* 1998a; Moormann *et al.,* 1999; Rogerson *et al.,* 2003). However, the mechanism by which these cytokines might cause IUGR is not known (Rogerson and Boeuf, 2007).

Malaria has been identified as a risk factor for pre-eclampsia, (a pathological condition that leads to LBW) (Sibai *et al.,* 2005) and hypertension in pregnancy in some studies (Sartelet *et al.,* 1996; Muehlenbachs *et al.,* 2006), howerever the report by Dorman *et al.,* (2002) did not associate these two conditions to malaria. A recent study showed an association between chronic placental

malaria and hypertension in primigravidae (Muehlenbachs *et al.,* 2006). There are no published data on the effect of malaria during placentation on cytotrophoblast invasion and spiral artery remodelling, but the peak prevalence of *P. falciparum* parasitaemia early in the second trimester overlaps with the period when spiral arteries undergo remodeling (complete by 20-22 weeks of gestation).

Thus, there is a window-in time during which malaria infection could indeed impair the establishment of an optimal placental blood flow. Trophoblast invasion of spiral arteries is vulnerable to factors causing activation of maternal cells in the uterine bed. In particular, TNF and interleukin 1b (both increased in placental malaria) dramatically increase secretion of monocyte chemokines MCP-1 by uterine decidua (Lockwood *et al.,* 2006). Activated macrophages have been shown to inhibit trophoblast invasion *in vitro* probably through TNF production (Bauer *et al.,* 2004; Regnault *et al.,* 2005). Monocyte chemokines have been reported to be increased in placental malaria (Abrams *et al.,* 2003; Suguitan *et al.,* 2003).

Maternal malaria infection during placental development could thus increase numbers of activated macrophages in the maternal decidua and reduce trophoblast invasiveness, impairing the remodeling of spiral arteries and leading to a sub-optimal placentation and a possible placental hypoxia.

2.6 IMMUNE RESPONSE TO MALARIA IN PREGNANCY

Pregnancy is an immunological balancing act in which the mother's immune system has to remain tolerant to the foetus and yet maintain immune competence for defense against microorganisms. Studies have documented a decrease in cell-mediated immune responses to malaria parasite antigens during pregnancy (Fievet *et al.,* 1997; Ricke *et al.,* 2000) and early

post-partum (Riley *et al.,* 1989; Diagne *et al.,* 2000) in association with increased susceptibility to malaria.

Repeated and continuous exposure to malaria parasites during infancy leads to premunition, an immune state defined as the symptomless persistence of a low number of parasites. The development of premunition is related to the acquisition of a repertoire of protective antibodies reactive against polymorphic molecules prominently exposed at the surface of the infected erythrocyte (Marsh *et al.,* 1989; Bull *et al.,* 1998). The acquisition of antibodies binding the VSA on infected erythrocytes plays a major role in the development of age and exposure-dependent immunity (Marsh *et al.,* 1989; Ofori *et al.,* 2002).

Immunity to malaria usually requires repeated exposure to the parasite to become long lasting. One reason for this is the capacity of the parasite to vary the antigens which are major targets for protective antibodies (Beeson and Duffy, 2005). It is now known that malaria-infected individuals living in endemic areas accumulate a broad spectrum of protective antibodies directed against variant surface antigens (VSAs) present on infected erythrocytes (IEs) (Marsh *et al.,* 1989; Bull *et al.,* 1998).

Women in their first pregnancy (primigravidae) are more likely to be infected with malaria and the consequences are more severe (Brabin, 1983). This probably reflects their lack of pre-existing antibodies specific for the novel variant surface antigens (VSA) expressed by CSA-binding placental parasites (Fried *et al.,* 1998; Ricke *et al.,* 2000; Beeson *et al.,* 2004). These antibodies are associated with decreased prevalence of placental infection (Fried *et al.,* 1998) and reduced risk of maternal anemia and infant low birth weight, the major complications of MIP (Duffy and Fried, 2003; Staalsoe *et al.,* 2004).

Epidemiological evidence suggests that women in low to moderate malaria transmission areas may need to experience at least three or four pregnancies before developing immunity that prevents malaria-associated low birthweight (Nosten *et al.*, 1991; Shulman *et al.*, 2001). This may suggest that several infections and exposure to several different PfEMP1 variants and/or other parasite molecules are required before functional immunity develops. It is uncertain how many different PfEMP1 variants need to be recognized by the host's immune system before an immune response that effectively prevents placental infection develops.

In the study conducted by Fievet and co-workers (2002), it was demonstrated that the acquisition of antibodies against CSA-adhering parasites correlated with the number of past pregnancies. Thus, the likelihood of having pregnancy-associated parasite-specific immunization during a previous parasitized pregnancy increases with parity. Anti-VSA antibodies accumulate with each pregnancy and protect the mother from placental malaria in subsequent pregnancies (Duffy and Fried, 2003; Staalsoe *et al.*, 2004).

Moreover, antibodies directed against CSA-adhering parasites and acquired during an infected pregnancy inhibited the cytoadherence of CSA-adhering parasites to human syncytiotrophoblasts (Staalsoe *et al.*, 2001). Moore *et al.*, (2000) proposed a model in which re-circulation of memory T lymphocytes from the intervillous blood to local lymphoid tissue facilitates the maintenance of local immunity.

Antibodies that bind CSA-selected parasites and block adhesion are not acquired by malaria-exposed males. There is a striking female-specific antibody response recognizing both *in vitro* CSA-selected parasites (Ricke *et al.*, 2000; Staalsoe *et al.*, 2001) and *P. falciparum* isolates taken from infected placentae at delivery (Salanti *et al.*, 2003; Staalsoe *et al.*, 2004; Khattab *et al.*, 2004).

Both humoral and cellular mechanisms may account for the lower susceptibility of multigravidae to malaria (Fievet *et al.,* 2002). The acquisition of antibodies to the surface of placental isolates correlates with protection from MIP and the targets of these antibodies are potential vaccine candidates (Staalsoe *et al.,* 2001; 2004).

IgG antibodies with specificity for the VSA expressed by placental parasites (VSA_{PAM}) are associated with protection from maternal anaemia, prematurity and low birth weight, which is the greatest risk factor for death in the first month of life (Khattab *et al.,* 2004). Antibody-dependent protection is primarily mediated by cytophilic IgG antibodies activating cytotoxic and phagocytic effector functions of neutrophils and monocytes. Malaria infection also involves elevated production of IgE antibodies. However, IgE-containing immune complexes are pathogenic rather than protective by crosslinking IgE receptors (CD23) on monocytes, leading to local overproduction of TNF, a major pathogenic factor in this disease (Perlmann and Troy-Blomberg, 2000).

T cells are essential for both acquisition and regulation of malaria immunity. The major T cells controlling blood stage infections are CD4+ of both the Thl and Th2 subsets. However, T cells carrying the γ6- receptor also contribute to this control. A balance between the cytokines produced by different cell types is critical for the course of infection, with interferon- γ (IFN-γ) having a key role in anti-malaria defense. Blood-stage infections are also under complex genetic control. Among the regulatory genes, those involved in elevated production of TNF are associated with increased risk of severe disease and death due to *P. falciparum* infection (Perlmann and Troy-Blomberg, 2000).

Recognition of placental parasites shows a parity- and sex- dependent pattern. The pan-reactive anti-CSA binding antibodies react with the surface of IEs in a sex-specific manner, such that

serum samples from malaria exposed males do not show any reactivity toward placental IE surface (Fried *et al.,* 1998; Ricke *et al.,* 2000). Placental infections at delivery in primiparous women appear to be sufficient to induce functional antibodies which can both recognize the surface of the infected erythrocytes as well as block their binding to CSA. The correlation between serum reactivities of placental field isolates from different geographic locations collected at different times is indicative of the conserved nature of the antigen(s) mediating pregnancy-associated malaria (Khattab *et al.,* 2004). The targets of antibodies that inhibit CSA binding are thought to be generated in a strain-independent manner, with the capacity of recognizing parasites from different geographic locations.

In a study in which placental isolates were used, it was shown that primigravid women lacked antibodies that were capable of inhibiting the adhesion of IEs to CSA (Fried *et al.,* 1998) but a significant level of anti-adhesion antibodies was detected in primiparous women whose placentae were found to carry malaria infections at delivery (Maubert *et al.,* 1999; O'Neil-Dunne *et al.,* 2001). Significant association between CSA-adhesion inhibitory antibodies and agglutination antibodies has been reported, but many samples were found to differ in their quality, on the one hand in strongly inhibiting adhesion, but on the other in being negative or only weakly positive in agglutination (Beeson *et al.,* 2004). In addition to agglutination and anti-adhesion assays, flow cytometric analysis has also been used successfully to detect antibodies in sera from pregnant women directed against the surface of *P. falciparum* IEs, previously selected to bind CSA (Ricke *et al.,* 2000; Khattab *et al.* 2004).

In a study cohort, primiparous as well as secundiparous women had the greatest risk of infection at delivery as well as during pregnancy. Primiparous women with infected placentas at delivery showed higher levels of VSA_{PAM}-specific IgG compared to women who had no malaria

infections at delivery. Placental isolates of Gabonese and Senegalese origin tested on plasma samples from Gabon showed parity dependency and gender specificity patterns. There was a significant correlation of plasma reactivity as measured by flow cytometry between different placental isolates. In the plasma of infected primiparous women, VSA_{PAM}-specific IgG measured by flow cytometry could be correlated with anti-adhesion antibodies measured by the inhibition of CSA binding (Khattab *et al.*, 2004).

Maternal malaria is associated with the sequestration in the placenta of *P. falciparum*-infected erythrocytes onto CSA, via the duffy binding-like (DBL)-γ3 domain of the PfEMP1(CSA) (DBL-γ3(CSA)). The production of antibodies against CSA-binding infected erythrocytes (IEs(CSA)) is correlated with resistance to maternal malaria in multiparous women. Costa *et al.* (2003) produced recombinant DBL-γ3(CSA) (rDBL-γ3(CSA)) in insect cells, corresponding to 2 variant DBL-γ3(CSA) subtypes that mediate binding to CSA in laboratory lines and placental isolates. Both recombinant cysteine-rich DBL-γ3(CSA) domains blocked IEs(CSA) binding to CSA. Immunization of mice, with the rDBL-γ3(CSA)-FCR3 and rDBL-γ3(CSA)-3D7 domains, resulted in the generation of antibodies recognizing homologous and heterologous rDBL-gamma3(CSA), a finding indicating conserved epitopes inducing a pan-reactive immune response. Mouse monoclonal antibodies (MAbs) against both recombinant proteins were pan-reactive with various IEs (CSA). One MAb efficiently inhibited and reversed IE(CSA) cytoadhesion to endothelial cells in vitro. Thus, DBL-γ3(CSA) is the target of inhibitory and pan-reactive antibodies. *Saimiri sciureus* monkeys immunized with FCR3-rDBL-γ3(CSA) developed pan-reactive and inhibitory antibodies, a finding suggesting that the development of a vaccine to prevent maternal malaria is feasible (Costa *et al.*, 2003).

Rapid clearance of parasitaemia following transfusion of IgG from malaria immune adults to clinically ill recipients illustrates that naturally acquired antibodies have a parasite clearing role in human malaria infection (Cohen *et al.,* 1961; Sabchareon *et al.,* 1991).

Antibodies targeting variant antigens on the surfaces of chondroitin sulfate A (CSA)-binding malaria-infected erythrocytes have been linked to protection against the complications of MIP. In Malawi, women in their first pregnancy with placental malaria produced significantly greater amounts of immunoglobulin G1 (IgG1) and IgG3 reactive with surface antigens of malaria-infected erythrocytes than uninfected women of the same gravidity. IgG1 and IgG3 levels in infected and control women in later pregnancies were similar to those in infected women in their first pregnancy. Levels of IgG2 and IgG4 were similarly low in infected and uninfected women of all gravidities. IgM that bound to the surface of CSA-adherent *P. falciparum* occurred in all groups of women and malaria-naive controls. There was a significant correlation between IgG1 and IgG3 levels, indicating that women usually produced both subtypes. Levels of IgG1 and IgG3 correlated with the ability of serum or plasma to inhibit parasite adhesion to CSA. Taken together, these data suggest that IgG1 and IgG3 dominate the IgG response to placental-type variant surface antigens. They may function by blocking parasite adhesion to placental CSA, but given their cytophilic nature, they might also opsonize malaria-infected erythrocytes for interaction with Fc receptors on phagocytic cells (Elliot *et al.,* 2005).

Binding of the Fc portions of cytophilic antibodies, immunoglobulin G1 (IgG1) and IgG3, to Fcγ receptors on phagocytic cells triggers a range of effector functions including phagocytosis, production of cytokines and chemokines, cytotoxicity and generation of reactive oxygen and nitrogen species. It is the cytophilic subtypes of antibodies targeting merozoite surface antigens that are associated with clinical and parasitological immunity (Mina-Osorio and Ortega, 2004).

There is evidence to suggest that the relatively conserved PfEMP1, VAR2CSA, expressed on the surfaces of CSA-binding IEs is a key target of antibodies associated with protection against MIP (Salanti *et al.,* 2004). Recombinant proteins corresponding to *var2csa* domains are recognized by antibodies in plasma from malaria-exposed donors according to gravidity and gender, and antibodies to these domains are associated with reduced risk of infant low birth weight (Salanti *et al.,* 2004). The isotype and subtype of an antibody confer specific functional activity.

Beeson *et al.,* (2004) have shown previously that placental malaria in primigravid Malawian women is associated with induction of antibodies that recognize CSA-adherent IEs of the It line CS2 and inhibit adhesion of CS2 IEs to CSA. CS2 is recognized by malaria-exposed sera in a gravidity- and gender-dependent manner (Beeson *et al.,* 2004), and it transcribes *var2csa* as the dominant *var* transcript (Duffy *et al.,* 2005).

Genetic polymorphism of the Fc receptor IIa for IgG (Fc gamma RIIa) determines IgG subclass binding. Previous studies have shown that individuals with the IgG1/3-binding Fc gamma RIIa-Arg/Arg131 genotype are relatively protected against high-density malaria, whereas individuals with the IgG2-binding Fc gamma RIIa-His/His131 genotype are at increased risk for developing cerebral malaria (Brouwer *et al.,* 2004). Among HIV-positive women, the frequency of the IgG2-binding Fcγ RIIa-His/His131 genotype was significantly higher in women with placental malaria than in women without placental malaria. The study by Brouwer *et al.,* (2004) suggests that the IgG2-binding Fcγ RIIa-His/His131 genotype is associated with enhanced susceptibility to placental malaria in HIV-positive women but not in HIV-negative women. Among HIV-negative women, there was no difference in the distribution of the Fcγ RIIa polymorphism by placental malaria status.

Elliot *et al.,* (2005) demonstrated that placental infection is associated with induction of cytophilic antibodies IgG1 and IgG3 specific for VSA expressed by the CSA-binding *P. falciparum* line CS2, in which the dominant *var* gene transcribed is *var2csa*. Both subtypes correlated with the ability of serum or plasma to inhibit adhesion of CS2 IEs to CSA.

2.7 INTERMITTENT PREVENTIVE TREATMENT OF MALARIA DURING PREGNANCY

Intermittent treatment of malaria during pregnancy (IPTp) is the treatment of asymptomatic pregnant women, regardless of their malaria infection status, with regularly spaced therapeutic doses of an effective antimalarial drug beginning after quickening (the time at which foetal movements are first felt by the mother) in the second trimester. Quickening in most women occur between 16 and 20 weeks of pregnancy. The objective of IPTp is to reduce the adverse effects of malaria on maternal anaemia and pregnancy outcome (Shulman and Dorman, 2003; Greenwood, 2004). In Nigeria, the strategy of malaria control in pregnancy employs the use of sulphadoxine-pyrimethamine (SP) for IPTp (IPTp-SP). Pregnant women should receive 2 doses of SP after quickening if they are HIV-negative and 3 doses of SP if HIV-positive after quickening in the second and third trimesters of pregnancy (FMOH, 2005a). Following the recommendation of at least 4 antenatal visits by pregnant women (3 of the 4 visits after quickening) (WHO, 2004), it is technically feasible to administer IPTp-SP through antenatal clinics as direct observed therapy.

The drug of choice for IPTp, sulfadoxine-pyrimethamine (SP), is documented to be safe after the first trimester of pregnancy (Newman *et al.,* 2003), cheap, easy to administer and good feasibility for use in programs, as it can be delivered as a single-dose treatment under observation by a health worker in an antenatal clinic thus ensuring compliance (FMOH, 2005a). The minimum

interval between IPTp-SP doses is 4 weeks (Rogerson *et al.,* 2000). WHO (2004) recommends that the first SP dose should be administered in the second trimester after quickening and the second dose of SP in the third trimester because it ensures that the placenta is cleared of parasites at the time of rapid foetal growth.

2.7.1 Pharmacokinetics of Sulfadoxine-Pyrimethamine

Although sulfadoxine-pyrimethamine has been used for decades, there are very few data on the pharmacokinetics in pregnancy, so the optimum dose has not been determined. The current information on pharmacokinetics of SP was determined in non-pregnant population (Nosten *et al.*, 2007). Both sulfadoxine and pyrimethamine are well absorbed from the gastrointestinal tract. Like other sulphonamides, sulfadoxine is widely distributed in the body. Peak plasma levels are achieved within 2 to 8 h. Pyrimethamine is distributed mainly to the kidneys, lungs, liver and spleen. Plasma protein binding is about 90% for both pyrimethamine and sulfadoxine. About 5% of sulfadoxine appears in the blood as acetylated metabolite, about 2-3% as the glucuronide. Pyrimethamine is transformed to several metabolites (IPCA, 2006).

Dosing for antimalarials in pregnancy has been based on extrapolations from studies in non-pregnant adults, yet there are many physiological changes in pregnancy that may have an impact on drug exposure, including altered drug absorption, distribution, metabolism, and clearance (Dawes and Chowienczyk, 2001). A significant increase in both intravascular and extravascular volume is also seen in pregnancy, leading to substantial changes in apparent volumes of distribution (Dawes and Chowienczyk, 2001). Significantly lower levels of sulphadoxine have been documented during pregnancy than in non-pregnant women (Green *et al.*, 2007; Karunajeewa *et al.*, 2009; Nyunt *et al.*, 2010). There has been less consistency in the results of pyrimethamine pharmacokinetics. While Nyunt *et al.,* (2010) reported an increase in

pyrimethamine level in pregnancy, two other studies reported pyrimethamine level to be similar or lower than the those in non-pregnant women (Green *et al.*, 2007; Karunajeewa *et al.*, 2009).

Both sulfadoxine and the pyrimethamine are excreted mainly by the kidneys. The apparent elimination half life of sulfadoxine ranged from 100 to 231 hours with a mean of 169 hours, whereas pyrimethamine half lives ranged from 54 to 148 hours with a mean of 111 hours. (Weidekamm *et al.*, 1982; IPCA, 2006). It is important to consider both the half-life of a drug and the existing level of resistance when choosing a drug for IPT. The role of drug elimination half-life in the evolution of parasite resistance has been reviewed and modeled (Hastings *et al.*, 2002a). Readily absorbed drugs with a long elimination half-life, such as mefloquine and SP, have multiple therapeutic advantages. Patient compliance is improved because these treatments are given either as a single dose, or as a short regimen, which can be directly observed by the clinic staff. Moreover, residual drug levels during the post-therapeutic period offer a certain protection against the reemergence of parasitemia for several weeks (up to 8 weeks for SP) and may help patients to recover from anemia, a major cause of ill health and death in areas of intense malaria transmission.

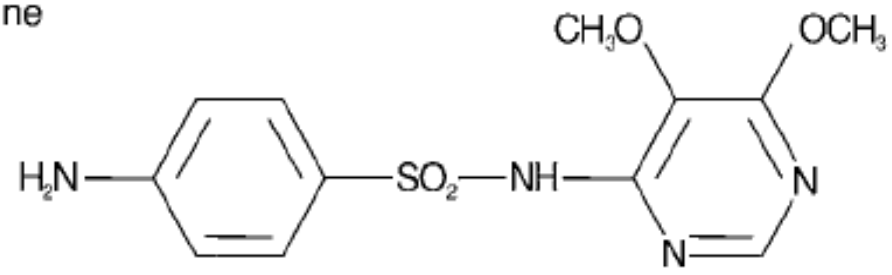
Drugs with long half-lives, such as SP, also increase parasite exposure to sub-therapeutic drug concentrations and thus, selection for resistant mutants. Infections that appear during the gradual decay of SP in the bloodstream of a treated patient are more likely to be pyrimethamine-resistant than those that appear after the drug has cleared (Watkins and Mosobo, 1993).

2.7.2 Mechanism of Action of Sulfadoxine-Pyrimethamine

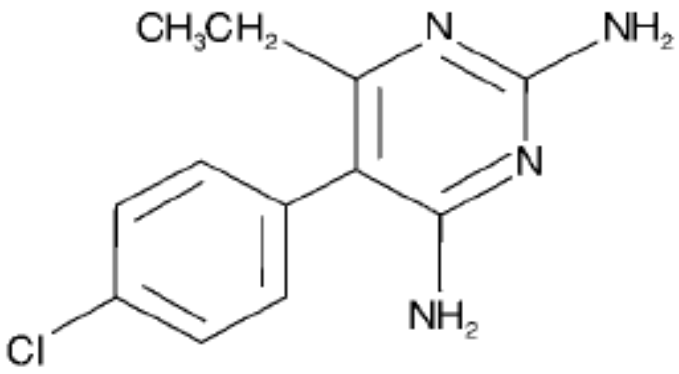
Sulfadoxine-Pyrimethamine (SP) is a combination of two antifolate antimalarial drugs that block two key enzymes in the folic acid biosynthetic pathway. Folic acid is needed for the biosynthesis of purines and pyrimidines and hence DNA synthesis and cell multiplication. (Bzik *et al.*, 1987;

Sibley *et al.*, 2001). Pyrimethamine [2,4-diamino-5-(p-chlorophenyl)-6-ethylpyrimidine] is a synthetic diaminopyridine that binds to and inhibits the bifunctional enzyme dihydrofolate reductase-thymidylate synthase (DHFR-TS) of plasmodia. This leads to a failure of nuclear division and subsequent cell death.

Sulfadoxine



Pyrimethamine



Sulfadoxine [N1-(5,6-dimethoxy-4-pyrimidinyl) sulfanilamide], a structural analog of p-aminobenzoic acid (PABA), is a long-acting sulfonamide that competitively inhibits dihydropteroate synthase (DHPS), an enzyme that necessary for the conversion of PABA to folic acid (Sibley *et al.*, 2001). This enzyme is also a component of the folate metabolic pathway and is upstream of DHFR, the enzyme targeted by pyrimethamine (Figure 2.2).

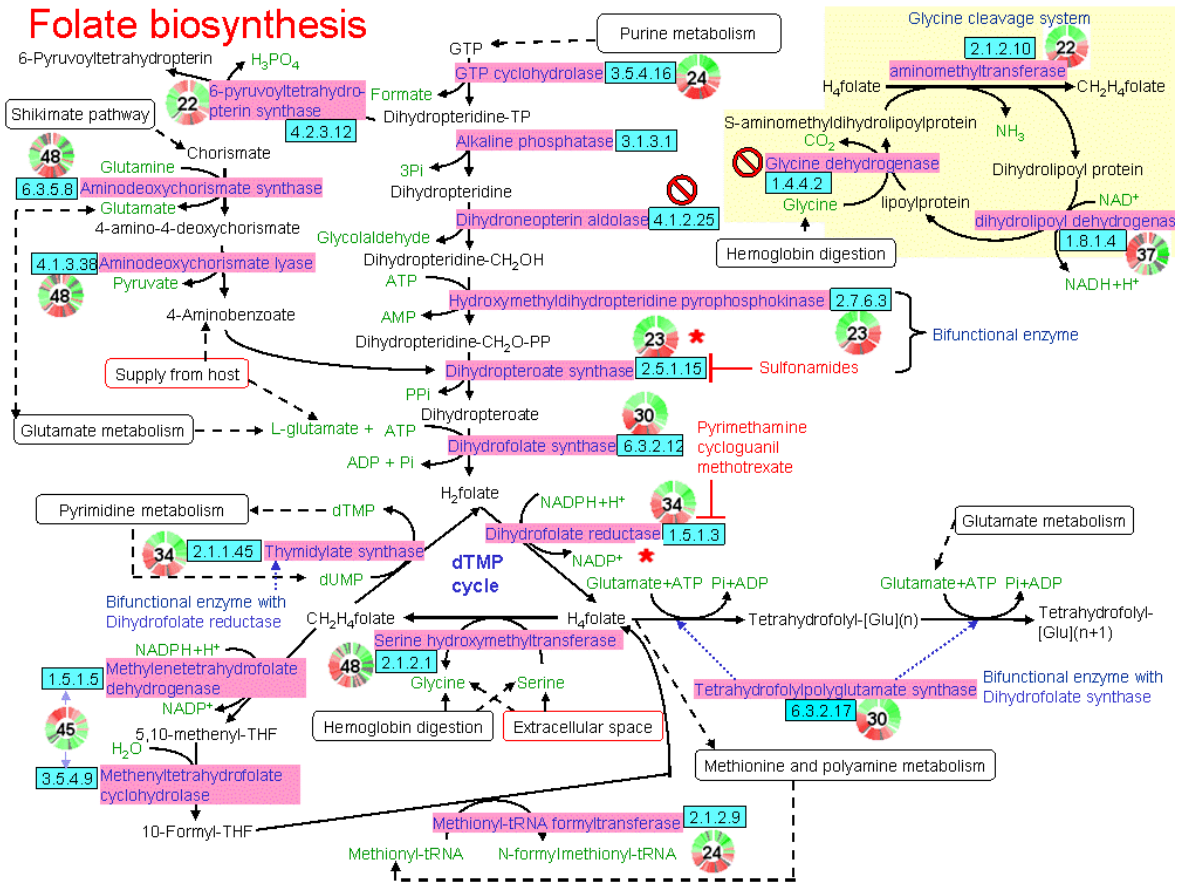


Figure 2.2: Folate Biosynthesis.

(Source: http://sites.huji.ac.il/malaria/maps/folatebiopath.html Accessed 2nd March 2009)

The combination of pyrimethamine and sulfadoxine thus offers a two-step synergistic blockade of plasmodial division. SP is schizonticidal and also active against the other asexual erythrocytic forms of susceptible plasmodia

2.7.3 Prospects of IPTp-SP

The IPTp-SP policy has good prospects with regards to its feasibility of implementation, compliance with dosing regimen, efficacy of IPTp-SP and even the presence of some level of resistance to SP.

2.7.3.1 *Implementation of IPTp-SP*

Technically, the implementation of IPTp-SP program is very feasible. The drug used, SP, is cheap (less than ₦100 for a full treatment dose) and widely available in virtually all patent medicine stores, pharmacies and clinics. The incorporation of IPTp-SP into the normal antenatal clinic care of pregnant women means that pregnant women do not need special appointments to obtain their SP dose. Also, the intake of SP dose can be directly observed by a health worker. If the WHO recommendation of at least four ANC visits (WHO, 2004) is adhered to by all pregnant women, then the recommended 2 and 3 doses of SP for HIV-negative and HIV-positive pregnant women (FMOH, 2005a) can be effectively administered. High ANC attendance (upto 70%) has been reported in many African countries (WHO, 2006).

In areas where ANC attendance is low, community-based delivery system is recommended. This involves the training and delivery of IPTp-SP through community health workers, patent medicine vendors and traditional birth attendants. This method is effective at delivering at least 2 doses of IPTp-SP to women who do not have easy access to health centers (Mbonye *et al.*, 2007).

2.7.3.2 *Compliance with IPTp-SP*

Unlike chemprophylaxis with chloroquine or pyrimethamine that requires weekly administration of the drugs, IPTp-SP only requires the pregnant woman to take the drug just twice or thrice beginning from the second trimester. Each treatment dose is taken once as a single-dose therapy. Thus IPTp-SP is more acceptable resulting in better compliance to the treatment regimen.

2.7.3.3 *Efficacy of IPTp-SP*

Monitoring the therapeutic and protective efficacy of SP is very important in determining the usefulness of IPTp-SP. Therapeutic efficacy of SP in children cannot be extrapolated to pregnant women because there is evidence that parasitological response is significantly better in pregnant women than in children treated with the same drug (Mutabingwa *et al.,* 2009). There is evidence that SP remains effective in preventing placental malaria, maternal anaemia and LBW in settings with high levels of SP resistance in children (ter Kuile *et al.,* 2007). IPTp-SP has been reported to be superior to chloroquine prophylaxis or as IPTp-CQ (Tiono *et al.,* 2009).

The administration of at least two therapeutic doses of IPTp-SP after the first trimester has been found to be effective in preventing maternal and placental malaria as well as improving pregnancy outcomes among parturient women in Nigeria (Falade *et al.,* 2007), Ghana (Hommerich *et al.,* 2007), Mali (Kayentao *et al.,* 2005), Mozambique (Challis *et al.,* 2004), Kenya (Parise *et al.,* 1998; van Eijk *et al.,* 2004a), Malawi (Rogerson *et al.,* 2000) and Gabon (Ramharter *et al.,* 2007).

2.7.3.4 *Resistance to Sulphadoxine-Pyrimethamine*

As with any drug-based intervention strategy, it is important to understand how implementation may affect the spread of drug-resistant parasites. Some researchers are of the opinion that drugs to which little or no resistance exists are not advisable for IPT in high-transmission areas (Prudhomme O'Meara *et al.,* 2006). This is because the administration of the drug to a large population of pregnant women would build up drug pressure within the population which may lead to the development of resistance. Thus SP has a good profile for use in IPT.

The World Health Organization (2005) recommendation in areas where the parasitological failure rate of SP is less than 50% in the general population countries should:

- Continue implementing or adopt a policy of at least two doses of IPT with SP.
- Implement also other control measures such as ITN and anaemia and malaria case management.
- Evaluate the impact of IPT on an ongoing basis.

In a national efficacy study conducted in 2004, day 14 efficacy of SP in South-west Nigeria in acute uncomplicated malaria was 75.6% (i.e. parasitological failure rate was ~25%) among children aged 6 months to five years (FMOH, 2005a). Thus, following the WHO guideline, Southwest Nigeria should fully adopt IPT with SP as the strategy of malaria control in pregnancy.

2.7.4 Challenges in the Implementation of IPTp-SP

The challenges faced in intermittent preventive treatment of MIP using SP are based on issues of resistance to SP, effect of daily folate supplementation, national coverage of IPTp-SP, adequacy of the number of doses of SP, timing of the administration of SP, adverse effects of SP, coverage at first trimester, attendance to antenatal clinics and co-infection with HIV

2.7.4.1 *Resistance to Sulfadoxine-Pyrimethamine*

Clinical trials of SP in six geopolitical zones of Nigeria in 2004 led to the removal of SP as the first line drug for treatment of uncomplicated malaria in the general population and the adoption of Artemisinin-based combination therapy (FMOH, 2005). Thus, there are concerns about how effective SP is as the drug of choice for intermittent preventive treatment of malaria in pregnancy. Reports of high levels of mutations associated with resistance to SP in Nigeria have further aggravated the fear that IPTp-SP may not be adequately protectpregnant women (Happi *et al.,* 2005, 2006). Possible alternatives to SP are being sought but currently none has been

found to be better than SP in terms of preventing maternal anaemia , adverse foetal outcome and acceptability (Kalilani *et al.*, 2007; Briand *et al.*, 2007; Smith *et al.*, 2010;).

2.7.4.2 *Effect of Daily Folate Supplementation*

In antenatal clinics, the practice is that pregnant women are placed on daily folate drugs (5 mg) because folate supplementation in pregnancy has been associated with reduction in anaemia and prevention of megaloblastic erythropoiesis (Tamaru and Picciano, 2006). The international guidelines recommend 0.4 or 0.6 mg of folate daily (Stolzfus and Dreyfuss, 1997) however the 5 mg tablet is more widely available and is being used daily. In their study, Ouma *et al.*, (2006) reported that the combined use of SP and daily folate supplementation at a dose of 5 mg compromised the efficacy of SP for the treatment of malaria parasitaemia in pregnant women while the use of 0.4 mg daily did not. Malaria parasites can utilize exogenous folate (the salvage pathway) as well as synthesize folate *de novo* for DNA synthesis (Krungkrai *et al*., 1989). Although biosynthesis seems to be the preferred method, (Wang *et al*., 2004), malaria parasites can switch to the salvage pathway when they cannot synthesize folate *de novo* if there is an abundance of folate. Thus, the abundance of folate will compromise the efficacy of SP (Ouma *et al.*, 2006; Metz, 2007; Nduati *et al.*, 2008).

2.7.4.3 *National Coverage of IPTp-SP*

The target set for national IPTp coverage at the African summit of Heads of States in Abuja in 2000 was 60% and 80% by 2005 and 2010 respectively (RBM, 2000). However, the coverage as at 2005 was just 17% (FMOH, 2007) (Figure 2.3).

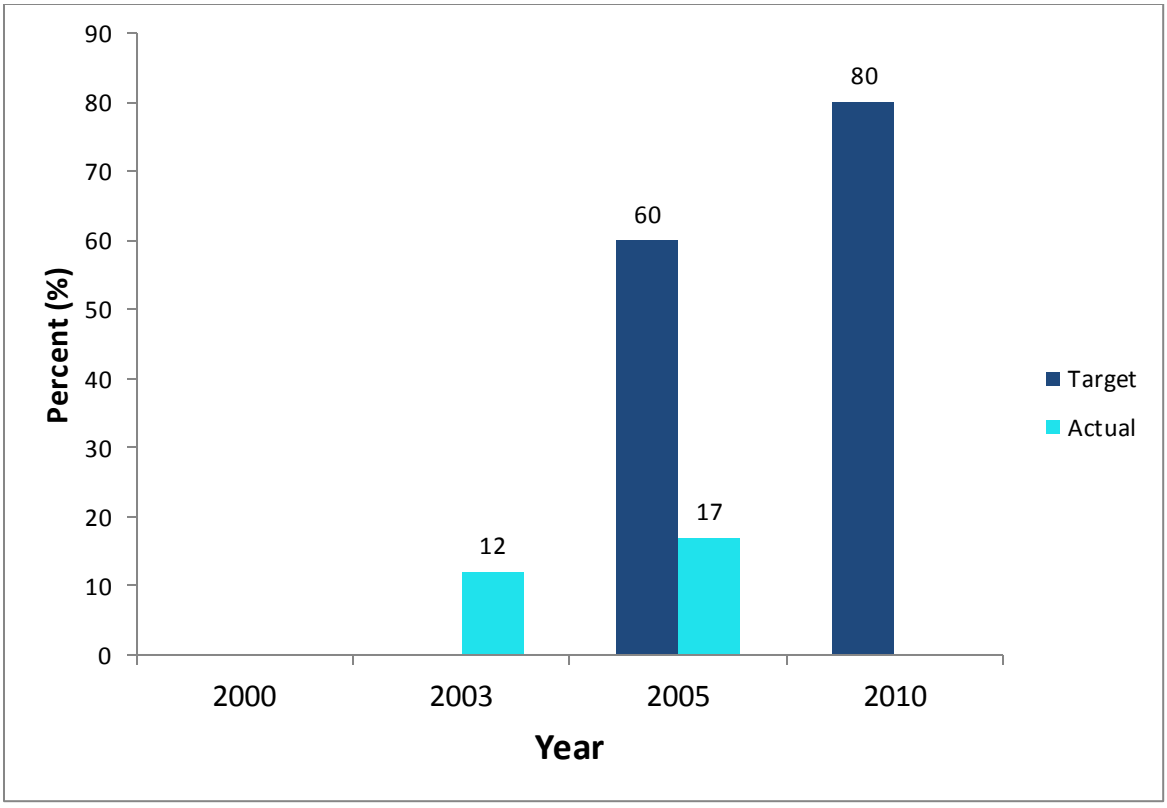


Figure 2.3: NATIONAL IPTp-SP COVERAGE VS ABUJA TARGET

Hill and Kazemgbe (2006) in their review attributed the low IPTp coverage and poor compliance in developing countries to factors such as:

- Perceptions of pregnant women on taking of SP
- Practices of health-care workers in the public sector that deter women from going to public hospitals
- Poor access and intermittent supplies of medications
- Irregular antenatal clinic attendance
- Inadequate clinic staff training

Training and motivation of health-care workers, as well as the use of simplified IPTp messages has been used to significantly improve the implementation of IPTp in some settings (Briand *et al.* 2007; Ouma *et al.*, 2007)

2.7.4.4 *Adequacy of the Number of Doses of IPTp-SP*

The Federal Ministry of Health of Nigeria recommends the administration of 2 doses of SP to pregnant women who are not infected with HIV, while for HIV-positive women, 3 doses of SP should be administered (FMOH, 2005b). For HIV-negative women, there are reports that maximum benefit is achieved by monthly administration of SP after quickening (Parise *et al.,* 1998; Filler *et al.,* 2006). The benefit of monthly SP doses appear to be dependent on the level of malaria endemicity. In an area of meso-endemicity in Zambia, monthly IPTp-SP was not more efficacious than the standard 2-dose regimen for the prevention of placental malaria or adverse birth outcomes (Hamer *et al*., 2007) whereas in hyper-endemic areas, monthly IPTp-SP was a better option (Filler *et al.,* 2006).

The intention to give the standard 2-dose regimen frequently results in mothers inadvertently receiving only 1 dose, something that rarely occurred among mothers for whom the intention was to give monthly doses of SP. Single-dose SP has been shown to be inferior to all other dosing regimens (Gill *et al*, 2007). Thus, at a programmatic level, monthly SP appears to be preferable to standard IPTp because it virtually eliminates the risk of inadvertently under-dosing pregnant women.

2.7.4.5 *Timing of IPTp-SP*

There is currently no precision regarding the best timing for IPTp-SP administration as it entirely depends on the timing and frequency of ANC visits of the woman. There is need to particularly protect women in late pregnancy because this is when both foetal growth and deleterious effects of malaria are most important (McGready *et al.,* 2004; Gamble *et al.,* 2006,).

In the absence of ANC visits in the two first trimesters, it could still be worthwhile to administer IPTp even only in the last month of pregnancy. Sufficient benefit is derived from 2-3 doses of

IPT, although a single dose is beneficial (White, 2005; Sirima *et al.,* 2006). Two studies that highlighted the relevance of a protection in late pregnancy reported that IPTp had a higher efficacy when the last dose was administered close to delivery (van Eijk *et al.,* 2004b; Filler *et al.,* 2006). There are conflicting reports on the administration of SP close to delivery (after 36 weeks). Although the study by Taylor and White (2004) did not report any adverse effect after administering SP beyond 36 weeks, caution should be exercised because there have been reports of an increased risk of neonatal kernicterus in babies of women who received SP after 36 weeks (Andersen *et al.* 1956; Forna *et al.,* 2006).

2.7.4.6 *Adverse Effects of SP*

SP has not been associated with adverse effects on the fetus and is well tolerated by mothers. The most common side-effects associated with sulfadoxine-pyrimethamine are gastrointestinal and cutaneous (when used as a prophylactic) (Taylor and White, 2004). There is a fatal risk of Steven-Johnson syndrome and toxic epidermal necrolysis associated with weekly prophylaxis with SP. These are very rare occurrences when interval of administration of IPTp is at least 4 weeks (Hamer *et al.,* 2007). The estimated risk of severe adverse reactions to intermittent administration of SP (monthly or less often) is 0%-6.3% (Brentlinger *et al.,* 2006). Passive surveillance for severe adverse events associated with SP and trimethoprim-sulfamethoxazole in Malawi found that life-threatening reactions (including Stevens-Johnson syndrome) occurred infrequently Fatal adverse reactions to SP were estimated to be 0.11 deaths/100,000 SP exposures (Gimnig *et al.,* 2006; Forna *et al.,* 2006). This, however, does not preclude the need for careful monitoring for serious reactions when SP is used for IPTp.

2.7.4.7 *Coverage at First Trimester*

The administration of SP in the first trimester is not advised because of potential teratogenic effect on the foetus (FMOH, 2005). Thus, protection against malaria afforded by IPTp-SP does not cover the pregnant woman in her first trimester. However, for those with malaria who become symptomatic, they are treated as indicated in section on *Case Management of Malaria Illness and Anaemia* (in this thesis).

2.7.4.8 *Attendance at Antenatal Clinics*

Although ANC attendance is high in most countries with IPTp policy (2.0-4.8 ANC visits per woman) (Marchesini and Crawley, 2004), it has not been sufficient to ensure a high IPTp coverage (Ndyomugyenyi and Katamanywa, 2010). Some of the identified reasons were: not given SP by the midwife for unknown reasons, SP stock-outs and irregular ANC attendance. One of the concerns about attendance to ANC is that multigravid women tend to come to ANC in their third trimester because they feel they are experienced and can easily buy the drugs they are given at ANC from a patent medicine vendor.

2.7.4.9 *Co-infection with HIV*

In immunocompromised women, there is a higher risk of adverse reactions to sulfonamides, conferred by HIV-related immunosuppression (Moore *et al* 1996). Thus, administering SP on a monthly basis may not be ideal in immunocompromised pregnant women. In a trial of twice-weekly SP for prevention of AIDS-related opportunistic infections, 6·3% (6/95) of patients required discontinuation of SP because of adverse reactions (Schurmann *et al.,* 2001). Fatal reactions to SP have been reported in HIV-infected individuals, including one participant in an intermittent preventive treatment trial conducted in Zambia (Raviglione *et al.,* 1988; Hamer *et al.,* 2005). In the same Zambian trial, a regimen of monthly preventive SP was associated with an

increased incidence of fatal sepsis in neonates born to trial participants, but this observation has not been replicated elsewhere (Hamer *et al.,* 2005).

The lower limit of estimated monthly SP risk to immunocompromised women is likely to be somewhat higher than the very low reported risk of adverse reactions to preventive SP in previous intermittent preventive treatment trials because of the known increase in adverse reactions to sulfonamides

Because of concerns about both effectiveness and safety, SP has been supplanted by other agents for the prevention of *Pneumocystis carinii* pneumonia in the USA (Allen *et al.,* 1992). The risk of substantial adverse reactions to intermittent (monthly or less frequently) SP in immunocompromised HIV-infected pregnant women has not been quantified, because no published intermittent preventive treatment trials have reported the CD4 cell counts, or other markers of immunosuppression, of HIV-infected participants. It is likely to fall substantially below the 6·3% level cited above because the study subjects were severely immunocompromised and SP was administered aggressively on a twice weekly basis (Schurmann *et al.,* 2001).

There are concerns raised by some workers that the standard IPT regimen used for HIV-positive pregnant woman may not be very effective in protecting them from placental and peripheral malaria parasitaemia. Rather a monthly administration of treatment dose of SP has been suggested (Parise *et al.,* 1998; Filler *et al.,* 2006). However, Hamer *et al.* (2005) failed to document any difference in efficacy of standard versus monthly IPTp-SP in HIV-positive women (Hamer *et al.,* 2005).

A study reported that a substantially greater reduction of placental parasitaemia was seen in HIV-infected women receiving three or more doses of SP compared with two doses, but in HIV-negative women the regimens were equivalent (Parise *et al.,* 1998). In a study carried out in

Malawi, monthly IPT with SP prevented placental parasitaemia significantly more effectively than two-dose SP in HIV-positive women, but not in HIV-negative women (Filler *et al.,* 2006). Where the benefits of standard two-dose SP in the HIV-infected woman are indeed reduced, immunosuppression and/or antimalarial drug resistance may be responsible (Mount *et al.*, 2004).

The risks of adverse reactions to SP is related to the frequency of SP dose and the degree of the immunocompromised state of the individual (Hamer *et al.,* 2007). The more frequent SP is given (less than monthly), the more the risk of adverse reactions developing; also the more immunocompromised an individual is, the more likely he is to develop adverse reactions to SP.

2.7.4.10 *Possible Alternative Drugs For IPTp*

The following properties are required of any drug that will replace SP in future when it will no longer be useful for IPTp:

- It must have a long-half life, as it has been suggested that the duration of prophylaxis rather the treatment effect is the most important determinant of IPTp efficacy;
- It must be safe during pregnancy and well-tolerated to ensure a high compliance with the treatment in women who are often asymptomatic when infected with malaria;
- The administration should be easy (ideally a single dose);
- It should be readily available and at an affordable cost (Briand *et al.,* 2007).

Mefloquine (MQ) is presently one of the most attractive options. It has a long half-life and is still highly efficacious in African countries (Briand *et al.,* 2007). Most studies have shown that MQ was safe for use in pregnancy (Steketee *et al.,* 1996; Vanhauwere *et al.,* 1998; Phillips-Howard *et al.,* 1998). A study has suggested that women who received MQ treatment had a significantly higher risk of stillbirth than women not treated or treated with other antimalarials (WHO, 1993).

Further studies have not confirmed this finding so far (Newman *et al.,* 2003). MQ has been associated with a range of side-effects, raising the question of the women's compliance with the treatment. In a clinical trial, in which Beninese women are randomized to receive SP or MQ (15mg/kg as a single intake) for IPTp only mild adverse events have been observed - such as vomiting, nausea and dizziness, and they resolved fast (NIH, 2006). Very few women have refused to take the second dose because of a poor tolerance of the first intake. The drug has been well-accepted in spite of its bitter taste, and there has been less than 1% of early vomiting (within one hour) after giving a fat snack before the women took the drug (as recommended by the manufacturer).

Whatever the alternative drug, the cost will be substantially higher than for SP or CQ. Mefloquine remains expensive even if its cost has recently declined. In the near future, lower cost and higher availability of the drug in African countries should make this option even more realistic.

Artemisinin combination therapies (ACT) are also being evaluated for IPTp. They have been shown to be highly efficacious and safe during pregnancy except when used in the first trimester. However, if the effect of IPTp is mainly prophylactic (White, 2005), then short-acting drugs would be expected to provide little benefit. Moreover, ACTs are still very expensive and less easily deliverable as they require multiple treatment doses that could not be given as a directly-observed therapy in the ANC clinic. Because the treatment needs to be administered several times, compliance might be low.

Other potential candidates, such as SP plus azithromycin and SP plus artesunate, have been evaluated and found to be safe and effective (Kalilani *et al.,* 2007). SP plus amodiaquine and chlorproguanil-dapsone are also being assessed for IPTp. Piperaquine (used in combinations with

other antimalarials rather than used alone) might be one of the very promising options for IPTp (Briand *et al.*, 2007).

2.8 MOLECULAR MARKERS OF ANTIMALARIA DRUG RESISTANCE

The emergence and spread of parasites resistant to antimalarial drugs continues to be a major public health problem in the management of *Plasmodium falciparum* infections in malaria-endemic countries A parasite strain is said to have developed resistance to a drug if it is able to survive or multiply in the presence of the minimum inhibitory concentration of the drug (Bloland 2001). Molecular surveillance is important in predicting shifts of drug resistance in *Plasmodium* populations following drug policy changes. Genetic mutations that confer some level of resistance to a drug are thought to arise randomly during replication (White, 2003). The emergence and spread of drug resistance depends on the number of mutations required to encode resistance and their effects on parasite fitness (White, 1999). Typically, high-level resistance to a particular drug requires multiple mutations that accumulate gradually over several parasite generations. Molecular markers of resistance have been useful in surveillance of resistance to antimalarial drugs having been associated with therapeutic failures. Usually, specific multiple point mutations in a gene make up a resistance marker for an antimalarial drug. It has been observed that treatment with SP strongly selects resistant parasite genotypes with triple *dhfr* gene mutations and quintuple mutations (triple *dhfr* plus double *dhps* mutation) (Dzinjalamala *et al.*, 2005).

2.8.1 Markers of Sulphadoxine-Pyrimethamine resistance

The increase in resistance to SP is associated with the stepwise acquisition of specific point mutations in the *dihydrofolate reductase (dhfr)* and *dihydropteroate synthase (dhps)* genes, which alter the drug-binding sites of SP (Hyde, 1989; Sibley *et al.*, 2001). Once formed, these

mutations spread across vast, continent-wide areas (Roper *et al.*, 2004). Table 2.2 lists the important amino acid substitutions observed in the *dhfr* and *dhps* genes that are associated with resistance to type 1 (pyrimethamine, chlorproguanil, trimethoprim) and type II antifolates (sulfonamides: sulfadoxine and dapsone) respectively (Hyde, 1990; Wang *et al.*, 1997 Kublin *et al.*, 2002).

Table 2.2: Amino acid substitutions in *dhfr* and *dhps* gene

Gene	Codon	Amino acid change	Code
Dhfr	16	alanine to valine	A16V
	50	cysteine to arginine	C50R
	50	cysteine to isoleucine	C50I
	51	asparagines to isoleucine	N51I
	59	cysteine to arginine	C59R
	108	serine to asparagine	S108N
	108	serine to threonine	S108T
	140	valine to leucine	V140L
	164	isoleucine to leucine	I164L
Dhps	436	serine to phenylalnine	S436F
	436	serine to alanine	S436A
	437	alanine to glycine	A437G
	540	lysine to glutamine	K540E
	581	alanine to glycine	A581G
	613	alanine to serine	A613S
	613	alanine to threonine	A613T

The rapid development of SP resistance is consistent with both monogenic and additive mechanisms. Conversely, the observation that CQ resistance took longer to develop suggests that the genetic basis for CQ resistance involves two or more genes. However, it is also consistent with multiple mutations on the same gene, all of them required (epistatic basis) for resistance to be achieved (Talisuna. 2004). S108N mutation in the *dhfr* gene seems to be the key mutation that confers resistance to pyrimethamine *in vitro* (Cowman *et al.,* 1988; Schönfeld *et al.,* 2007; Noranate *et al.,* 2007). Noranate *et al.* (2007) observed that mutation at codon 59 was not detected as a single or double mutant haplotype, but only in the triple mutant haplotype. Other mutations that confer a selective advantage, which arise after the codon 108 mutation are additive, increasing the level of pyrimethamine resistance. N51I and C59R mutations appear to confer higher levels of *in vitro* pyrimethamine resistance when they occur with the S108N mutation (Sims *et al.,* 1999; Mbaisi *et al.,* 2004); whereas I164L in combination with S108N, C59R and (or) N51I has been found in *P. falciparum* strains that are highly resistant to both pyrimethamine and cycloguanil (Basco *et al.,* 1995; Mkulama *et al.,* 2008). I164L *dhfr* mutation has been reported in Tanzania (Hastings *et al.,* 2002b). S108T plus A16V mutation also confer parasite resistance to cycloguanil, the active form of proguanil (Peterson *et al.,* 1990; Mkulama *et al.,* 2008).

The mutations in the *dhps* gene modulate parasite tolerance to the sulphonamides. Resistance to sulphadoxine is associated with the presence of the following substitions: serine to alanine at position 436 (S436A), serine to phenylalanine at position 436 (S436F), alanine to glycine at position 437 (A437G), alanine to glycine at position 581 (A581G), alanine to threonine or serine at position 613, and lysine to glutamate at position 540 (K540E) (Triglia *et al.,* 1997).

Quintipule mutations (three *dhfr* and two *dhps)* are most significantly associated with therapeutic SP resistance (Kublin *et al.,* 2002; Happi *et al.,* 2005; Schönfeld *et al.,* 2007). It has been suggested that the sequence of mutations occurs in a stepwise fashion, with selection for mutations in *dhfr* gene probably occurring first and the *dhps* gene mutations following later (Sibley *et al.,* 2001).

For the purposes of molecular surveillance of SP resistance, *Pfdhfr* triple mutation has been suggested to be an early molecular marker for SP resistance in Tanzania (Mugittu *et al.,* 2004). However, other workers are of the opinion that the best marker of SP resistance in the field is the presence of quintuple mutations, three mutations in *dhfr* (S108N plus C59R plus N51I) and two mutations in *dhps* (A437G plus K540E) genes (Kublin *et al.,* 2002; Happi *et al.,* 2005).

2.8.2 Aminoquinoline resistance markers

The level of susceptibility of *P. falciparum* strains to quinoline antimalarial drugs such as chloroquine, amodiaquine and mefloquine has been attributed to mutations in *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt*) gene and *P. falciparum* multidrug resistance 1 (*Pfmdr1*) gene. These markers have been used to monitor development of resistance in *P. falciparum* isolates (Duraisingh *et al.,* 2000; Happi *et al.,* 2006).

Chloroquine (CQ) resistant *P. falciparum* appeared in the late 1950s and early 1960s in South America and Southeast Asia (Su *et al.,* 1997). The origin of CQ resistance in Africa has been traced to the spread of resistance from the Thai-Cambodian border region (Hayton and Su, 2004; Mita *et al.,* 2009) but was first reported in Nigeria in 1985 (Ekanem, 1985).

Chloroquine, a 4-aminoquinoline, is a diprotic weak base at low pH and, therefore, accumulates to high levels in the acidic digestive vacuole of erythrocytic stage parasites (Yayon *et al.,* 1985).

CQ and other quinolines are thought to form non-covalent complexes with heme. CQ binding interferes with the heme sequestration process, leading to the buildup of toxic drug-heme complexes that lead to the killing of parasites (Chou *et al.,* 1980; Orjih *et al.,* 1994). Replacement of the positively charged lysine by an uncharged threonine at codon 76 (K76T) of the *Pfcrt* gene, a digestive vacuole membrane transporter protein, removes the electrostatic charge on this protein which then prohibits the protonated and positively charged CQ drug molecule from pore entry (Sanchez *et al*., 2007).

Pfcrt gene is located on chromosome 7 and encodes a 48.6 kDa protein containing 424 amino acids located in the digestive vacuole membrane in erythrocytic stage parasites (Fidock *et al.,* 2000; Cooper *et al.,* 2002). PfCRT belongs to the drug and metabolite transporter superfamily (Martin and Kirk, 2004). There is a general agreement that a lysine to threonine substitution in codon 76 (K76T) of the *pfcrt* gene is necessary but probably not sufficient change for resistance to be acquired (Djimde *et al.,* 2001). Further amino acid differences in *pfcrt* codons 72, 74 and 75 are evidence of this. CQ-resistant parasites from South America carry the *pfcrt* 72-76 haplotype of Ser-Val-Met-Asn-Thr (**SVMNT)**, but those from Africa and many parts of Asia have the haplotype Cys-Val-Ile-Glu-Thr (**CVIET)**. The *pfcrt* 72-76 haplotype of the wild-type (i.e. CQ sensitive) parasites is Cys-Val-Met-Asn-Lys (**CVMNK)** (Mita *et al.,* 2009). The K76T substitution has been found in every *in vitro*-tested CQ-resistant parasite from around the world (Fidock *et al.,* 2000; Wootton *et al.,* 2002; Plowe, 2003; Tatfeng *et al.,* 2008).

The *P. falciparum* multidrug resistance 1 (*Pfmdr1*) gene was identified in 1989 and is located on chromosome 5. It encodes a glycoprotein termed P-glycoprotein homolog 1 (Pgh 1). Important polymorphisms occur at five amino acid positions, 86, 184, 1034, 1042, and 1246 (Foote *et al.,* 1990). Some field studies (Babiker *et al.,* 2001; Nagesha *et al.,* 2001; Mita *et al.,* 2006) and a

transfection experiment (Reed *et al.,* 2000) have shown an association between the mutation at position 86 from asparagine to tyrosine (N86Y) and CQ resistance. Meanwhile, other *in vitro* and *in vivo* chloroquine sensitivity studies have cast doubt on this association (Haruki *et al.,* 1994; Pillai *et al.,* 2001; Thomas *et al.,* 2002; Jalousian *et al.,* 2008). *Pfmdr1* mutations are thought to be compensatory in response to the deleterious effects caused by mutations in *pfcrt* (Adagu and Warhurst, 2001; Laufer *et al.,* 2006; Mita *et al.,* 2006).

Mutations in *pfcrt* as well as copy number and point mutations in the *pfmdr1* gene tend to proportionally affect the parasite sensitivity to mefloquine, halofantrine and artemisinin, while often inversely affecting sensitivity to CQ (Duraisingh *et al.,* 2000; Price *et al.,* 2004; Sisowath *et al.,* 2009). These observations are supported by the localization of Pgh1, the *pfmdr1* gene product, to the digestive vacuole membrane (Cowman *et al.,* 1991). Mutations in *pfmdr1* are thought to be compensatory in support of PfCRT functional changes, although Pgh1 does not appear to be directly associated with producing CQR (Hayton and Su, 2004). Selection for mefloquine resistance has been associated with a decreased resistance to CQ and an amplification of the *pfmdr1* gene copy numbers. In areas subjected to mefloquine pressure, wild-type *pfmdr1* 86N was found more frequently (Reed *et al.,* 2000).

Cross-resistance between CQ and amodiaquine has been reported both *in vitro* (Childs *et al.,* 1989; Basco and Bras, 1993) and *in vivo* (Sowunmi and Salako, 1992; Sowunmi *et al.,* 2001; Schellenberg *et al.,* 2002). *P. falciparum* isolates expressing *pfcrt* 76T allele have been shown to retain sensitivity to amodiaquine while showing a reduced susceptibility to monodesethyl amodiaquine, the active metabolite of amodiaquine (Sidhu *et al.,* 2002). The combination of *pfcrt* 76T and *pfmdr1* 86Y mutations has been associated with amodiaquine treatment failure (Happi *et al.,* 2006). Thus, the simultaneous presence of *Pfcrt* 76T and *pfmdr1* 86Y has been

suggested as a potential marker for amodiaquine resistance (Tinto *et al.,* 2008; Djimde *et al.,* 2008; Tekete *et al.,* 2009).

Association between *pfcrt* and *pfmdr1* mutations and susceptibility to quinine has not been observed (Rungsihirunrat *et al.,* 2009).

2.9 INSECTICIDE-TREATED NETS

The use of insecticide-treated nets (ITN) has been recommended for pregnant women regardless of HIV status (Rabkin *et al.,* 2003; WHO, 2005). Since IPTp does not cover the first trimester and most antimarials are contra-indicated at this period, the use of ITN from the first trimester becomes very necessary. Sleeping under an ITN protects the pregnant woman from malaria (Gamble *et al.,* 2007). Their use should be encouraged for women throughout pregnancy and during the postpartum period.

The use of ITN has been associated with reduction in cases of low birth weight, incidence of malaria parasitaemia and maternal anaemia in pregnant women (ter Kuile *et al.,* 2003; Njagi *et al.,* 2003). In Kenyan children, a single dose of SP combined with regular net use was found to be as effective as multidose SP for the prevention of anaemia; this approach has not yet been evaluated in pregnant women (Desai *et al.,* 2007). Regular insecticide-treated net use, although not yet studied specifically in HIV-infected women, is expected to reduce the burden of malaria during pregnancy even among those who do not adhere to the full course of recommended antenatal visits. Thus, getting pregnant women to use ITN would serve as an important strategy to control MIP.

Other methods which may be used to prevent contact with mosquitoes include the use of mosquito repellents on skin or clothing or sleeping in a room with burning mosquito-repellent coils or tablets.

2.10 CASE MANAGEMENT OF MALARIA ILLNESS AND ANAEMIA

Pregnant women infected with *P. falciparum* must be treated without delay, whether or not they are symptomatic. By clearing the placenta of parasites, prompt treatment reduces the adverse effects of malaria infection on the fetus. The relative efficacy and safety of antimalarials during pregnancy are poorly documented; therefore, most antimalarials (with the exception of quinine, proguanil, chloroquine, and clindamycin) must be avoided during the first trimester unless there is a clear benefit for the mother (Nosten *et al.,* 2007).

As part of routine antenatal care, pregnant women should receive iron and folic acid supplements for anemia as well as screeining for anemia. Those with moderate to severe anemia and who live in areas where the risk of *P falciparum* infections is high, should receive an ACT (even in the absence of parasitaemia) in the last two trimesters and quinine (or another effective antimalarial) in the first trimester (WHO, 2004).

Current recommendations for case management (WHO, 2006) for Uncomplicated falciparum malaria

First trimester

- First episode: quinine 10 mg/kg three times a day for 7 days, preferably with clindamycin 5 mg/kg three times per day for 7 days.
- Subsequent episodes: repeat treatment with quinine and clindamycin (as above); ACT that is locally effective; or artesunate 2 mg/kg per day for 7 days with clindamycin (as above).

Second and third trimesters

- First episode: ACT that is locally effective or artesunate plus clindamycin (as above).
- Subsequent episodes: artesunate plus clindamycin as above; or quinine plus clindamycin as above.

Severe malaria

- Artesunate 2·4 mg/kg intravenously at hour 0, 12, and 24 and continued every 24 h until the patient can tolerate oral artesunate 2 mg/kg per dose, for a total of 7 days, and clindamycin 5 mg/kg three times daily for 7 days.

Or

- Intravenous quinine: loading dose 20 mg/kg given over 4 h, then 10 mg given 8 h after the loading dose was started, followed by 10 mg/kg every 8 h for 7 days. Once the patient has recovered sufficiently to tolerate oral medication both quinine 10 mg/kg and clindamycin 5 mg/kg, three timesdaily should be continued for 7 days.

Non-falciparum malaria

- Chloroquine phosphate (one tablet contains 250 mg salt, equivalent to 155· mg base). Dose is 10 mg/kg base once a day for 2 days followed by 5 mg/kg base on third day.
- For chloroquine-resistant *P vivax*, amodiaquine, quinine, or artemisinin derivatives can be used.

CHAPTER THREE

MATERIALS AND METHODS

3.1 EQUIPMENT AND REAGENTS

- Whatmann No.1 filter paper spotted with blood
- Vacuum pump
- Harris Uni-core hole punch
- 96 well 1ml plate
- Water bath
- Eppendorf Centrifuge 5810R (Eppendorf AG, Germany)
- 96 well PCR plate
- Rotorgene 6® Real Time PCR Machine (Corbett Research, Pty Ltd)
- Tourniquet (Bioscientia, Germany)
- CX21 Olympus Microscope (Olympus Corporation, Tokyo, Japan)
- New Improved Neubauer chamber
- 22 x 22 mm Cover slip (Heinz Herenz, Hamburg, Germany
- 4ml EDTA vacutainer bottles (BD Vacutainer)
- 2ml Syringe with needle (Dana-Ject, Zheijiang Kindly Medical Devices & Plastics Co. Ltd,China
- Haematocrit centrifuge and reader (Hawksley , England)
- Cristaseal (Hawksley, England)
- 1-20µl adjustable micropipette (P20 Pipetman, Gilson, France)
- 1-1000µl adjustable micropipette (P1000 Pipetman, Gilson, France)
- Microscope slide (Marienfeld Laboratory Glassware, Paul Marienfeld GmbH and Co. KG, Germany)
- Latex hand gloves (Neogloves Latex Examination gloves, Neomedic Ltd, UK)

- Oakton pH/mV/^{o}C meter (Eutech Instruments, Singapore)
- Questionnaire (See appendix 1)
- CX21 Olympus Microscope (Olympus Corporation, Tokyo, Japan)
- New Improved Neubauer chamber ()
- 22 x 22 mm Cover slip (Heinz Herenz, Hamburg, Germany).
- 2ml Syringe with needle (Dana-Ject, Zheijiang Kindly Medical Devices & Plastics Co. Ltd,China
- Heparinized Capillary tubes (Hawksley, England)
- Oakton pH/mV/^{o}C meter (Eutech Instruments, Singapore)
- Saponin (Sigma-Aldrich, USA)
- Phosphate buffer
- Chelex
- PCR buffer (Bioline)
- Taq DNA polymerase (Bioline)
- Deoxynucleotide triphosphates (dTTP, dATP, dGTP, CTP) (Bioline).
- TaqMan probes - Joe, Rox and Fam
- Methylated Spirit (LNL Laboratory Reagent)
- DELE Absorbent Cotton wool BP (Ayo-Ayodele Pharmaceutical Chemists Ltd, Kwara, Nigeria
- Immersion oil
- Glacial acetic acid (BDH Chemicals Ltd, Poole, England)
- Crystal violet
- Giemsa Stain (Powder) (Avondale Laboratories, Oxon, England)

- Methanol (BDH Chemicals Ltd, Poole, England)
- Glycerol (BDH Chemicals Ltd, Poole, England)
- Tourniquet (Bioscientia, Germany)
- Methylated Spirit (LNL Laboratory Reagent)
- DELE Absorbent Cotton wool BP (Ayo-Ayodele Pharmaceutical Chemists Ltd, Kwara, Nigeria
- Immersion oil (QCA)
- Potassium dihydrogen phosphate (BDH Chemicals Ltd, Poole, England)
- Dipotassium hydrogen phosphate (BDH Chemicals Ltd, Poole, England)
- Glacial acetic acid (BDH Chemicals Ltd, Poole, England)

3.2 METHODS

3.2.1 Study Design and Sites

The study was conducted in three phases. The first phase which was carried out between May 2007 and February 2008 was a cross-sectional survey of pregnant women to determine baseline information on MIP and factors associated with increased risk of malaria among pregnant women. The second phase, which was also carried out between May 2007 and February 2008, was a longitudinal study to determine the equivalence of the standard two-dose IPTp-SP to experimental monthly dose IPTp-SP and efficacy of SP used in intermittent preventive treatment of MIP. The third phase, which was carried out between May 2009 and December 2009, was a descriptive study to determine the distribution of antimalaria drug resistant haplotypes of *P. falciparum* isolates from pregnant women.

The study sites were located in Lagos State, South-west Nigeria (latitude: 6^{o} 27' 11" longitude: 3^{o} 23' 45"). In the South, Lagos state stretches for 180 km along the coast of the Atlantic Ocean.

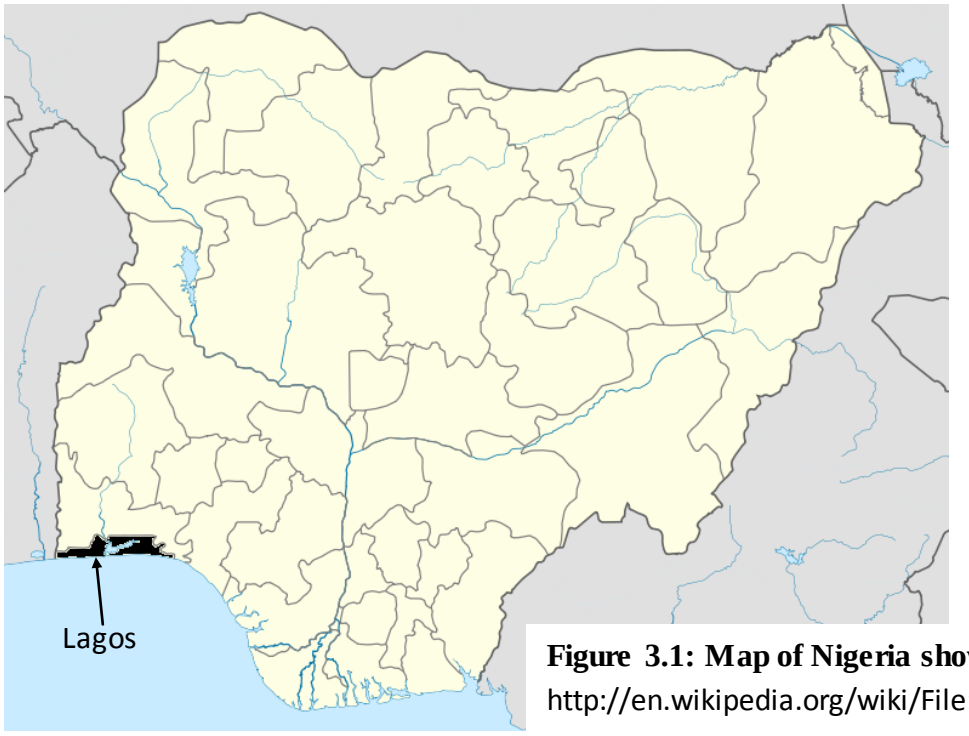


Figure 3.1: Map of Nigeria showing Lagos. Source: http://en.wikipedia.org/wiki/File:Nigeria_location_map.svg

The field study took place simultaneously in two sites in Lagos state: (a) Ajeromi General Hospital, Ajegunle, a secondary public health care facility; and (b) St. Kizito Primary Healthcare Center, Lekki, a not-for-profit primary health care facility. These facilities were selected because of the large number of patients that seek medical care from them. In both facilities, daily attendance at antenatal clinics was greater than 40 pregnant women. Routine antenatal care included the monitoring of body weight, blood pressure, haemoglobin level, urinalysis, fundal height and foetal heart beat. Health education was carried out by midwives on each clinic day. The women received haematinics and weekly pyrimethamine prior to this study. HIV counseling and testing along with screening for sexually transmitted diseases were preconditions for enrollment for antenatal services at the hospitals used for this study.

Malaria microscopy, determination of PCV and total leukocyte count were carried out in the Tropical Diseases Research Laboratory of the Department of Medical Microbiology and Parasitology, College of Medicine of University of Lagos, Nigeria. The molecular studies were carried out in the Immunology Department of the London School of Hygiene and Tropical Medicine, London, United Kingdom.

3.2.2 Ethical Considerations

Approvals to conduct this study were obtained from: (1) Research Grants and Experimentation Ethics Committee, College of Medicine of the University of Lagos, Lagos; (2) Research and Ethics Committee, Lagos University Teaching Hospital, Lagos; and (3) Institutional Review Board of Nigerian Institute of Medical Research, Lagos. Permissions were obtained from the hospitals were the studies were carried out. All the research participants gave informed consent. Patients that declined to participate were not denied access to the available routine care.

Generally, the study was conducted in line with the principles of Good Clinical Laboratory Practice and the 1994 Helsinki Declaration.

3.2.3 Prevalence and Determinants of Malaria in Pregnancy in Lagos

Pregnant women attending study sites ANCs who consented to the study were recruited.

Sample size determination: The total number of pregnant women recruited in the first phase of the study was one thousand and eighty four (1084); minimum study population was three hundred and eighty four (384) as estimated using Statcalc software of Epiinfo 6 (Center for Disease Control, Atlanta) for Population survey or Descriptive study using random sampling. The assumptions were:

- Lagos population size = 9,013,534 (National Census, 2006)
- Population of pregnant women 2006 = 396,595 (4.4% of the Lagos population) (UNFPA http://www.unfpa.org/emergencies/manual/9a5.htm. Accessed 19th September 2006)
- Population of pregnant women in 2007 (Growth of 2.34%) = 405,875
- Expected frequency of MIP = 10%
- Worst acceptable frequency of MIP = 7.0%
- Confidence level = 95%

Demographic data, information on history of fever during current pregnancy, treatment and preventive measures adopted were collected using interviewer-administered semi-structured questionnaire (Appendix 1). Malaria diagnosis by microscopy and total leukocyte count were carried out as stated above.

3.2.4 Collection of Samples

Venous blood samples (~2 ml) were collected from the participants at recruitment and at each monthly antenatal visit from recruitment. At delivery, placental blood samples were collected by

making an incision on the placenta tissue (maternal surface) and blood collected with a 2ml syringe. Two slides of thin and thick films and four spots on Whatmann No.3 filter paper were made of all blood samples collected.

3.2.5 Diagnosis of Malaria By Microscopy

Malaria diagnosis by microscopy was carried out in accordance with WHO protocol (WHO, 1991b) with minor modifications. For thick film, 12µL of blood taken with an adjustable micropipette (P20 Pipetman, Gilson) was spread over a diameter of 15mm, while 2µL of blood taken with an adjustable micropipette (P20 Pipetman, Gilson) was used for thin film. Duplicate slides were made and labeled appropriately. Thin film was fixed with absolute methanol and air dried. MBFs were stained after 24 hours with 3% Giemsa stain (pH 7.2). The stained slides were read by two competent microscopists. Discordant readings were resolved before the final result was taken. The mean parasite counts of the two readers were taken provided the percentage discrepancy of the two readings was less than 20%.

The absolute parasite density per µl of blood was calculated using the formula below.

$$\frac{\text{\# of parasites counted} \times \text{Total leukocytes count}}{\text{\# of leucocytes counted}}$$

3.2.6 Determination of Total Leukocyte Count

The total leukocyte count determination was carried out using then improved Neubauer Chamber as described by Baker and Silverton (1985). 1:20 dilution of whole blood was made with Turk's solution (2% Acetic acid tinged with gentian violet) to lyse the red cells leaving the stained leukocytes. The diluted sample was loaded onto a New Improved Neubauer Chamber and the leukocytes counted. The final leukocyte count was calculated using the formula below.

Leukocyte count/µl of blood = $\frac{\text{no. of leukocytes counted} \times \text{Dilution factor (20)}}{\text{No. of areas counted (4)} \times \text{Depth of counting chamber (0.1mm)}}$

3.2.7 Determination of Packed cell volume

The Packed cell volume was determined by filling a heparinized capillary tube (Hawksley, England) up to 75% with a well mixed anticoagulated blood, sealed at one end with Cristaseal, (Hawksley, England), spun at 13000 rpm for 5 mins in a Hawksley haematocrit centrifuge (Hawksley, England). The percentage of the packed cells was read with a Hawksley Haematocrit Reader (Hawksley, England). The PCV values were classified as follows: Normal values (≥33%); Mild anaemia (30-32.9%); Moderate anaemia (21-29.9%); and Severe anaemia (<21%) (WHO, 1989).

3.2.8 Protective Efficacy Of IPTp-SP And Equivalence Of Two Dose IPTp-SP To Monthly IPTp-SP

There were two major arms of this study, **Arm A** (2 doses of SP) and **Arm B** (monthly dose of SP).

Sample size determination: Population of pregnant women in Lagos is approximately 405,875. Assuming a worst cure rate of 50% for SP at 95% confidence interval, precision of 10% and a dropout rate of 10%, a minimum sample size of 60 was needed for each arm, giving a total of 120 pregnant women. (WHO, 2001).

The **inclusion criteria** were: (a) the pregnancy must be in the second trimester; (b) no history of allergic reaction to sulphonamides; (c) the pregnant woman must be HIV negative (determined by the hospital). The **exclusion criteria** were: (a) positive test for venereal diseases, diabetes, and hypertension (essential or pregnancy-induced determined by the hospital); (b) Severe anaemia (PCV $\leq$ 20%); (c) multiple pregnancy (determined by an ultrasound scan report)

Pregnant women were recruited at booking and were given study numbers and personal identifiers removed to ensure confidentiality. Access to study records was restricted only the principal investigator. The pregnant women were randomly assigned into two arms: **Arm A** - those that received the standard 2 doses of SP; and **Arm B -** those that received monthly dose of SP. A total of 259 pregnant women in their second trimester were recruited into this study: 122 (47.1%) in Arm A (2-dose IPTp-SP) and 137 (52.9%) in Arm B (Monthly IPTp-SP). In Arm A, the study participants were given a treatment dose of sulphadoxine-pyrimethamine (3 tablets each containing 500mg sulphadoxine and 25mg pyrimethamine) in the second trimester and another treatment dose in the third trimester. In Arm B, the participants received a treatment dose of SP monthly. All the participants were clinically monitored at each visit before delivery.

In both arms parasitaemia and other clinical parameters such as blood pressure, body weight, fundal height and foetal heart beat were monitored monthly till delivery. Those that missed appointment days were traced by phone and appointments rescheduled within one week else they were traced to their homes. The administration of SP was by directly observed treatment (DOT) method. Drugs and water were provided at no cost to the study participants. At each monthly visit, the nurses and doctors conducted their routine medical checkups on the pregnant women. The month of recruitment was termed Month 0 (M0). Subsequent follow-up months were termed M1, M2, M3 and M4.

The indicators used in this study were protective efficacy of SP, parasite clearance and delivery outcome. The protective efficacy of SP was defined as the ability of SP to prevent the malaria infection in pregnant women who were aparasitaemic over a period of time. The parasite clearance was assessed by the proportion of women who were parasitaemic who became aparasitaemic after the intake of SP. Pregnancy outcome was assessed based on two criteria: a) if the pregnancy resulted in the birth of a live baby or not; and b) the birth weight of the live baby. Low birth weight was defined as birth weight less than 2.5kg.

3.2.9 Molecular typing of antimalarial resistance markers

Blood spots of fifty four (54) of the samples positive for *P. falciparum* malaria from the prevalence study and the parasitologic monitoring of women on IPTp-SP were used for molecular typing of resistance markers.

3.2.9.1 *Malaria parasite DNA Extraction From dried blood spots*

A section of blood spot was cut directly into a 1ml 96 well plate using a sterile Harris Uni-Core hole punch (~2mm diameter). 1mL 0.5% saponin in 1x phosphate buffered saline (PBS) was added into the well and incubated overnight at 37^{o}C to release haemoglobin, leaving parasite

DNA on the paper. After a brief centrifugation (4000 rpm for 5 mins) in a plate centrifuge (Eppendorf Centrifuge 5810R) the saponin solution and debris are removed using a vacuum pump. Then 1mL 1x PBS was used to wash the paper twice. 150µL 6% chelex suspension was added to the 96 well plate and plate covered with foil and heat sealed. The plate was incubated in a water bath at 100^{o}C for 25 mins to release parasite DNA from paper. The plate was centrifuged in a plate centrifuge for 2 mins to spin down chelex. The DNA supernatant (~100µL) was transferred to a new plate and stored at -20^{o}C, while chelex and filter paper were discarded (Plowe *et al.*, 1995)

3.2.9.2 *Amplification of Pfcrt gene*

A multiplex real-time polymerase chain reaction (qPCR) assay using the Rotorgene 3000 platform (Corbett Research, Australia) was used to genotype the *Pfcrt* gene as described by Sutherland *et al.*, (2007). Briefly, pfcrt DNA was amplified from each sample using primers pfcrt F (TGG TAA ATG TGC TCA TGT GTT T) and pfcrt R (AGT TTC GGA TGT TAC AAA ACT ATA GT) in the presence of each of the three double-labelled probes, TaqMan probes (MWG, Germany), representing the wild-type and the two most common resistance-associated haplotypes at codons 72-76 of pfcrt. The probes were CVMNK (50FAM-TGT GTA ATG AAT AAA ATT TTT GCT AA-BHQ1), CVIET (50JOE-TGT GTA ATT GAA ACA ATT TTT GCT AA-BHQ1), and SVMNT (50ROX-AGT GTA ATG AAT ACA ATT TTT GCT AA-BHQ2). The reaction conditions were 95ºC for 6 mins; (95ºC for 15 secs, 55 ºC for1 min) x45 cycles. Each probe consists of a fluorophore reporter molecule and a quencher dye covalently linked to either the 3´ or 5´ end of a 20 to 30 bp oligonucleotide. The proximity of the fluorescent reporter fluorophore to the quencher prevents the reporter from fluorescing. During the PCR extension step, the 5’ → 3’ exonuclease activity of Taq polymerase cleaves the fluorescent reporter from

the probe. The resultant fluorescent signal from the free reporter is then measured. Their different fluorescent molecules enabled the detection of the haplotypes present in each sample. Samples were considered positive for a particular genotype if a CT value of 35 cycles or fewer was obtained in at least two independent PCR experiments. The sequence-specific positive control DNA samples, 3D7 (CVMNK), Dd2 (CVIET), and 7G8 (SVMNT) obtained from the Malaria Research and Reference Reagent Resource (MR4, Manassas, Vermont, USA) were kindly provided by Dr Colin Sutherland of the Immunology Unit, London School of Tropical Medicine and Hygiene. Nuclease-free water was used as a negative control.

3.2.9.3 *Amplification of Pfmdr1 and dhfr gene fragments*

Amplification of the pfmdr1 gene was performed in three fragments (FR 1, FR 2 and FR 3). The first fragment contained codons 86 and 184; second fragment contained codons 1034 and 1042; and third fragment contained codon 1246. The reaction primers and conditions for FR1 were as described by Humphrey *et al.* (2007), while FR2 and FR3 were as described by Humphreys *et al.*, (2007). The following primers and cycling conditions were used. **FR 1** : Primary reaction primers F: AGGTTGAAAAAGAGTTGAAC and R: ATGACACCACAAACATAAAT; reaction conditions were 94ºC for 3 min/[94ºC for 30 s, 45ºC for 1min 72ºC for 1 min] x 30 cycles/ 72^{o}C for 5 min. The Nested reaction primers were F: ACAAAAAGAGTACCGCTGAAT and R: AAACGCAAGTAATACATAAAGTC; reaction condition were the same as primary PCR. **FR 2** primers for the primary reaction were F: GCATTTTATAATATGCATACTG and R: GGATTTCATAAAGTCATCAAC; reaction conditions were 94^{o}C for 3mins/ [94^{o}C for 30s, 55^{o}C for 1 min/ 65^{o}C for 40s] x 30 cycles/ 65^{o}C for 5mins/ 15^{o}C for 5mins. The nested reaction primers were F: GGTTTAGAAGATTATTTCTGTAA and R: GGATTTCATAAAGTCATCAAC; reaction conditions were the same as the primary reaction. **FR3** primary reaction primers were F: CAAACCAATCTGGATCTGCAGAAG and R:

CAATGTTGCATCTTCTCTTCC; the reaction conditions were 94^{o}C for 3mins/ [94^{o}C for 30s, 56^{o}C for 1min, 65^{o}C for 50s]x30cycles/ 65^{o}C for 5mins/ 15^{o}C for 5mins. The nested reaction primers were F: GATCTGCAGAAGATTATACTG and R: CAATGTTGCATCTTCTCTTCC; the reaction conditions were the same as primary reaction.

The primers used for sequencing of the three *Pfmdr1* gene fragments were: FR 1 (F: ACAAAAAGAGTACCGCTGAAT and R: AAACGCAAGTAATACATAAAGTC), FR 2 (F: GGTTTAGAAGATTATTTCTGTAA and R: GGATTTCATAAAGTCATCAAC) and FR 3 (F: GATCTGCAGAAGATTATACTG and R: CAATGTTGCATCTTCTCTTCC). The sequencing reaction conditions were 96°C 1 min [96°C 30 s-50°C-60°C 4 min] × 26 cycles

Amplification of *dhfr* gene involved primers and cycling conditions described by other workers (Sutherland *et al.*, 2009; Pearce *et al.*,2003). In brief, parasite DNA extracts from blood spots were amplified in a polymerase chain reaction. Primary reaction (650 bp) primers were F_dhfr_M1 TTT ATG ATG GAA CAA GTC TGC and R_dhfr_M7 CTA GTA TAT ACA TCG CTA ACA. The reaction conditions were 93°C for 5 min, [94°C for 30s, 54°C for 60s, 65°C for 60s] x 41cycles, 65°C for 5min, 15°C for 5min. Nested reaction (594 bp) primers were primers were F_dhfr_M3 TGA TGG AAC AAG TCT GCG ACG TT and R_dhfr_M9 CTG GAA AAA ATA CAT CAC ATT CAT ATG. The reaction conditions were 95^{0}C for 5min, [93^{o}C for 30s, 56^{o}C for 30s, 68^{0}C for 75s] x 30 cycles, 75^{o}C for 5min, 4^{o}C hold

The PCR products of nested reactions were separated by gel electrophoresis on a 1.2% agarose gel stained with ethidium bromide to identify amplified bands of DNA under ultra-violet illumination. Amplicons from nested PCR reactions were purified using the QIAquick PCR Purification Kit (QIAGEN, UK) according to manufacturer's instructions and subjected to di-deoxy fluorescent sequencing (BigDye 3.1, Applied Biosystems, UK) using conditions and

sequencing primer pairs described elsewhere (Pearce *et al.,* 2003) or as shown in Table 3. The sequence of amplified DNA products was determined using ABI PRISM 3730 Genetic Analyser (Applied Biosystems, UK). Chromas Lite® (Technelysium, Australia) was used to analyse the sequence results. The DNA base sequence was converted to peptide sequences using a web-based six frame nucleotide to peptide sequence conversion tool, Transeq® (http://www.ebi.ac.uk/Tools/emboss/transeq/ index.html). Detection of single nucleotide polymorphism (SNP) was carried out by aligning the sample protein sequences with reference protein sequence of the pfmdr1 and pfdhfr of standard 3D7 (wild) strain of *P. falciparum* using the online multiple alignment tool, ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

3.2.10 Data Analysis

The data generated from the study were analyzed using EPIINFO^{TM} Version 3.5.1 statistical software (CDC USA, 2008). Tests for associations and differences were carried out by chi-square analysis, Fischer Exact test and analysis of variance where appropriate. Test of statistical significance was set at P value less than 0.05 at 95% confidence interval.

CHAPTER FOUR

RESULTS

4.1 PREVALENCE AND DETERMINANTS OF MALARIA IN PREGNANCY IN LAGOS

A total of 1084 pregnant women participated in the study: 583 in St. Kizito Primary Health Center Lekki and 501 in Ajeromi General Hospital Ajegunle. Their demographic characteristics are summarized in Table 4.1.1. The women had a mean age of 27.4±4.8 years and majority of them 567 (52.3%) were in their second trimester. Most of the pregnant women, 694(64.1%) had secondary education. The major occupation of the study participants was trading, 359 (33.1%) followed by full-time housewives 317 (29.2%) and artisans 189 (17.4%). Only a few of them were highly skilled professionals 24(2.2%). The women that possessed any type of bed net were 325 (30.0%); of which 230(70.8%) were insecticide-treated nets (Table 4.1.1).

There was a direct correlation (r = 0.54) between age and gravidity showing lower gravidity for younger women. A high proportion 33(84.6%) of the 39 women in age group 15-19 years were primigravidae; while 71(84.5%) of the 84 women in age group >34 years were multigravidae (Table 4.1.2).

The *Plasmodium* species found in the infected women were mostly single species infection of *Plasmodium falciparum* alone 76 (91.6%) and *P. malariae* 4(4.8%). The others were mixed infection of *P. falciparum and P. malariae* 3(3.6%) (Figure 4.1.1).

The prevalence of malaria was 7.7% (n=83) while the prevalence of anaemia in pregnancy was 53.2% (n=577). Malaria infection was significantly associated with anaemia status (P<0.001). The mean PCV value was significantly lower (P <0.001) in pregnant women who were parasitaemic, 30.3±5.3% than those who were aparasitaemic (32.2±4.0%). The proportion of pregnant women who had malaria infection was lowest, 25(4.9%) in the 507 women with normal PCV values (≥33.0%) and highest 3(42.9%) in the 7 women with severe anaemia (PCV<21%).

However, there was no correlation (r=0.01) between PCV and parasite density in this study population (Table 4.1.3).

The total mean malaria parasitaemia was 5,419.5±17,522.2 parasites/µl. Malaria prevalence was significantly higher in age group 15-19 years (20.5%) (χ^2=13.36, df=4, P=0.010); while the age group >34 years had the least mean parasitaemia. There was an association (P<0.001) between the mean PCV values and age of the women. The lowest mean PCV of 28.9±5.0% was seen in the youngest age group 15-19 years. Although there seemed to be an inverse relationship between the age of the women and the proportion of anaemic women (PCV<33.0%), the relationship was not statisitically significant (P=0.080) (Table 4.1.4).

Malaria prevalence was not significantly associated with either the gestation age (trimester) at the time of booking (χ^2 = 1.10, df = 2 P = 0.577) or educational status (χ^2 = 3.08, df = 2, P= 0.215), However anaemia was significantly associated with both gestation age (χ^2= 17.49, df=2, P<0.001) and educational status (χ^2=14.05, df=2, P=0.001) of the pregnant women. Second trimester pregnant women had the highest proportion of anaemia, 335(59.1%) of the 567 women, while those with primary education had the highest proportion of anaemia, 72 (69.9%) of the 104 women (Table 4.1.5).

The difference in mean parasitaemia and proportion of women with malaria infection was not significantly different (P>0.05) when compared with gravidity. However, malaria infection rate was highest in primigravidae (9.2%), while the mean parasitaemia was highest in the secundigravidae 8121.6 parasites/µl of blood (Figure 4.1.2).

The mean PCV values increased with gravidity but was not significant (P=0.382). Severe anaemia was observed in 0.4%, 0.7% and 0.8% in secundigravidae, primigravidae and

multigravidae respectively. There was no significant association between anaemia status and gravidity (P=0.106) (Table 4.1.6).

Table 4.1.7 shows the malaria preventive methods adopted by the pregnant women in this study and the proportion with malaria. The use of insecticide sprays 885 (81.6%) was the most common malaria preventive method adopted by these women. Although 7.2% of the women that used ITN alone were infected with malaria parasites, combination with chemoprophylaxis (4.5%) or insecticide sprays (1.3%) resulted in a much lower proportion with malaria. However, only the combination of insecticide spray and ITN use showed a statistically significant reduction of parasitaemia (RR=0.15, 95% C.I. 0.02-1.09; P = 0.025). None of the women that used a combination of the three methods had malaria parasitaemia. The combined use of insecticide sprays and chemoprophylaxis did not significantly reduce the proportion of women with malaria parasitaemia (RR = 0.95, 95% CI 0.55-1.66; P=0.869).

Table 4.1.1: Demographic Characteristics Of The Pregnant Women Who Participated In The Study

Character		n(%)
Age group (years)	15-19	39(3.6%)
	20-24	266(24.5%)
	25-29	434(40.0%)
	30-34	261(24.1%)
	>34	84(7.7%)
Gravidity:	Primigravidae	415 (38.3%)
	Secundigravidae	270 (24.9%)
	Multigravidae	399 (36.8%)
Trimester:	1st	235 (21.7%)
	2nd	567 (52.3%)
	3rd	274 (26.0%)
Occupation:	Artisan	189 (17.4%)
	Trader	359 (33.1%)
	Civil servant	50 (4.6%)
	Housewife	317 (29.2%)
	Professional	24 (2.2%)
	Student	91 (8.4%)
	Teacher	54 (5.0%)
Education:	Primary	103 (9.5%)
	Secondary	694 (64.1%)
	Tertiary	286 (26.4%)
Bed nets:	Any net (possession)	325 (30.0%)
	Any net (use)	161 (14.9%)
	ITN (possession)	230 (21.2%)
	ITN (use)	125(11.5%)

Table 4.1.2: Age Distribution Of The Study Participants By Gravidity

AGE GROUP (YEARS)	GRAVIDITY			TOTAL
	Primigravidae	Secundigravidae	Multigravidae	
15-19	33 (84.6%)	3 (7.7%)	3 (7.7%)	**39**
20-24	155 (58.3%)	64 (24.1%)	47 (17.7%)	**266**
25-29	159 (36.6%)	147 (33.9%)	128 (29.5%)	**434**
30-34	64 (24.5%)	47 (18.0%)	150 (57.5%)	**261**
>34	4 (4.8%)	9 (10.7%)	71 (84.5%)	**84**
TOTAL	**415 (38.3%)**	**270 (24.9%)**	**399 (36.8%)**	**1084**

$\chi 2 = 242$, df = 8 P < 0.001

Correlation coefficient (r) (Age and Gravidity) = 0.54

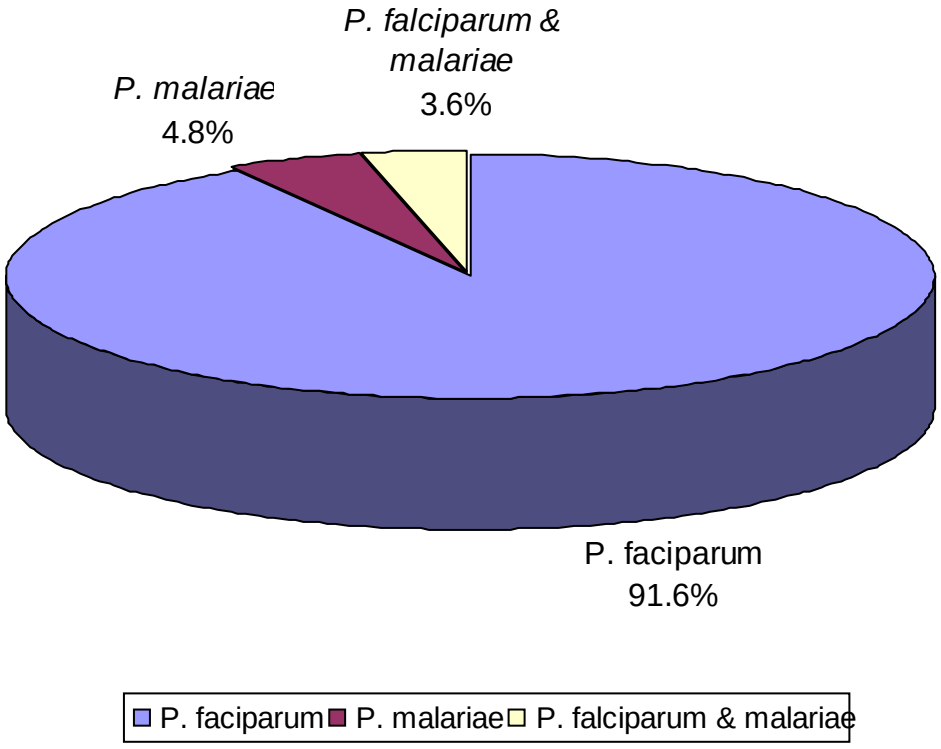


Figure 4.1.1: Prevalence of *Plasmodium*

Table 4.1.3: Relationship between Packed Cell Volume and malaria Infection in pregnant women

Malaria parasitaemia	n	Mean PCV±SD	PCV group			
			Normal	Anaemia		
				Mild	Moderate	Severe
Positive	83(7.7%)	30.3 ± 5.3	25 (4.9)	17(5.8)	38(13.7)	3(42.9)
Negative	1001(92.3%)	32.2 ± 4.0	482 (95.1%)	275(94.2)	240(86.3)	4(57.1)
Total	**1084**	**32.0±4.2**	**507**	**292**	**278**	**7**
Statistics		$\chi 2 = 14.0$; df=1 P < 0.001	$\chi^2 = 33.20$ Df = 3, P <0.001			

Correlation coefficient (r) = 0.02, P=0.225 (Parasite density vs PCV)

Table 4.1.4: Comparison of Parasitaemia and Packed Cell Volume With Age of The Pregnant Women.

Age Group	Malaria Parasitaemia		Packed Cell Volume		
(Years)	No. Positive	Mean Parasitaemia	Mean PCV	Normal (≥33%)	Anaemia (<33.0%)
15-19	8(20.5%)	6,028.7 ± 7142.2	28.9±5.0	12 (30.8)	27(69.2)
20-24	26(9.8%)	4,176.0 ± 11,249.6	31.7±4.0	112(42.1)	154(57.9)
25-29	29(6.7%)	7,741.6 ± 25,056.4	32.2±4.1	211(48.6)	223(51.4)
30-34	14(5.4%)	4,702.2 ± 15,528.4	32.2±4.0	129(49.4)	132(50.6)
>34	6 (7.1%)	101.5 ± 38.9	32.9±4.3	43(51.2)	41(48.8)
Total	**83(7.7%)**	**5,419.5±17,522.2**	**32.0±4.2**	**507(46.8)**	**577(53.2)**
Statistics	χ^2=13.36	χ^2= 8.51	F=7.34	χ^2= 8.33	
DF	**4**	**4**	4, 1079	4	
P	0.010	0.075	< 0.001	0.080	

Table 4.1.5: Comparison Of Malaria Infection and Anaemia With Gestation Age (Trimester) And Educational Status Of The Study Group

Character	Malaria infection		PCV status	
	Positive	Negative	Normal	Anaemic
Trimester				
First (n=235)	16(6.8%)	219(93.2%)	131 (55.7%)	104 (44.3)
Second(n=567)	48 (8.5%)	519(91.5%)	232 (40.9%)	335(59.1%)
Third (n=282)	19 (6.7%)	263(93.3%)	144 (51.1%)	138 (48.9)
	χ^2=1.10, df=2, P=0.577		χ^2= 17.49, df=2, P<0.001	
Education				
Primary (n=104)	12(11.7%)	91 (88.3%)	31(30.1%)	72(69.9%)
Secondary (n=695)	53 (7.6%)	642 (92.4%)	329(47.3%)	366(52.7%)
Tertiary (n=286)	18 (6.3%)	268(93.7%)	147(51.4%)	139(48.6%)
	χ^2=3.08, df=2, P=0.217		χ^2=14.05, df=2, P=0.001	

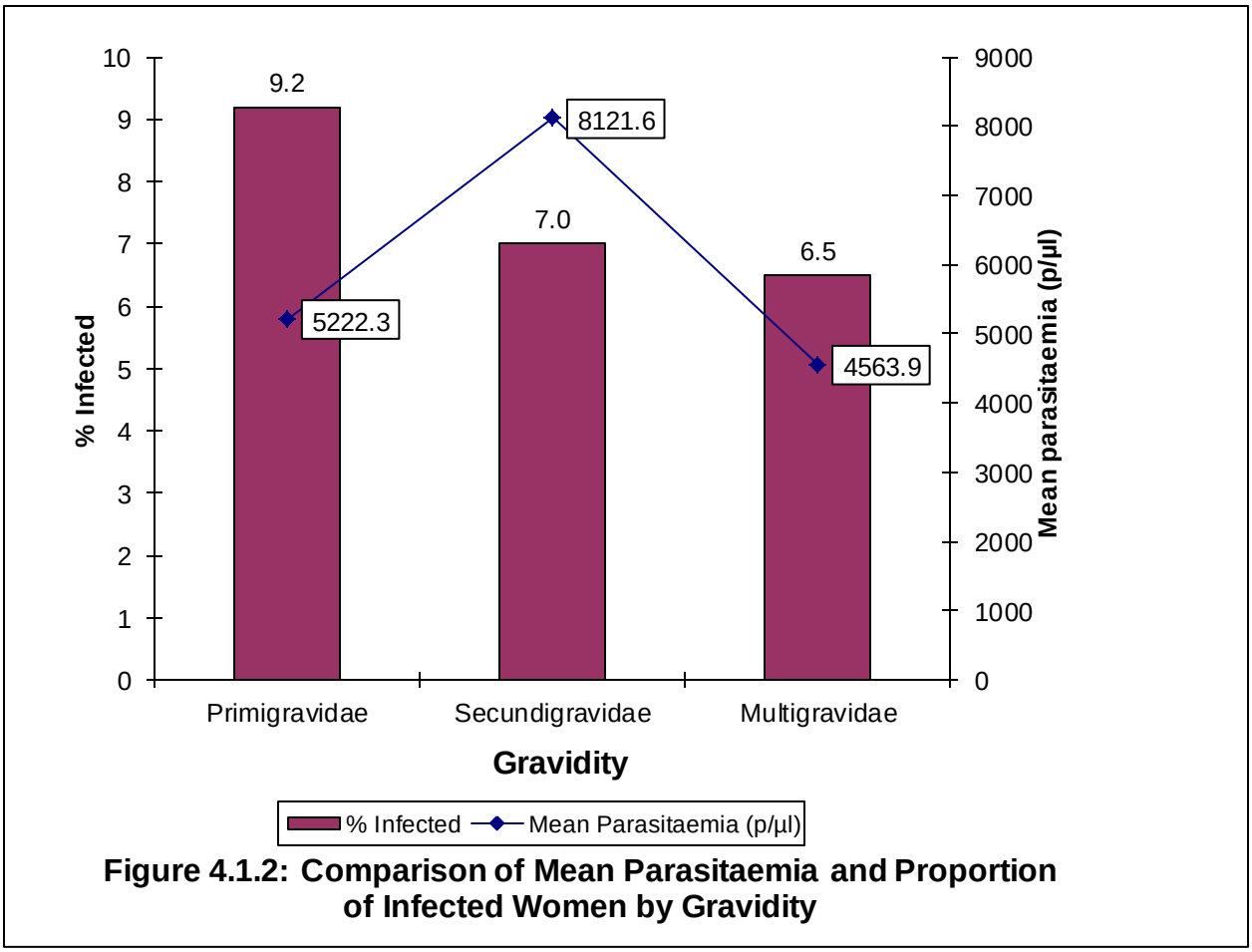


Figure 4.1.2: Comparison of Mean Parasitaemia and Proportion of Infected Women by Gravidity

χ^2 = 2.20, df = 2, P= 0.333 (For proportion with malaria infection)

Kruskal Wallis Test = 2.120, df = 2, P = 0.3464 (For Mean Parasitaemia)

Table 4.1.6: Association between Packed Cell volume and gravidity of the pregnant women at booking for antenatal clinic care.

Gravidity	n	Mean PCV ± SD	PCV group			
			Normal	Mild	Moderate	Severe
Primigravidae	415	31.8 ± 4.3	188(45.3)	106(25.5)	118(28.4)	3(0.7)
Secundigravidae	270	32.0 ± 4.0	127(47.0)	64(23.7)	78(28.9)	1(0.4)
Multigravidae	399	32.2 ± 4.1	192(48.1)	122(30.6)	82(20.6)	3(0.8)
Total	**1084**	**32.0±4.2**	**507(46.8)**	**292(26.9)**	**278(25.6)**	**7(0.6)**
Statistics		F = 0.964 df = 2; 1081 P = 0.382	χ^2 = 10.49; df = 6; P = 0.106			

Table 4.1.7: Relative Risk of Malaria Parasitaemia In Relation To Malaria Prevention Methods Adopted By The Pregnant Women

Preventive Methods	n (% of total)	Proportion with malaria	Risk ratio (95% C.I.)	P value
a) Insecticide sprays	885 (81.6%)	51 (5.8%)	2.79 (1.84-4.22)	<0.001*
b) Chemoprophylaxis	243 (22.4%)	21 (8.6%)	0.85(0.53-1.37)	0.512
c) Bed net Use				
Any net	161 (14.9%)	9 (5.6%)	1.43 (0.73-2.81)	0.285
ITN	125 (11.5%)	9 (7.2%)	1.07 (0.55-2.09)	0.838
d) Insecticide sprays + ITN use	80 (7.4%)	1 (1.3%)	6.53 (0.92-46.33)	0.025*
	22 (2.0%)	1 (4.5%)		0.487
e) Chemoprophylaxis + ITN use	190 (17.5%)	14 (7.4%)	1.70 (0.25-11.66)	0.989
f) Insecticide sprays + chemoprophylaxis	11 (1.0%)	0 (0%)	1.05 (0.60-1.82)	
g) Insecticide sprays + Chemoprophylaxis + ITN use			0	

* Statistically significant (P<0.05).

4.2 PROTECTIVE EFFICACY OF IPTp-SP AND EQUIVALENCE OF TWO DOSE IPTp-SP TO MONTHLY IPTp-SP

The study participants in the two arms of the study had similar baseline characteristics (P>0.05). The mean age of the women in Arms A and B were 26.5 ±4.2years and 27.9 ±4.9 years respectively.

In Arm A, the mean PCV was 32.3±3.7% with 29 (23.8%) being anaemic (i.e. PCV <33%); while in Arm B the mean PCV was 32.4±3.7% with 29 (21.2%) of the women being anaemic (Table 4.2.1).

In Arm A, all the 122 (100%) women received 2 doses of SP. In Arm B, 85 (62.0%) received 4 SP doses; and 52 (38.0%) got 5 doses of SP depending on their gestation age at recruitment. The number of SP doses received by the women were similar across the age groups (P = 0.361) and gravidity (P = 0.756) in Arm B (Table 4.2.2).

At enrolment (M0), 5(4.1%) and 3(2.2%) of the study participarnts had parasitaemia in Arms A and B respectively. The mean parasitaemia reduced by M1 in both Arms and cleared by M2 in Arm B. In Arm A, 1 (0.8%) of the women had parasitaemia (3365 parasites/µl) at the M2. The rate of clearance of parasitaemia, measured by both the reduction in mean parasitaemia and proportion of infected persons by M1 was similar (P>0.05) in both arms of the study (Table 4.2.3).

Of the 251 aparasitaemic pregnant women recruited at Month 0, 247 (98.4%) remained aparasitaemic by M1. The protective efficacy of SP in Arm A was 98.3% and Arm B was 98.5% at M1 (P=0.636). Similar results was obtained at M2 (P = 0.466). However, none of the

women in the monthly IPTp-SP (Arm B) developed parasitaemia after M1; while a woman became parasitaemic at Month 2 in the 2-Dose IPTp-SP group (Arm A). (Figure 4.2.1).

Of the 3 women that had parasitaemia at M0 in Arm B parasite clearance was 100% by M1; whereas in Arm A, of the 5 women with parasitaemia at M0, parasite clearance was 80% (4 out of 5) (Figure 4.2.2).

A total of 12 placental blood samples were collected from women in Arm B. None (0.0%) of the samples had malaria parasitaemia.

The result of pregnancy outcome was obtained from 156 of the 259 women studied. The birth outcome was assessed based on the proportion of live birth and weight of the babies. There was a total of 149 (95.5%) live births and 7 (4.5%) deaths. Of the dead babies, 4 were from women in Arm A and 3 from women in Arm B. There was no statistical association (P=0.178) between the proportion of deaths and the IPTp schedule. The mean weight of the babies with LBW was 1.9±0.1 kg and 2.0±0.4 kg (range 1.4 - 2.3 kg) in Arms A and B respectively; while the babies with normal birth weights had a mean weight of 3.3±0.4 kg and 3.3±0.4 kg in Arms A and B respectively. The proportion of LBW was similar in Arm A, 2 (4.1%) and Arm B 5 (5.0%) (P=0.581) (Table 4.2.4).

The women whose babies died were between 20 and 29 years of age. In Arm A (2-Dose IPTp-SP), 3 (12.5%) of the women in age group 25-29 years lost their babies, while 1 (7.7%) of those in age group 20-24 years lost her baby. In Arm B (monthly IPTp-SP), 2 (8.3%) of the women in age group 20-24 years had dead babies; while 1 (3.0%) of those in age group 25-59 years had a dead baby. Overall, the association between age and the study group of the women whose babies died was not statistically significant (P=0.270) (Table 4.2.5).

The IPTp schedule was not significantly associated (P = 0.907) with gravidity of the women whose babies died. In Arm A, 2(9.5%) of the women whose babies died were primigravidae, while 1 (6.7%) secundigravid and 1 (5.9%) multigravid lost their babies. One woman in each of the three gravidity groups lost a baby each in Arm B (Table 4.2.6).

The age of the women who had LBW babies was not significantly associated with study arm (P=0.208). In Arm A, 2 (4.1%) women had LBW babies: 1 (8.3%) in 20-24 years age group, and 1 (4.8%) in 25-29 years age group. In Arm B, 1 (3.1%) of the 5 (5.0%) of the women who had LBW babies was in 25-29 years age group, 2 (6.1%) in age group 30-34 years and 2 (18.2%) were >34 years old (Table 4.2.7). The gravidity of the women who had LBW babies did not differ significantly in the study groups (P=0.472) (Table 4.2.8).

Table 4.2.1: Baseline Characteristics of The Study Participants

	Arm A	**Arm B**
Number	122	137
Mean Age (years)	26.5 ±4.2	27.9 ±4.9
Gravidity		
Primigravidae	47 (38.6%)	56 (40.9%)
Secundigravidae	33 (27.0%)	35 (25.5%)
Multigravidae	42 (34.4%)	46 (33.6%)
Mean PCV(%)	32.3±3.7%	32.4±3.7%
PCV <33%	29 (23.8%)	29 (21.2%)
PCV ≥33%	93 (76.2%)	108 (78.8%)

Table 4.2.2: Number Of Doses Of IPTp-SP Received In Relation To Age And Gravidity

Character	Arm A	Arm B			Statistics
	2 doses	4 doses	5 doses	Total	(Arm B)
Age (years)					
15-19	4 (100)	3 (100)	0 (0.0)	3	χ^2 =4.35;
20-24	35 (100)	16 (50.0)	16 (50.0)	32	df = 4;
25-29	54 (100)	32 (66.7)	16 (33.3)	48	P = 0.361
30-34	26 (100)	26 (61.9)	16 (38.1)	42	
>34	3 (100)	8 (66.7)	4 (33.3)	12	
Gravidity					
Primigravidae	47 (100)	35 (62.5)	21 (37.5)	56	χ^2=0.559;
Secundigravidae	33 (100)	20 (57.1)	15 (42.9)	35	df=2;
Multigravidae	42 (100)	30 (65.2)	16 (34.8)	46	P=0.756
TOTAL	122 (100)	85 (62.0)	52 (38.0)	137	

Table 4.2.3: Comparison Of The Monthly Malaria Parasitaemia In The Two Study Arms.

Month	Arm A			Arm B			P
	n	Mean Parasitaemia	Range	n	Mean Parasitaemia	Range	
0	5	777.4	111 - 2,063	3	691.7	127 - 1,664	0.881
1	3	298.3	149-390	2	110.5	40 - 181	0.248
2	1	3,365		0	0		
3	0	0		0	0		
4	0	0		0	0		

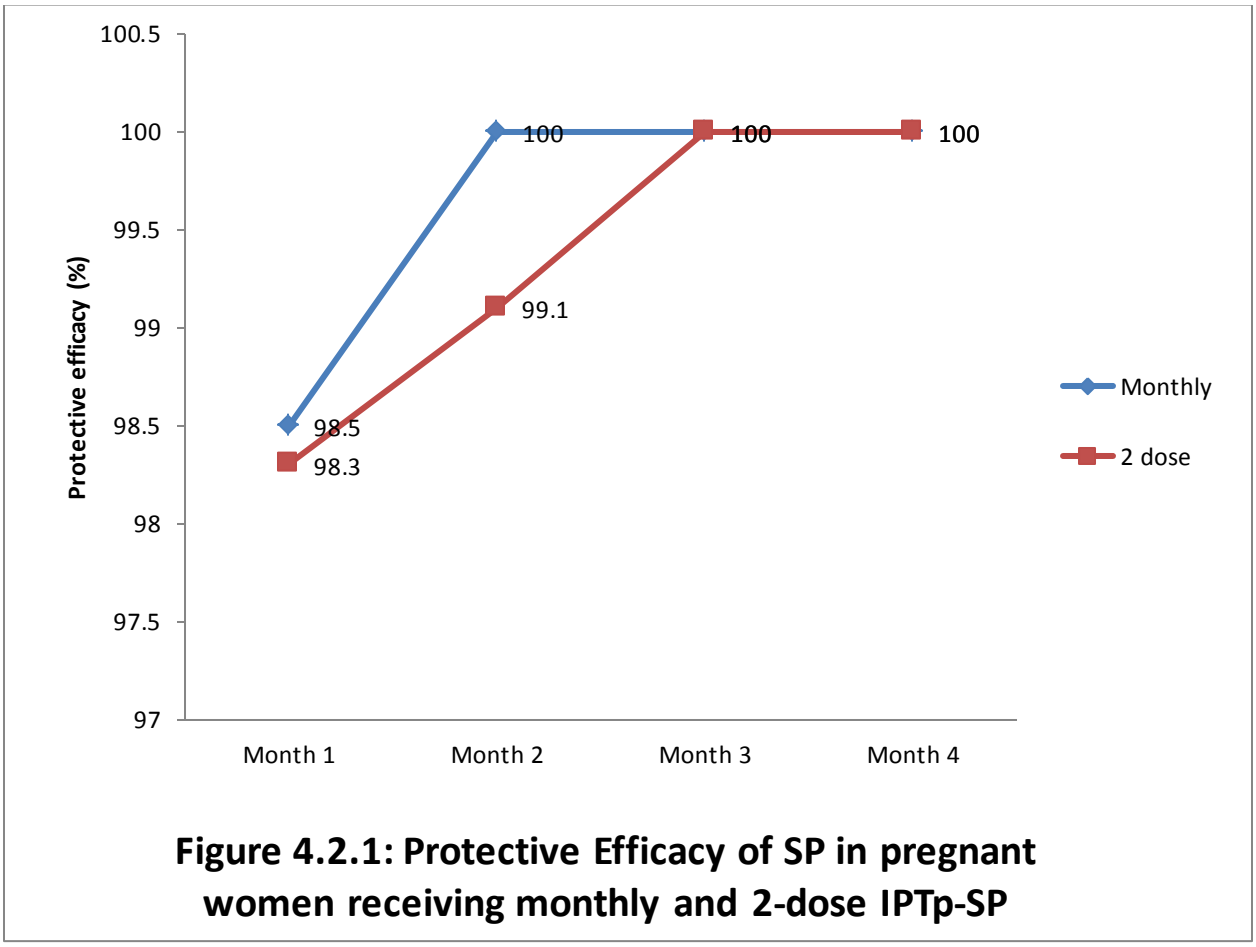


Figure 4.2.1: Protective Efficacy of SP in pregnant women receiving monthly and 2-dose IPTp-SP

Month 1: Fisher Exact P=0.636

Month 2: Fisher Exact P = 0.466

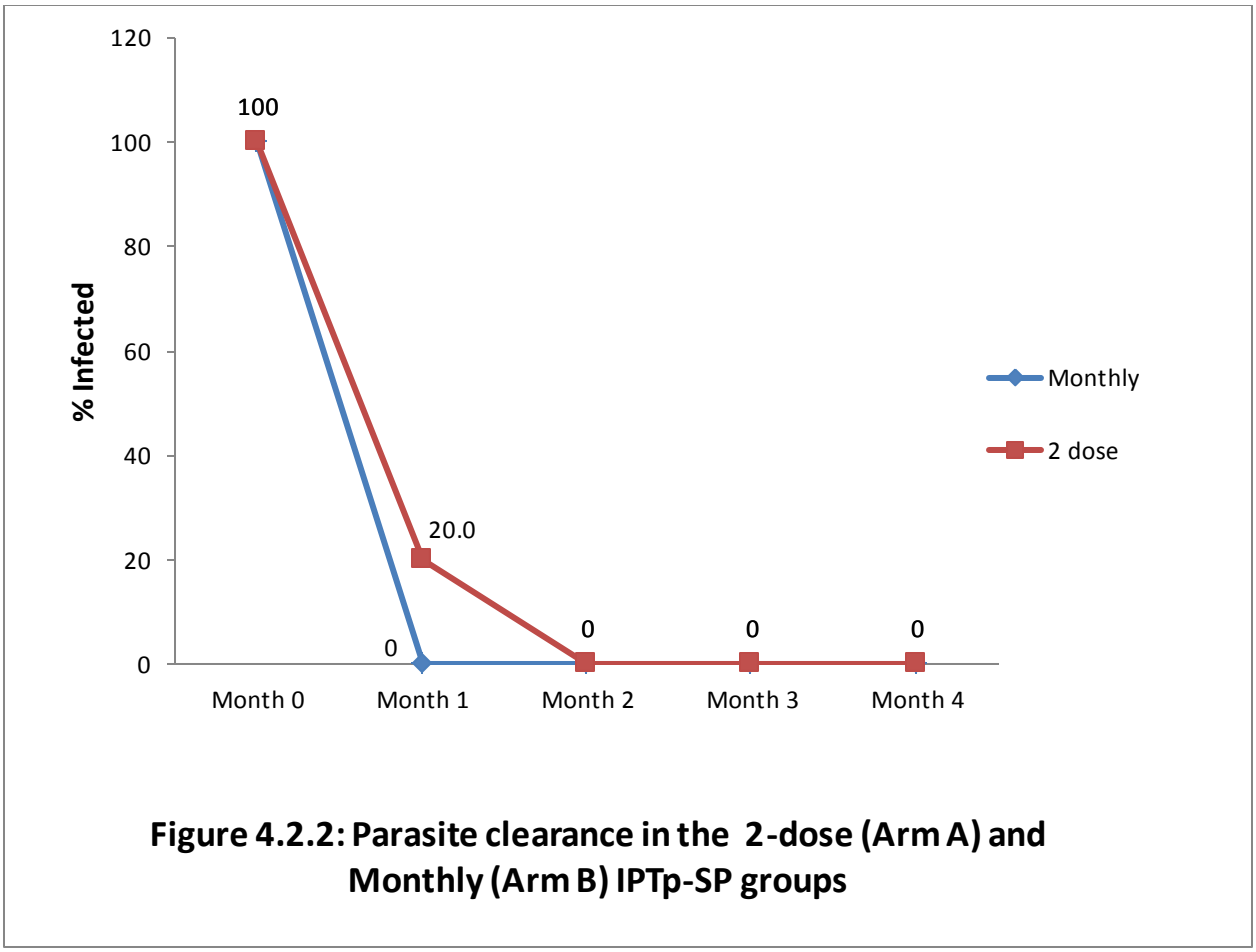


Figure 4.2.2: Parasite clearance in the 2-dose (Arm A) and Monthly (Arm B) IPTp-SP groups

Fisher Exact P = 0.625

Table 4.2.4: Pregnancy Outcome of Women In The Two Study Arms

Pregnancy Outcome	STUDY ARMS		TOTAL	P
	Arm A	Arm B		
Live Birth	49 (92.5)	100 (97.1)	149 (95.5)	0.178*
Dead	4 (7.5)	3 (2.9)	7 (4.5)	
Stillbirth	3	3	6	
Miscarriage	1	0	1	
Baby weight				
Low (<2.5 kg)	2 (4.1)	5 (5.0%)	7 (4.7)	0.581*
Normal (≥2.5kg)	45 (95.9)	95 (95.0)	142 (95.3)	

* Fisher Exact Test

Table 4.2.5: Comparison of Live Births With Age of the Study Participants

	DEAD		ALIVE		
Age (years)	**Arm A**	**Arm B**	**Arm A**	**Arm B**	**TOTAL**
15-19	0 (0.0)	0 (0.0)	2 (100.0)	2 (100.0)	4
20-24	1 (7.7)	2 (8.3)	12 (92.3)	22 (91.7)	37
25-29	3 (12.5)	1 (3.0)	21 (87.5)	32 (97.0)	57
30-34	0 (0.0)	0 (0.0)	13 (100.0)	33 (100.0)	46
>34	0 (0.0)	0 (0.0)	1 (100.0)	11 (100.0)	12
Statistics	χ^2=1.22; P=0.270		χ^2=5.43; df=4; P=0.246		

Table 4.2.6: Comparison of Live Births with Gravidity of the Study Participants

Gravidity	DEAD		ALIVE		TOTAL
	Arm A	Arm B	Arm A	Arm B	
Primigravidae	2 (9.5)	1 (2.4)	19 (90.5)	40 (97.6)	62
Secundigravidae	1 (6.7)	1 (3.6)	14 (93.3)	27 (96.4)	43
Multigravidae	1 (5.9)	1 (2.9)	16 (94.1)	33 (97.1)	51
Statistics	χ^2=0.194; df=2;P=0.907		χ^2=0.043; df=2;P=0.979		
Total	4 (7.5)	3 (2.9)	49 (92.5)	100 (97.1)	156

Table 4.2.7: Comparison of Birthweight with Age of Women in the Two Study Groups

	Low Birth Weight		**Normal Birth Weight**	
Age (years)	Arm A	Arm B	Arm A	Arm B
15-19	0 (0.0)	0 (0.0)	2 (100)	2 (100)
20-24	1 (8.3)	0 (0.0)	11 (91.7)	22 (100.0)
25-29	1 (4.8)	1 (3.1)	20 (95.2)	31 (96.9)
30-34	0 (0.0)	2 (6.1)	13 (100.0)	31 (93.9)
>34	0 (0.0)	2 (18.2)	1 (100.0)	9 (81.8)
Total	**2 (4.1)**	**5 (5.0)**	**47 (95.9)**	**95 (95.0)**
P	χ^2=4.55; df=3; P =0.208		χ^2=4.039; df=4; 0.401	

Table 4.2.8: Comparison of Birthweight With Gravidity Of Women In The Two Study Groups

Gravidity	Low Birth Weight		Normal Birth Weight	
	Arm A	**Arm B**	**Arm A**	**Arm B**
Primigravidae	1 (5.3)	2 (5.0)	18 (94.7)	38 (95.0)
Secundigravidae	1 (7.1)	1 (3.7)	13 (92.9)	26 (96.3)
Multigravidae	0 (0.0)	2 (6.1)	16 (100)	31 (93.9)
Total	**2 (4.1)**	**5 (5.0)**	**47 (95.9)**	**95 (95.0)**
Statistics	χ^2=1.28; df=2; P=0.526		χ^2=0.043; df=2; P=0.979	

4.3 MOLECULAR TYPING OF ANTIMALARIAL RESISTANCE MARKERS

A subset of 54 blood spots of samples slide positive for *P falciparum* obtained from pregnant women in Lagos were analyzed for the haplotypes of *Pfcrt* and *pfmdr1* genes of *P. falciparum*. Figures 4.3.1 and 4.3.2 show the exponential amplification of *CVIET* and CVMMK haplotypes respectively.

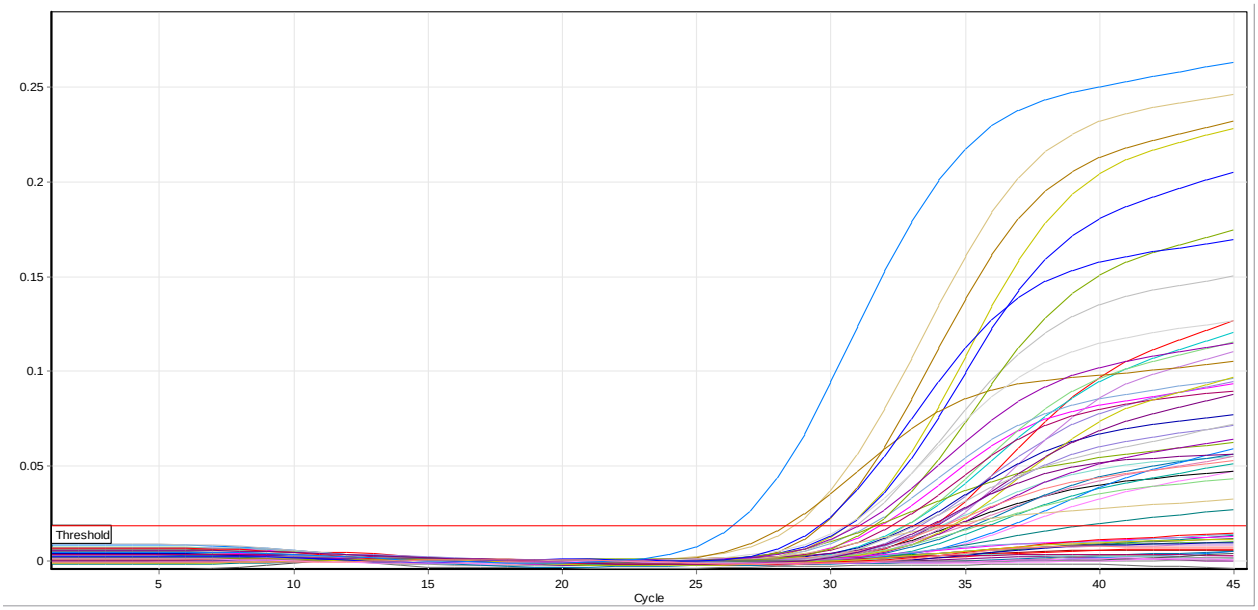


Figure 4.3.1: Graph of the exponential amplification of *CVIET* haplotype of *Pfcrt* gene by real time PCR

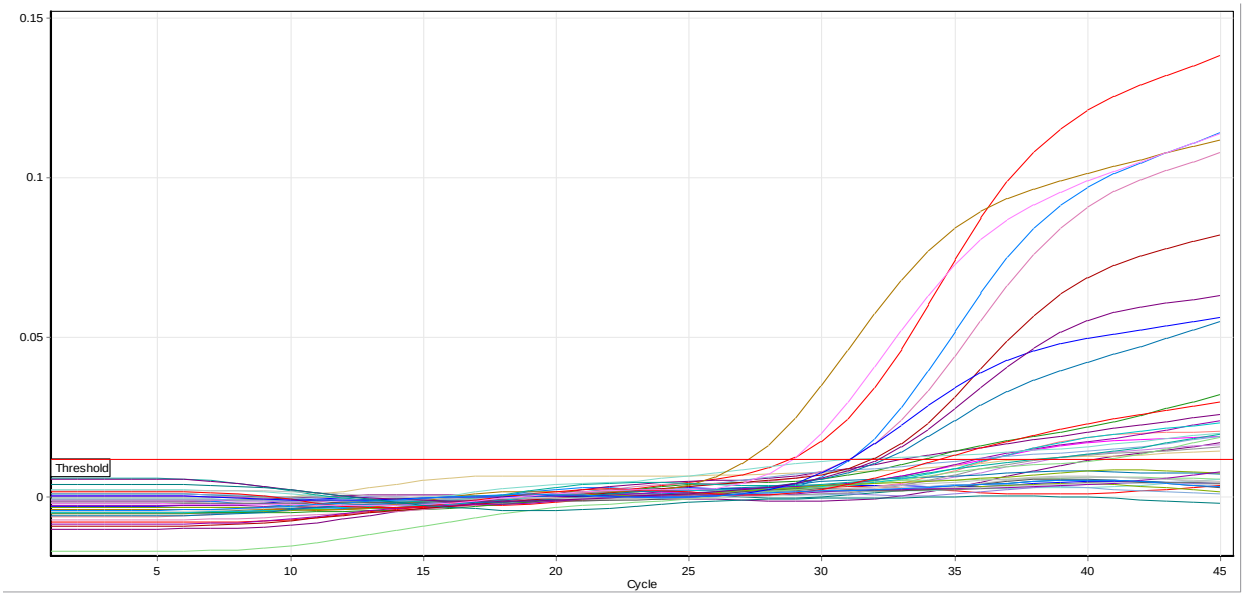
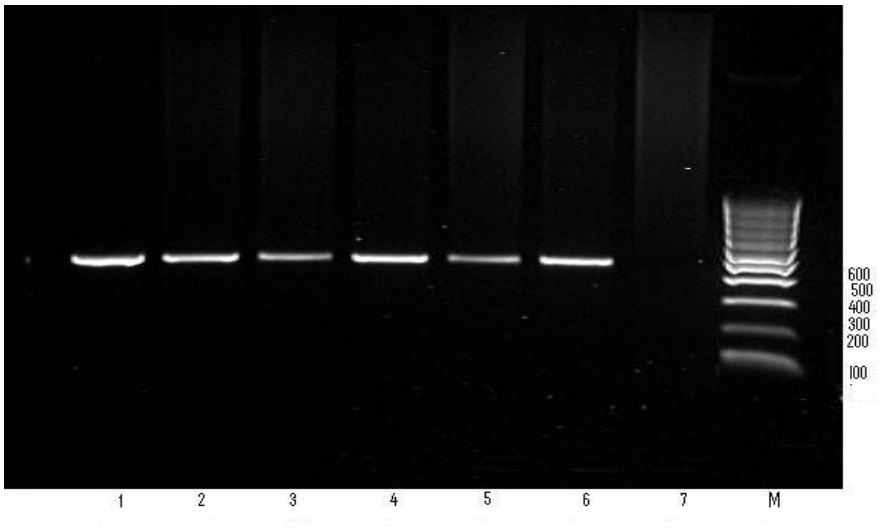


Figure 4.3.2: Graph of the exponential amplification of *CVMNK* haplotype of *Pfcrt* gene by real time PCR

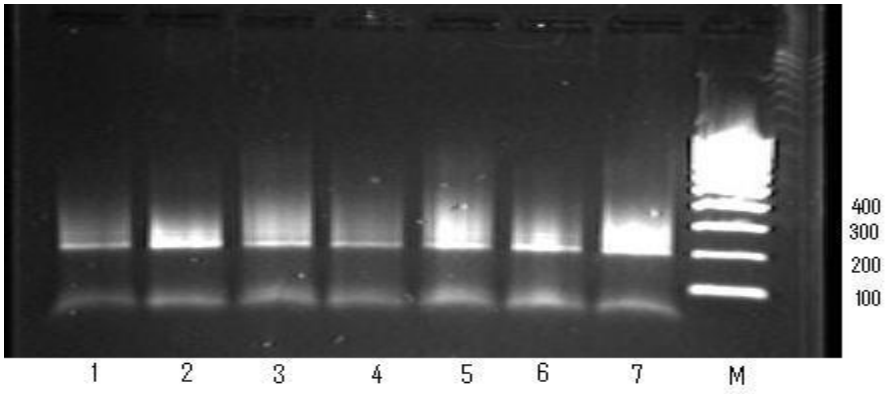
Table 4.3.1 summarises the frequency of haplotypes *pfcrt*, *pfmdr1* and *dhfr* genes. The *Pfcrt* haplotypes were CVMNK (wild) 13(24.1%), CV**IET** (mutant) 29(53.7%) and CVMNK+CVIET 12(22.2%). Thus, the CVIET haplotype was found in 41 (75.9%) of the samples. The **S**VMN**T** haplotype of *Pfcrt* was not found in any of the samples. The haplotypes of pfmdr1 gene of *P. falciparum* isolates from pregnant women were: NYSND (wild) 15(53.6%), **Y**YSND 5(17.9%), N**F**SND 6(21.4%), **YF**SND 2(7.1%). A total of 15 blood spots with *P. falciparum* isolates were analyzed for haplotypes of dhfr. The haplotypes of *dhfr* gene were: ACNCSVI (wild) 4(26.7%), AC**I**C**N**SVI 1(6.7%) and AC**IRN**VI (triple mutant) 10 (66.7%). The I164L mutation was not observed in this study.

Figure 3 shows the gel resolution of *Pfmdr1* gene fragments before sequencing. In the *Pfmdr1* gene, point mutation in codon 86 resulting in the amino acid change from asparagine (N) to tyrosine (Y) was seen in 7 (25.0%) of the 28 samples, while at codon 184 amino acid change

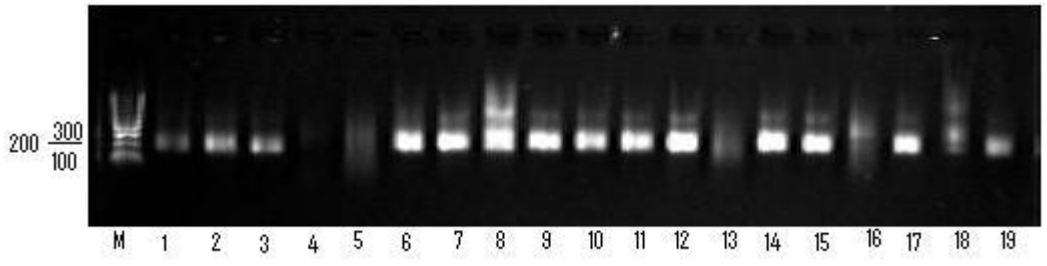
from tyrosine (Y) to phenylalanine (F) was observed in 8 (28.6%) of the 28 samples. No mutation was observed at codons 1034, 1042 and 1246 of the *Pfmdr1* gene (Table 4.3.1).



Fragment 1 (534 bp)



Fragment 2 (234bp)



Fragment 3 (194bp)

Figure 4.3.3: The gel photograph of pfmdr1 fragments 1, 2 and 3 resolved on 1.2% agarose gel (Lane M= 100bp Molecular Marker)

There was no significant association (P>0.05) between the *Pfcrt* and *Pfmdr1* haplotypes and gravidity of the pregnant women from which the parasites were isolated. However, the *CVMNK* haplotype of *Pfcrt* gene was highest among the primigravidae 10(34.5%) compared to secundigravidae 0 (0%) and multigravidae 3 (17.6%). The wild haplotypes of pfmdr1 86N and 184Y were the predominant haplotypes in all the isolates of *P. falciparum* irrespective of gravidity of the women (Table 4.3.2).

The level of peripheral parasitaemia was not significantly associated with either *Pfcrt* haplotypes *or Pfmdr1* haplotypes (P>0.05) (Table 4.3.3).

In this study, 47 (87.0%) did not take any chemoprophylaxis before sample collection. The presence of the mutant *CVIET* haplotype was high irrespective of the use of chemoprophylaxis or the type of drug used for chemoprophylaxis. The *CVIET* haplotype was present in 3 (75.0%) of those that used chloroquine (CQ), 2(66.6%) of those that used sulphadoxine-pyrimethamine (SP) and 76.6% of those that did not use any chemoprophylaxis. Thus, there was no significant association between the use of chemoprophylaxis and the *Pfcrt* haplotype (P=0.778). However, there was a significant association between *Pfmdr1* N86Y haplotype and chemoprophylaxis. (P = 0.025). The *Pfmdr1* 86Y (mutant) haplotype was found in 2 (100%) of those that took CQ, while 4 (100%) of those that took SP had the 86N (wild) haplotype. Majority of those that did not take any prophylaxis 17 (77.3%) also had the *Pfmdr1* 86N haplotype (Table 4.3.4).

Table 4.3.1: Distribution of haplotypes of *Pfcrt, Pfmdr1 and dhfr* genes of *P. falciparum* isolates from pregnant women in Lagos.

Pfcrt (n=54)	
CVMNK (wild)	13 (24.1%)
CVIET (mutant)	29 (53.7%)
CVMNK + *CVIET*	12 (22.2%)
SVMNT (mutant)	0 (0.0%)
Pfmdr1 (n=28)	
NYSND (wild)	15 (53.6%)
YYSND (single mutant)	5(17.9%)
NFSND (single mutant)	6(21.4%)
YFSND (double mutant)	2(7.1%)
Dhfr (n=15)	
ACNCSVI (wild)	4(26.7%)
ACICNVI (double mutant)	1(6.7%)
ACIRNVI (triple mutant)	10(66.7%)

Table 4.3.2: Relationship between *Pfcrt* and *Pfmdr1* haplotypes and gravidity of the study group

	Haplotype	GRAVIDITY			TOTAL	statistics
		Primi	Secundi	Multi		
Pfcrt	***CVMNK***	10 (34.5)	0 (0)	3 (17.6)	13 (24.1)	χ^2=7.83; df = 4; P=0.098
	CVIET	11 (37.9)	7 (87.5)	11 (64.7)	29 (53.7)	
	CVMNK/CVIET	8 (27.6)	1 (12.5)	3 (17.6)	12 (22.2)	
	SVMNT	0	0	0	0	
	TOTAL	**29**	**8**	**17**	**54**	
Pfmdr1 N86Y	**N**	12 (80.0)	1 (100)	8 (66.7)	21 (75.0)	χ^2=0.98; df=2; P=0.613
	Y	3 (20.0)	0 (0)	4 (33.3)	7 (25.0)	
	TOTAL	**15**	**1**	**12**	**28**	
Pfmdr1 Y184F	**Y**	10 (66.7)	1 (100)	9 (75.0)	20 (71.4)	χ^2=0.64; df=2; P=0.726
	F	5 (33.3)	0 (0)	3 (25.0)	8 (28.6)	
	TOTAL	**15**	**1**	**12**	**28**	

Key:

Primi = Primigravidae

Secundi = Secundigravidae

Multi = multigravidae

Table 4.3.3: Relationship Between *Pfcrt, Pfmdr1* Haplotypes and the Level of Peripheral Parasitaemia

Parasite density (p/ul)	PFCRT				Pfmdr1 N86Y			Pfmdr1 Y184F		
	CVIET	*CVMNK*	*CVMNK/CVIET*	TOTAL	N	Y	TOTAL	F	Y	TOTAL
Gametocytes only	1 (20.0)	1 (20.0)	3 (60.0)	5	3 (100)	0 (0)	3	1 (33.3)	2 (66.7)	3
1-500	15 (53.6)	8 (28.6)	5 (17.9)	28	9 (60.0)	6 (40.0)	15	3 (20.0)	12 (80.0)	15
501-1000	3 (75.0)	1 (25.0)	0	4	1 (100)	0 (0)	1	0 (0)	1 (100)	1
1001-5000	9 (75.0)	1 (8.3)	2 (16.7)	12	5(83.3)	1 (16.7)	6	3 (50.0)	3 (50.0)	6
>5000	1 (20.0)	2 (40.0)	2 (40.0)	5	3 (100)	0	3	1 (33.3)	2 (66.7)	3
TOTAL	29	13	12	54	21	7	28	8	20	28
Statistics	x2=10.72; df=8; P=0.218				x2=4.36; df=4; P=0.360			x2=2.36; df=4; P=0.671		

Key

Pfcrt: *CVMNK*= Wild; *CVIET* = Mutant;

Pfmdr1 86: N= Wild; Y= Mutant

Pfmdr1 184: Y= Wild F =Mutant

Table 4.3.4: Effect of previous chemoprophylaxis on the *Pfcrt and Pfmdr1* haplotypes of *P. falciparum* isolates from pregnant women

	Haplotype	Prophylactic drug			TOTAL	statistics
		CQ	SP	None		
Pfcrt	*CVIET*	3 (75.0)	1 (33.3)	25 (53.2)	29	χ^2=1.78; df=4; P=0.778
	CVMNK	1 (25.0)	1 (33.3)	11 (23.4)	13	
	CVMNK/CVIET	0	1 (33.3)	11 (23.4)	12	
	TOTAL	**4**	**3**	**47**	**54**	
pfmdr1 N86Y	N	0	4 (100)	17 (77.3)	21	χ^2=7.39; df=2; P=0.025
	Y	2 (100)	0	5 (22.7)	7	
	TOTAL	**2**	**4**	**22**	**28**	
pfmdr1 Y184F	F	0	0	8 (36.4)	8	χ^2=3.05; df=2; P=0.217
	Y	2 (100)	4 (100)	14 (63.6)	20	
	TOTAL	**2**	**4**	**22**	**28**	

Key:

SP = Sulphadoxine-pyrimethamine

CQ = Chloroquine

CHAPTER FIVE

DISCUSSION

5.1 PREVALENCE AND DETERMINANTS OF MALARIA IN PREGNANCY IN LAGOS

The frequency distribution of *Plasmodium* species (Figure 4.1.1) seen in this study showed that *P. falciparum* with a frequency 95.2% (as mono and mixed infection) was the most prevalent *Plasmodium* species in Lagos. This points to the enormity of the risk of MIP because *P. falciparum* is the only species that sequesters in the placenta (Duffy and Fried, 2001) and as such, is responsible for the adverse effects to the foetus. Steffen *et al.,* (2003) reported that 80-95% of malaria infections in tropical Africa are caused by *P. falciparum*, while the national figures from the Federal Ministry of Health Nigeria shows that *P. falciparum* infection accounts for about 98% of malaria cases in Nigeria.

In this study, the prevalence of MIP was 7.7% (95% C.I. 6.2-9.4%) (Table 4.1.3). The large differences in the reported prevalence rates of malaria in Nigeria, especially in SouthWest Nigeria (8.4% to 72.0%) (Table 2.1) may be attributed to the varying skill and experience of laboratory personnel involved in the various processes of malaria microscopy. In this study, there was strict adherence to standard protocols for blood film preparation, staining with 3% Giemsa stain and reading of the stained slides (WHO, 1991b), thus ensuring the production of clear, well stained slides, thereby reducing errors due to artefacts. Inaccurate laboratory diagnosis of malaria is not peculiar to Nigeria. Mwanziva *et al.,* (2008) reported a bad case in Tanzania where <1% of the slides read as malaria positive by clinic microscopists were confirmed by trained research scientists.

Over-reporting of malaria cases (false positives) create the impression that the efforts to control malaria by the Government and other agencies like the Roll Back Malaria programme, WHO,

UNICEF and many other non-governmental agencies are not working. Whereas, in countries with good surveillance and high intervention coverage, up to 50% reduction in malaria episodes and deaths have been recorded between 2000 and 2006 (WHO, 2008). The factors reported in other studies (Bates *et al.,* 2004; Prudhomme O'Meara *et al.,* 2006; Ohrt *et al.,* 2007) that contribute significantly to the accuracy of malaria microscopy results are: training, experience, motivation and available facilities. The consequences of over-reporting of malaria cases include: inaccurate National data and difficulty in assessing the impact of malaria control programmes; unnecessary treatment with antimalarial drugs, thereby increasing the chances of antimalarial drug resistance developing and wasting of scarce resources.

The prevalence of anaemia in pregnancy was 53.2% in this study. This corresponds with the estimate by the WHO that anaemia in pregnancy in developing countries can be as high as 61% (WHO, 1994). Similar results have been reported in Delta state 56.1%, (Oboro *et al.,* 2002); Gombe Northeast Nigeria 51.8% (Bukar *et al.,* 2008), and Mozambique, 58% (Liljestrand *et al.,* 1986). However the prevalence of anaemia in this study is much lower than the 62.4% in Ogun State (Idowu *et al.,* 2008) 72.0% in Yobe state (Kagu *et al.,* 2007); and higher than the 35.3% (Anorlu *et al.,* 2006), and 32.4% in SouthEast Nigeria (Ekejindu *et al.,* 2006). The two studies that had low anaemia rates also had much smaller sample sizes which may have affected the results.

In this study, anaemia was not associated with age of the pregnant women and gravidity, though there was a reduction in the proportion with anaemia as gravidity increased (Table 4.1.6). This is in contrast with the previous reports that primigravidae are more likely to be anaemic than other gravidites (Idowu *et al.,* 2005; Anorlu *et al.,* 2006). Educational status and gestational age of the women were significantly associated with anaemia (Table 4.1.5). The more educated

women were more likely to be aware of nutritional requirements during pregnancy as well as be more able to afford diets rich in folic acid and iron or their supplements and prevent infections (Idowu *et al.,* 2005). Study participants in their second trimester were more anaemic, 225(59.1%), than those in the first, 104(44.3%), and third trimesters, 138(48.9%). This is consistent with the physiologic observation that haemodilution during pregnancy is worse at the second trimester (Milman, 2008).

The proportion of pregnant women with malaria infection was highest (9.2%) among the primigravidae and lowest in multigravidae (6.5%) though the difference between the various gravidities was not significant in this study (P=0.333) (Figure 4.1.2). Primigravidae have been reported by many studies to be at highest risk and multigravidae at lowest risk of malaria infection by many studies (Tako *et al.,* 2005; Tayo *et al.,* 2009). This was the case in this study. However, the mean parasitaemia was highest in secundigravidae in this study (Figure 4.1.2). This is in contrast to the report of Marielle and co-workers (2003) in Gabon where the parasite density was highest among primigravidae. However, in their study, 64% of the women were teenagers and the mean age of the primigravidae was 19±3.3 years which explains the difference in reports. In this study young maternal age was also identified as a significant risk factor for MIP. The prevalence of malaria in the age group 15-19 years was 20.5% (Table 4.1.4). This finding was similar to previous reports by other authors (Bouyou-Akotet *et al.,* 2003; Tako *et al.,* 2005) where young maternal age < 25 years was reported to be a more important risk factor than gravidity.

Primigravids have been reported to be at greatest risk of MIP because they lack the specific immunity to placental malaria which is acquired from exposure to malaria parasites during pregnancy (Staalsoe *et al.,* 2004; Elliot *et al.,* 2005). In pregnancy, the infected erythrocytes

express parasite-derived immunologically and functionally unique subset of variant surface antigens (var2csa) not found in non-pregnant individuals (Hviid and Salanti, 2007). Exposure to these antigens allows the pregnant woman to acquire specific immunity, which accumulates with subsequent infection and subsequent pregnancies (Beeson and Duffy, 2005). Thus, multigravidae are expected to be at less risk of MIP. This was confirmed in this study with multigravidae having the least mean parasitaemia (4563.9 parasites/µl) and lowest prevalence of malaria (6.5%) compared to primigravidae and secundigravidae (Fig. 4.1.2). It has been reported in Cameroun that antibodies to merozoite surface protein 1-19 ($MSP1_{19}$) may also play a role in reducing placental malaria (Taylor *et al.,* 2004). Thus, the older women being more exposed to malaria infections would have higher levels of antibodies to $MSP1_{19}$ antigens. However, there was an unexpected increase in the proportion of infected women in age group >34 years. A similar finding was reported by Marielle *et al.* (2003) in Gabon and Adam *et al.* (2005) in Eastern Sudan. Nevertheless, it must be noted that this age group had the lowest mean parasitaemia (101.5±38.9 parasites/µl) (Table 4.1.4).

The similar malaria prevalence rates in secundigravidae (7.0%) and multigravidae (6.5%) seen in this study (Figure 4.1.2) suggests that there is no difference in the level of specific immunity to placental malaria. This may probably be due to many challenges with malaria parasites during a pregnancy period, thereby allowing the woman to develop sufficient immunity by the end of a first pregnancy.

This is the first study to report on the effect of insecticide spray on MIP in Nigeria. The use of insecticide spray was very common, 885(81.6%) among the study participants inspite of the emphasis on the use of insecticide treated nets (ITN) in the National guidelines for control of

MIP (FMOH, 2005). The use of insecticide spray had a significant impact in reducing malaria infection among pregnant women (P<0.001) (Table 4.1.5).

ITN coverage in this study population, 125 (11.5%) (Table 4.1.1) was far from the target for the year 2005 of 60% of pregnant women sleeping under an ITN set at the Abuja Summit in 2000 by African Heads of States (Roll Back Malaria, 2000). The similar prevalence of malaria in women using ITN (7.2%) and in those using any net (5.6%) suggests that strict adherence to sleeping under bed nets may be a problem. This can also be deduced from Table 4.1.1 which showed a difference of almost 50% between net ownership and net use. There were complaints of excessive sweating (data not shown) caused by poor ventilation and made worse by the near absence of public power supply to power electric fans and air conditioners. Some other studies have also reported that net ownership does not necessarily translate to usage (Opiyo *et al.*, 2007).

The use of bed net, whether treated or not did not have a significant impact (P=0.838) on malaria infection in the women, although fewer ITN users were positive for malaria (7.2%) than non-users (7.7%). This is consistent with the study of Marchant *et al.* (2002) where a non-significant but modest impact of ITN on MIP was reported. However, Gamble *et al.* (2006) in a systematic review of trials of treated nets concluded that ITN use substantially reduced the risk of MIP. The proposed reasons for the conflicting results on efficacy of ITN are: a) compliance with sleeping under a bed net; and b) the complex vector populations with exophagic/exophilic and early biting behavior. The high rate of usage of insecticide spray, 885(81.6%) in this study may also be responsible for the very low rate of usage of insecticide-treated nets. The use of the combination of insecticide spray and ITN resulted in a significant reduction (P=0.025) of malaria infection, while none (0%) of the 11 women that used a combination of insecticide

spray, ITN and chemoprophylaxis had malaria infection. The use of chemoprophylaxis did not significantly reduce malaria infection among the pregnant women (RR = 0.85, 95% C.I. 0.53 - 1.37) (Table 4.1.5).

5.2 PROTECTIVE EFFICACY OF IPTp-SP AND EQUIVALENCE OF TWO DOSE IPTp-SP TO MONTHLY IPTp-SP

Sulphadoxine-pyrimethamine had an overall protective efficacy of 98.4% in this study (Figure 4.2.1). This signifies that SP is effective in preventing malaria infection among pregnant women in Lagos despite reports of high parasitolgical failure rate of SP, 24.4% reported in children in south west Nigeria (FMOH, 2005). A similar finding was reported by Nyunt *et al.* (2010) where 1 of the 98 pregnant women who were on IPTp-SP became parasitemic (Protective efficacy of 99.0%), none developed clinical malaria during the period of follow-up and also none of the patients tested positive for *P. falciparum* on assessment of placental parasitaemia. It has been reported that parasitological response is significantly better in pregnant women than in children treated with the same drug (Mutabingwa *et al.,* 2009). The absence of parasitaemia after Month 1 in Monthly IPTp-SP and Month 2 in 2-Dose IPTp-SP agrees with the findings of other investigators that the administration of at least two therapeutic doses of IPTp-SP after the first trimester is effective in preventing maternal and placental malaria as well as improving pregnancy outcomes among parturient women as reported in Ghana (Hommerich *et al.,* 2007), Mali (Kayentao *et al.,* 2007), Mozambique (Challis *et al.,* 2004), Kenya (Parise *et al.,* 1998; van Eijk *et al.,* 2004), Malawi (Rogerson *et al.,* 2000) and Gabon (Ramharter *et al.,* 2007).

The low parasitaemia seen during follow-up months among women who were parasitaemic at M0, and in women who became parasitaemic after the first dose of SP, suggest that SP also had

a suppressive effect on parasite multiplication (Table 4.2.3). The reasons for the high protective efficacy in spite of reported high treatment failure among children (FMOH 2005b) can be attributed to the presence of acquired immunity which assist the drug in clearing malaria parasites while also providing a prophylactic effect (Bloland, 2001). Kalanda *et al.* (2006) reported that pregnant women (all gravidae) are more likely to be sensitive than children to both chloroquine and sulphadoxine-pyrimethamine. The greater drug sensitivity in pregnant women probably indicates differences in host susceptibility rather than parasite resistance. SP was seen to be effective in clearing parasites in pregnant women (Table 4.2.3). The disappearance of parasitaemia after dosing with SP and the non-appearance of parasitaemia in the months that parasitaemia was monitored attests to the usefulness of SP as a therapeutic and prophylactic antimalarial drug during pregnancy in the study area.

The proportion of women who became parasitaemic during the follow-up visits for monthly monitoring of parasitaemia 3 (2.5%) and 2 (1.5%) in Arms A and B respectively were similar in M1 (P=0.445) (Table 4.2.3). The only pregnant woman that had parasitaemia at M2 did not receive SP at M1. The relatively high parasitaemia (3,356 parasites/µl), albeit without signs and symptoms, is thought to be due to the presence of parasitaemia below the limit of detection by microscopy at Month 1 which increased to the level seen in M2. Alternatively, it may be an infection acquired after screening for malaria parasitaemia in M1. Nevertheless, on administration of a therapeutic dose of SP, this parasitaemia cleared by the next month's visit, which suggests that the level of SP in the woman's blood was not able to prevent the multiplication of the parasites It is known that in the presence of drug resistance, the drug level needed to either suppress or prevent infection is increased (Bloland, 2001).

Based on parasitaemia, the monthly dosing was not significantly better than the standard 2-dose regimen (P >0.05) (Table 4.2.3). A similar finding was reported by Hamer and colleagues (2007), where the monthly dosing was not more efficacious than 2-dose regimen in an area where malaria transmission was mesoendemic. However, Filler and co-workers (2006) reported that monthly IPTp-SP was a better option than 2-dose IPTp-SP in areas hyperendemic for malaria.

In this study, though the proportion of deaths was higher in the 2-dose IPTp-SP, 4 (7.5%) than in monthly IPTp-SP, 3 (2.9%), the difference was not statistically significant (P=0.178) (Table 4.2.4). Thus, the death rate was not necessarily influenced by any of the dosing regimens. The proportion of deaths in the two arms of the study was not associated with either gravidity or age of the women (P>0.05) (Table 4.2.5). This is an indication that adequate protection of the mothers from malaria infection during pregnancy removed the increased vulnerability of primigravidae and young pregnant women to miscarriage and stillbirth who, hitherto, were more vulnerable to adverse effects of malaria infection during pregnancy.

The effect of the different dosing regimens on low birth weight was similar (P =0.581) (Table 4.2.4). A similar finding was reported in Zambia by Gill and colleagues (2007) and Hamer and colleagues (2007). Both studies did not find any significant difference in the effect of 2 or more doses of SP on LBW and placental malaria. The absence of malaria parasitaemia in the peripheral blood of the women on monthly IPTp-SP and 2-Dose IPTp-SP except for a woman with parasitaemia in 2-Dose IPTp-SP in Month 2 (Table 4.2.3) was attributed to the therapeutic and prophylactic action of SP. Since SP was effective in preventing as well as clearing peripheral parasitaemia, it can be said to have the same effect on the placental parasitaemia. Of

the 12 women assessed for placental parasitaemia in monthly IPTp-SP, none (0%) had malaria parasites. The lack of association between LBW and dosing regimen is thought to be due to the similar efficacy of the two dosing regimen in the study population. On further analysis comparing the effect of age (Table 4.2.7) and gravidity (Table 4.2.8) of the women that had LBW babies to the IPTp-SP regimens, it was found that there was no significant association (P>0.05) between either their age or gravidity and IPTpSP schedule. Thus, it was demonstrated that IPTp-SP reduced the vulnerability of primigravidae and young mothers to the associated adverse effects of MIP.

5.3 MOLECULAR TYPING OF ANTIMALARIAL RESISTANCE MARKERS

This is the first study to report on the *Pfcrt* haplotypes in samples from pregnant women in Nigeria. The haplotype of *Pfcrt* gene described by the protein sequence in codons 72-76 has been shown to determine the susceptibility of *P. falciparum* to chlorquine. The CVIET haplotype (mutant) of *Pfcrt* gene has been identified to be the central determinant of chloroquine resistance in West Africa (Mita *et al.,* 2009). The *Pfmdr1* haplotype is defined by the protein sequence at codons 86, 184, 1034, 1042 and 1246 (Price *et al.,* 1999; Dlamini *et al.,* 2010). The NYSND haplotype (wild) had the highest frequency 15(53.6%). The mutations at positions 86 and 184 did not appear to be additive i.e. the two are not necessary for increased resistance to aminoquinolones hence the similar frequencies of the single mutations, YYSND 5(17.9%) and NFSND 6(21.4%). The double mutant haplotype YFSND had the least frequency 2(7.1%) (Table 4.3.1). Whereas in *dhfr* gene where it has been documented that the mutations are additive, the triple mutant haplotype, ACIRNVI had the highest frequency, 10 (66.7%).

In this study, the *CVIET* haplotype of *Pfcrt* gene was observed in 75.9% of the isolates and *Pfmdr1* 86Y (mutant) in 25.0% of the *P. falciparum* isolates from pregnant women. In Senegal,

the mutant *Pfrct* 76T was reported in 82.3% of the pregnant women taking chloroquine prophylaxis (Bertin *et al.,* 2005). Thus, there is an indication of sustained chloroquine pressure in Lagos for the *CVIET* haplotype to have a frequency of 75.9%. This suspicion is supported by the observation in the phase one of this study that 20% percent of the pregnant women had taken chloroquine prophylaxis before booking at the various antenatal clinics. The high frequency of *CVIET* haplotype (75.9%) found in pregnant women in Lagos suggests that prophylaxis with CQ during pregnancy would not be effective and should be actively discouraged because it leads to a strong selection at the PfCRT locus (Bertin *et al.* 2005; Niang *et al.*, 2008). Alternatively, the policy on intermittent preventive treatment of malaria during pregnancy with SP should be implemented at all levels of healthcare in Nigeria. The withdrawal of CQ from the open market should be considered to restrict access to CQ. Re-emergence of CQ-sensitive parasites with CVMNK haplotype of *Pfcrt* gene have been reported following withdrawal of CQ in Malawi (Kublin *et al.,* 2003; Laufer *et al.,* 2006), Gabon (Schwenke *et al.,* 2001), Brazil (Gama *et al.,* 2009), and China (Wang *et al.,* 2005). The decrease in the CQ-resistant parasite population is likely to be due to a fitness cost incurred as a result of drug resistance (Mita *et al.,* 2009).

In studies carried out in non-pregnant population in SouthWest Nigeria, *Pfcrt 76T* and *Pfmdr1 86Y* were reported in 74.0% and 36.0% respectively in pre-treatment *P. falciparum* isolates from children in Oshogbo, Osun State, Nigeria (Ojurongbe *et al.,* 2007). Folarin *et al.* (2008) reported a frequency of mutant *Pfcrt 76T*, *pfmdr1 86Y* and *pfmdr1 184F* alleles in 60%, 33% and 14% of the isolates respectively in children in Ibadan, Oyo state. In an earlier study also in Ibadan by Happi *et al.* (2006), *Pfcrt* 76T, *pfmdr1* 86Y were reported in 78% and 57% of pretreatment samples from children. In Angola, *Pfcrt* 76T was reported at a frequency of 93.9%

and *Pfmdr1* 86Y was 61.3% (Figueiredo *et al.,* 2008). Since the spread chloroquine resistance is from East to West Africa, it is not surprising that the frequency of *Pfcrt* 76T is higher than what is found in Nigeria. In Swaziland, *CVIET* haplotype increased in frequency from 70% in 1999 to 83% in 2007 (Dlamini *et al.,* 2010). This report showed that the CQ-resistant strains increase with time in the presence of sustained chloroquine use.

Amodiaquine has been reported to select the mutant *pfmdr1 86Y* in The Gambia (Duraisingh *et al.,* 1997), Kenya (Holmgren *et al.,* 2006) and Nigeria (Happi *et al.,* 2006). Thus, the low level of *Pfmdr1 Y86* in this study (25.0%) suggests that the parasites would still be sensitive to amodiaquine for some time. Therapeutic use of drugs such as artesunate-amodiaquine combination in pregnancy is expected to still be effective in Lagos. Continued molecular surveillance is therefore recommended to monitor the *Pfmdr1* haplotypes.

Selection for mefloquine resistance has been associated with a decreased resistance to CQ and an amplification of the *pfmdr1* gene copy numbers. In areas subjected to mefloquine pressure, wild-type *pfmdr1* 86N was found more frequently (Reed *et al.,* 2000). Although the *pfmdr1* 86N was present in 75% of the isolates in this study, a concrete statement on the resistance cannot be made because the *pfmdr1* gene copy numbers was not determined.

The lack of association (P>0.05) between gravidity and the *Pfcrt or Pfmdr1* haplotypes of the *P. falciparum* isolates from pregnant women in Lagos (Table 4.3.2) shows that the gravidity of the women did not exert a significant selective pressure on the *Pfcrt or Pfmdr1* haplotypes. This is consistent with the report that the immune system of semi-immune individuals is able to clear both drug-resistant and sensitive parasites (Bloland, 2001).

The level of parasitaemia was also not significantly associated with the *Pfcrt or Pfmdr1* haplotypes of the *P. falciparum* isolates (P>0.05) (Table 4.3.3). Since the immune system does not exert a selective pressure on *Pfcrt or Pfmdr1* haplotypes, the replication of existing haplotypes does not appear to lead to a change in haplotype.

The association between chemoprophylaxis and *Pfmdr1* N86Y needs to be further investigated because of the small number of persons that used CQ (2) and those that used SP (4) for chemoprophylaxis. The use of CQ prophylaxis has been associated with the selection of *Pfcrt* 76T haplotype (Bertin *et al.,* 2005) but this was not seen in this study probably due to the small number of pregnant women that took CQ prophylaxis (Table 4.3.4). Majority of the women 47 (87.0%) did not take any antimalarial chemoprophylaxis prior to recruitment in this study.

The three *dhfr* haplotypes observed ACNCSVI, 26.7%; ACICNVI, 6.7%; ACIRNVI, 66.7%. The triple mutant ACIRNVI, the most prevalent haplotype, is associated with pyrimethamine therapeutic failure (Happi *et al.,* 2005). Thus, antimalaria chemoprophylaxis with pyrimethamine in Lagos is not adviced. Key mutations such as 16V + 108T and 164L (Peterson *et al.,* 1990; Mkulama *et al.,* 2008) associated with high resistance to cycloguanil, the active form of proguanil, were not observed in this study.

CHAPTER SIX

CONCLUSION

6.1 CONCLUSION

The prevalence of malaria in pregnancy was not as high as most reports in Lagos. This correlates with the use of standard protocols and quality assurance procedures. Over half of the population were anaemic, thoug very few of the women were severel anaemic.

The use of insecticide spray was the preferred malaria prevention strategy by the pregnant women prior to booking at antenatal clinics; and this was associated with a reduction in the rate of malaria infection among pregnant women.

Intermittent preventive treatment of malaria during pregnancy with sulphadoxine-pyrimethamine was effective in protecting pregnant women from malaria infection with a protective efficacy of 98.4% in the first month. Monthly administration of SP to pregnant women may not be necessary in Lagos as monthly IPTp-SP was not found to be superior to the standard 2-dose regimen in either the protection of pregnant wome from malaria infection or in improving the birth outcome. IPTp-SP nullified the vulnerability to adverse effects of malaria in pregnancy associated with young maternal age and first pregnancy.

High levels of CQ-resistant haplotypes of *pfcrt* gene and pyrimethamine-resistant haplotypes of *dhfr* gene, but no cygloguanil-resistance conferring mutations in the dhfr gene were observed among *P. falciparum* isolates from pregnant wome in Lagos. The low level of mutations in the *pfmdr1* gene indicates the continued efficacy of amodiaquine against malaria in pregnancy.

6.2 CONTRIBUTIONS TO KNOWLEDGE

1. This study has contributed data on the protective efficacy of IPTp-SP in Lagos. Also, the equivalence of monthly IPTp-SP to the standard 2 dose IPTp-SP in Lagos was established.

2. The high level of mutations that code for chloroquine resistance (*Pfcrt* CVIET, 75.9%) and pyrimethamine resistance (*dhfr* ACIRNVI, 66.7%) from *P. falciparum* isolates from pregnant women provides evidence for the discontinued use of chloroquine and pyrimethamine for malaria prophylaxis during pregnancy in Lagos. The absence of mutations that encode for cucloguanil resistance (*dhfr* 16V+108T, and 164L) indicate the continued efficacy of cycloguanil in Lagos.

3. A reliable baseline data for planning and assessment of impact of malaria intervention among pregnant women in Lagos was estabiliched with the 7.7% prevalence of MIP in Lagos. This study draws attention to the need to adhere to standard protocols to obtain reproducible and acceptable prevalence rates.

RECOMMENDATIONS

1. Quality assurance procedures should be employed in the entire process of malaria diagnosis by microscopy from slide preparation to reading of stained slides to ensure the validity of results. Certification of malaria microscopists should be a criteria for engaging in malaria research and routine malaria diagnosis.
2. National coverage of IPTp-SP should be scaled up to protect all pregnant women, except those allergic to sulphonamides. The use of sulphadoxine-pyrimethaminee should be restricted to protection of pregnant women from malaria to reduce the drug pressure effects that lead to the selection of parasites resistant to SP.
3. Continuous monitoring of the protective efficacy of SP should be conducted to determine when to replace SP with another drug. Meanwhile, possible alternatives to SP for IPT should be sought.
4. Chloroquine should be withdrawn completely from the open market. This will facilitate the decrease in CQ-resistant parasites and eventually chloroquine, which is safe even in early pregnancy, will be used again to protect and treat malaria in pregnancy.
5. The use of pyrimethamine (S*unday Sunday medicine)* for chemoprophylaxis of malaria in pregnancy should be actively discouraged.

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APPENDIX 1

INTERMITTENT PREVENTIVE TREATMENT OF MALARIA IN PREGNANCY IN LAGOS STATE, NIGERIA

PATIENT QUESTIONNAIRE

Study ID ……..

1. Name: …….

2. Address: …….

3. Age: ……

4. Is this your first pregnancy?1. Yes [ ] 2. No [ ]

5. How old is your pregnancy ……

6. If No, How many times have you been pregnant? ……..

7. How many children do you have? ……

8. Have you had any miscarriage or still birth? 1.Yes [ ] 2. No [ ]

9. If Yes, did you have malaria at that time? 1.Yes [ ] 2. No [ ]
3. Can't remember [ ]

10. Have you had malaria/fever during **this** pregnancy? 1.Yes [ ] 2. No [ ]

11. If Yes, how often have you had malaria/fever? 1. Once [ ] 2. Once a week [ ] 3. Once in 2 weeks [ ] 4. once a month [ ]

12. When was the last time you had malaria? 1) This week [ ] 2) Last week [ ]
3) 2 weeks ago [ ] 4) 3 weeks ago [ ] 5) A month ago [ ] 6). Other (specify)…….

13. What symptoms do you experience when you have malaria? ……

14. Where were you treated? 1. At home [ ], 2. Pharmarcy/chemist [ ], 3. Hospital [ ]

15. Where you given antimalarial drug to be taking regularly? 1.Yes [ ] 2. No [ ]

16. If Yes, how often do you take these drugs? 1. Every week [ ] 2. Every month [ ]
3. When I feel sick [ ]

17. Do you know the name of the drug? 1.Yes [ ] 2. No [ ]

18. If Yes, what is the name of the drug? …….

19. How many tablets do you take at a time? ……

Personal protection from malaria

20. Are you taking any antimalarial drug to prevent malaria? 1.Yes [ ] 2. No [ ]

21. What type of drug are you taking? 1. Sulphadoxine-pyrimethamine [ ] 2. Chloroquine [ ] 3. Pyrimethamine [ ] 4. Proguanil [ ] 5. Other (specify) ……

22. Do you have a bednet at home? 1. Yes [ ] 2. No [ ]

23. Is the net treated with insecticide? 1. Yes [ ] 2. No [ ]

24. Do you sleep under the net? 1. Yes [ ] 2. No [ ]

25. Do you use insecticide sprays? 1. Yes [ ] 2. No [ ]

26. How often do you spray your home? 1. Every day [ ] 2. Twice a week [ ]
3. Once a week [ ] 4. Twice a month [ ] 5. Once a month [ ]

APPENDIX 2

COLLEGE OF MEDICINE

UNIVERSITY OF LAGOS
P.M.B. 12003, LAGOS, NIGERIA

Provost: **STEPHEN O. ELESHA,** *MB,BS (Lagos), DABPATH (USA), FACP (USA), FWACP (Lab. Medicine), FNMCPATH.*

Telephone: LAGOS 5453760-74 (15 Lines)
Fax: 234-01-5851432.

Deputy Provost: **R.O.ABIDOYE,** *M.A. (Columbia), Ph.D (Howard)*

College Secretary: **O.O. AMODU (MRS.),** *B.A. (Hons.) PGDPA (Ife), MINIM.*

CM/COM/8/VOL.XIX

November 8, 2006

Mr. Chimere O. Agomo
(Ph.D Student)
c/o Dr. Wellington O. Oyibo
Department of Med. Micro. & Para.
CMUL

Dear Mr. Agomo

RE: ETHICAL APPROVAL

The Research Grants and Experimentation Ethics Committee of the College met on **November 7, 2006** and considered your application for Ethical Clearance for the research Project entitled **"Intermittent Presumptive Treatment with Sulphadoxine-Pyrimethamine During Pregnancy and Plasmodium Falciparum Genetic Diversity in Peripheral, cord, and Placental Blood of HIV infected and non-infected women."**

On behalf of the Committee, I hereby convey **Ethical approval** for the protocols and urge you to forge ahead with the research.

Thank you.

Yours sincerely,

8/11/06

Prof. R.O. Abidoye
Chairman, Research Grants & Experimentation Ethics Committee

APPENDIX 3

LAGOS UNIVERSITY TEACHING HOSPITAL

PRIVATE MAIL BAG 12003, LAGOS, NIGERIA

Chairman:
PROF. B. C. UMERAH.
MB FRCR, FICS, FMCR, FWACS.

Director of Administration:
AYO OLAGUNJU B.Sc. (Hons), MNIM, AHAN, ANIPR.

Chief Medical Director
PROF. AKIN OSIBOGUN
MBBS (Lagos), MPH (Columbia) FMCPH, FWACP

Chairman, Medical Advisory Committee:
PROF. J. K. RENNER, F M C (Paed.)

Tel: 234 - 1 - 5850737, 5852187, 5852209, 5852158, 5852111

28th March, 2007

Ref. NO. ADM/DCST/221/Vol.10

Mr. Chimere O. Agomo,
(Ph.D Student),
C/o Dr. Wellington O. Oyibo,
Department of Medical Micro. & Parasitology,
C.M.U.L.

APPROVAL OF RESEARCH & ETHICS COMMITTEE

I wish to refer to your request in respect of the above stated matter.

Approval has been granted to you to continue with the study titled "INTERMITTENT PRESUMPTIVE TREATMENT WITH SULPHADOXINE-PYRIMETHAMINE DURING PREGNANCY AND PLASMODIUM FALCIPARUM GENETIC DIVERSITY IN PERIPHERAL, CORD, AND PLACENTAL BLOOD OF HIV INFECTED AND NON-INFECTED WOMEN".

Wishing you all the best in your study.

Thank you.

PROF. C. U. ODUM,
CHAIRMAN, RESEARCH & ETHICS COMMITTEE.

APPENDIX 4

INSTITUTIONAL REVIEW BOARD

NIGERIAN INSTITUTE OF MEDICAL RESEARCH

6, Edmond Crescent Off Murtala Muhammed Way, P. M. B. 2013 Yaba, Lagos.
Tel: 01-4823123, 01-7744723, 08050254484, 08033460947 Fax: 01-4823123, 234-1-3425171
E-mail: nimr_irb@yahoo.com *Website:* www.nimr-nig.org
Secretariat: Room 207, Biochemistry Division, Research Block, NIMR

30th 01-08

PROJECT TITLE: **INTERMITTENT PREVENTIVE TREATMENT OF MALARIA IN PREGNANCY IN LAGOS STATE, NIGERIA**

APPROVAL LETTER.

The above named proposal has been adequately reviewed; the protocol and safety guidelines satisfy the conditions of NIMR IRB, policies regarding experiments that use human subjects.

Therefore the study under its reviewed state is hereby approved by Institutional Review Board, NIMR.

DR. P. U. AGOMO
Name of vice IRB chairman

30/01/08
Signature & Date of IRB vice Chairman

DR. A.A ADEIGA
Name of IRB Member

30/1/08
Signature & Date of IRB Member

This approval is given with the investigator's Declaration as stated below;
By signing below I agree/certify that:

1. I have reviewed this protocol submission in its entirety and that I am fully cognizant of, and in agreement with, all submitted statements.
2. I will conduct this research study in strict accordance with all submitted statements except where a change may be necessary to eliminate an apparent immediate hazard to a given research subject.
   - I will notify the IRB promptly of any change in the research procedures necessitated in the interest of the safety of a given research subject.
   - I will request and obtain IRB approval of any proposed modification to the research protocol or informed consent document(s) prior to implementing such modifications.

30/01/08

3. I will ensure that all co-investigators and other personnel assisting in the conduct of this research study have been provided a copy of the entire current version of the research protocol and are fully informed of the current (a) study procedures (including procedure modifications); (b) informed consent requirements and process; (c) potential risks associated with the study participation and the steps to be taken to prevent or minimize these potential risks; (d) adverse event reporting requirements; (e) data and record-keeping; and (f) the current IRB approval status of the research study.

4. I will respond promptly to all requests for information or materials solicited by the IRB or IRB Office.

5. I will submit the research study in a timely manner for IRB renewal approval.

6. I will not enroll any individual into this research study until such time that I obtain his/her written informed consent, or, if applicable, the written informed consent of his /her authorized representative (i.e., unless the IRB has granted a waiver of the requirement to obtain written informed consent).

7. I will employ and oversee an informed consent process that ensures that potential research subjects understand fully the purpose of the research study, the nature of the research procedures they are being asked to undergo, the potential risks of these research procedures, and their rights as a research study volunteer.

8. I will ensure that research subjects are kept fully informed of any new information that may affect their willingness to continue to participate in the research study.

9. I will maintain adequate, current, and accurate records of research data, outcomes, and adverse events to permit an ongoing assessment of the risks/benefit ratio of research study participation.

10. I am cognizant of, and will comply with, current federal regulations and IRB requirements governing human subject research including adverse event reporting requirements.

11. I will make a reasonable effort to ensure that subjects who have suffered an adverse event associated with research participation receive adequate care to correct or alleviate the consequences of the adverse event to the extent possible.

12. I will ensure that the conduct of this research study adheres to Good Clinical Practice guidelines.

MR. CHIMERE O AGOMO
Principal Investigator Name

30/1/08
Principal Investigator signature and Date