MAKERERE UNIVERSITY

SULFADOXINE/PYRIMETHAMINE INTERMITTENT PRESUMPTIVE TREATMENT RELATIONSHIP WITH NEWBORN, MATERNAL *PLASMODIUM FALCIPARUM* INFECTION AND IMMUNITY

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ABSTRACT

Introduction

Malaria in endemic areas affects mainly pregnant women and children below five years. Infants below six months are generally protected from severe infection because of anti-parasite antibodies transferred from mother and presence of fetal haemoglobin. The adults are protected by the time dependent exposure to parasites leading to production of antibodies. To prevent the adverse effects of pregnancy malaria, in 1998 WHO adopted a strategy for prevention which includes effective case management, use of insecticide treated nets and intermittent presumptive treatment (IPTp). Roll Back Malaria Partnership recommends use of self-reported data where data on directly observed therapy is not available to determine IPTp coverage yet self-reported data has been found to be prone to bias. The main aim of the study was to determine the effect of using IPTp on proportions of antibodies to selected *P. falciparum* blood stage antigens transferred from mother to baby. In addition to determine the validity of self-reported IPTp use during pregnancy.

Methods

In a cross sectional study 290 mothers were recruited at delivery after informed written and oral consent. Data on demographic and obstetric history was collected using interviewer administered questionnaire. Participants were asked if they took IPTp during pregnancy and drug given and when it was administered. Blood from the mother was drawn aseptically within four hours prior to delivery and after delivery venous cord blood was drawn. This was used to make thick blood smears and serum kept at -70^{o}C till the analysis for sulfadoxine and antibody levels. Presence or absence of sulfadoxine in maternal blood at delivery was compared to self-reported IPTp use. To determine the congenital exposure and antibody transfer maternal and

cord sera were tested for IgG and IgM antibodies against Glutamine Rich Protein (GLURP), Merozoite Surface Protein (MSP3), Merozoite Surface Protein 3a (MSP3a) and Histidine Rich Protein (HRPII). Then determined how the proportion of antibody transferred and congenital immune priming was affected by use of IPTp by the mother during pregnancy.

Results and Conclusion

There was only slight agreement between self-reported IPTp use during pregnancy and finding sulfadoxine in blood at delivery with kappa statistics of 0.03. The more educated and older mothers were more likely to report IPTp use during pregnancy. The sero-prevalance of GLURP in the mothers was 72.8%, HRPII 92%, MSP3 71% and 83.8% for MSP3a. Using IPTp during pregnancy did not affect the levels or sero-positivity of the tested antibodies in the mothers and babies. The proportions of antibodies transferred were not affected by IPTp use; however mothers with high antibody levels transferred less compared to their counterparts. The prevalence of congenital malaria in the study population was 2.4%. The percentage of newborns with evidence of malaria immune priming was between three and 33% depending on the different representative antigen (GLURP, HRPII MSP3 and MSP3a). Using IPTp during pregnancy was found to be protective of congenital malaria and immune priming.

Data presented in this thesis describes the effect of IPTp use during pregnancy on maternal and neonatal infection and immunity to *P. falciparum* blood stage infection. In addition, validation of self-reported IPTp use during pregnancy is described. It opens new possible research areas to determine how immune priming may affect the immunity in infants.

LIST OF PUBLICATIONS

This thesis is compiled from the following four scientific paper and manuscripts;

1. Fatuma Namusoke, Niloofar Rasti, Fred Kironde, Mats Wahlgren, and Florence Mirembe. Malaria Burden in Pregnancy at Mulago National Referral Hospital in Kampala, Uganda. *Malaria Research and Treatment* 2010;2010(10):4061/2010/913857.

2. Fatuma Namusoke**,** Mohammed Ntale**,** Mats Wahlgren**,** Fred Kironde and Florence Mirembe. Validity of self –reported use of sulfadoxine/pyrimethamine intermittent presumptive treatment during pregnancy (IPTp): a cross-sectional study. *Malaria Journal* 2012, 11:310 doi: 10 1186/ 1475-2875-11-310.

3. Fatuma Namusoke, Mattias Engorstrom, Mats Wahlgren, F. Mirembe and F.Kironde. Maternal transfer of *P. falciparum* IgG antibodies to newborn following Intermittent Presumptive Treatment use during pregnancy. *Manuscript*.

4. Fatuma Namusoke, Mats Wahlgren, Florence Mirembe and Fred Kironde. Use of sulfadoxine/pyrimethamine Intermittent Presumptive Treatment in Pregnancy affects prenatal immune priming to malaria-Cross-sectional study. *Manuscript.*

DECLARATION

I Fatuma Namusoke (Dr) declare that this piece of work is original and has never been presented for award of degree. I set up the study site, protocol training of the research assistants and was the overall clinical supervisor of the studies. I personally did the ELISA, malaria microscopy and Polymerase Chain Reaction for all samples with the help of staff from biochemistry laboratory in the College of Health Sciences. I analysed the data with the help of a statistician and drafted all the manuscripts .To the best of my knowledge this work has not been submitted before to any university for the award of a degree.

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DEDICATION

My husband Isa Kabenge (Don ICK) and our children, Rayhaan, Aydin and Azraa

Fatuma Namusoke

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All gratitude goes to the Almighty Allah for all my achievements in life. My parents Hajji M. Musoke and Hajat Yudaya M. Musoke who natured me and emphasized the importance of education. It makes me happy to know that I come to the end of this work when we are all still close together. You have always inspired and encouraged me. Mum special thanks for taking care of our babies when we were all away. My sister Mariam Namusoke, for looking out for the children during those busy days.

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LIST OF ABBREVIATIONS

ANC:	Antenatal Clinic
EIR:	Erythrocyte infective Rate
GLURP:	Glutamine Rich Protein
HIV:	Human Immunedeficiency Virus
HPLC:	High performance liquid chromatography
HRPII:	Histidine Rich Protein II
IgE:	Immunoglobulin E
IgG:	Immunoglobulin G
IgM:	Immunoglobulin M
IPTp:	Intermittent presumptive treatment
iRBC:	infected Red Blood Cells
ITNs:	Insecticide treated nets
MOI:	Multiplicity of infection
MSP3:	Merozoite Surface Protein 3
MSP3a:	Merozoite Surface Protein 3a
PAM:	Pregnancy Associated Malaria
PCR:	Polymerase chain reaction
RBM:	Roll Back Malaria
SDX:	Sulfadoxine
SP:	Sulfadoxine Pyrimethamine
WHO:	World Health Organisation
WOA:	Weeks of ammenorrhea

CHAPTER ONE: INTRODUCTION

1.1 Background

1.1.1 Global Malaria burden

Malaria remains a major global public health burden causing over six hundred and sixty deaths annually. More than 90% of malaria deaths occur in Sub-Saharan Africa where the children below five years and pregnant women are the main victims (WHO 2012). The annual mortality and morbidity due to malaria increased globally after the Global Malaria Eradication Programme was abandoned in 1969 (Najera *et al*., 2011). Deaths due to malaria every year increased steadily from 1980 (995,000) to peak in 2004 (1,817,000) and then decreased steadily to 1,238,000 in 2010 (WHO, 2005; WHO, 2011). The recent decrease in deaths has been attributed to increase in access to preventable measures like insecticide treated nets in the susceptible population. Cumulatively Global Fund investments that increased insecticide treated nets/ Indoor residual spraying (ITN/IRS) coverage in 2002-2008 is estimated to have prevented 240,000 deaths. Effective prioritization of ITN/IRS scale-up in countries where malaria is a major cause of child deaths has been suggested as key in saving greater number of lives with available resources (Akachi & Atun, 2011).

Despite the general decrease in burden, malaria is still a global threat. In non-malaria endemic countries like United States, centres for disease control and prevention (CDC) reported 14% increase in the number of reported malaria cases between 2009 and 2010. These infections were caused by *Plasmodium Falciparum*, *Plasmodium Vivax, Plasmodium Malariae*, and *Plasmodium Ovale* were identified in 58%, 19%, 2%, and 2% respectively (Mali *et al.,* 2012).

Malaria control and elimination are under the constant threat of the parasite and mosquito vector developing resistance to antimalarials and insecticides respectively. It has been suggested that, the prospects of malaria eradication, rest heavily on the outcomes of research and development of new and improved tools (Mendis *et al.,* 2009). An estimated US$1 billion are distributed to the 1.4 billion people exposed to stable P. *falciparum* malaria risk annually. Although this is thought to be broadly

adequate, it's distribution is not proportionate to malaria burden in the respective countries and yet the financial commitment by the international community is inadequate (Snow *et al.,* 2008).

Malaria is a disease of poverty affecting mainly the rural poor population. Malaria is estimated to consume up to 40% of public health expenditure of these poor countries, causing in Africa an estimated US $12 billion loss in gross domestic product (GDP) every year. Uganda was estimated to lose an equivalent of US$ 49,825,003 of GPD in 2003 due to malaria, which translates to US$1.93 per person (Orem *et al.*, 2012). Malaria leads to poverty through increase in household expenditure, loss of time to work .On the other hand poverty predisposes people to malaria because of lack of bed nets which may be costly, poor housing facilities and failure to get timely treatment. Teklehaimanot *et al.*, 2008 have emphasised the need to address the social and economic conditions that fuel malaria transmission if malaria is to be reduced or even eliminated.

The biggest burden of malaria lies in the sub-Saharan Africa, Latin America, Asia and to a less extent the Middle East and some parts of Europe are affected by malaria (WHO, 2011). Across malaria endemic areas in Africa, malaria contributes 25-35% of outpatient and 20-45% of all the hospital admissions (WHO, 2005). In malaria endemic areas pregnant women, children, and immune-compromised individuals have the highest morbidity and mortality associated with malaria.

1.1.2 Global pregnancy malaria burden

It is estimated that more than 54 million women living in malaria endemic areas and 70.5 million in areas with low transmission or *P. vivax* transmission only areas become pregnant every year (Dellicour *et al.*, 2010). In Africa, approximately 25 million pregnant women are at risk of *Plasmodium falciparum i*nfection every year, and one in four women are said to have evidence of placental infection at the time of delivery (Dellicour *et al.*, 2010).

Malaria infection during pregnancy has adverse consequences for both the woman and foetus. The direct effects of pregnancy malaria on the mother include anaemia, cerebral malaria, pulmonary oedema and kidney failure (Steketee *et al.*, 2001). Pregnancy Malaria is responsible for an estimated 10,000 maternal and 200,000

infant deaths annually (Desai *et al.*, 2007). In the foetus malaria causes abortion, still birth, low birth weight, intrauterine fetal growth restriction and premature delivery (Shulman *et al.*, 2003). In sub-Saharan Africa malaria is said to account for 2-15% of cases of maternal anaemia (Guyatt *et al.*, 2001). Low birth-weight is associated with a marked increase in infant mortality and morbidity and malaria is estimated to contribute 30% of preventable low birth weight. Between 75,000 and 200,000 infant deaths are associated with pregnancy malaria annually (Steketee *et al.*, 2001).

Sequestration of *P. falciparum* parasites may lead to alterations in the syncytiotrophoblast that could lead to fetal exposure to parasites (Fried *et al.*, 1996). Congenital malaria in malaria endemic areas has been reported to be rare (1-5%) in some studies (Ouedraogo *et al.*, 2012) and not others (Uneke, 2007).

Development of the immune system in the human fetus starts as early as 10 weeks of gestation and increases progressively to birth (Holt & Jones, 2000). Therefore exposure of the fetus to parasites leads to immune response. It has been suggested that fetal priming (exposure to parasites/antigens) may influence the number and severity of malaria infections in an infant (Metenou *et al.*, 2007). Immunoglobulins G are the only antibodies that cross the placenta, presence of IgM in cord blood signifies exposure to the particular antigen in-utero (Gouling *et al.*, 2003). Data on the burden of congenital malaria and immune priming of the foetus by malaria parasites and factors associated in this setting is scanty.

Young women, primigravidae and HIV positive women are more susceptible to malaria during pregnancy than their counterparts (Shulman *et al.*, 2003). Maternal age has been shown to be an independent predictor of parasiteamia during pregnancy (Rogerson *&* Meshnick, 2007). HIV and malaria in pregnancy are mutually aggravating infections where pregnant mothers with HIV are more prone to getting malaria and its complications (Steketee *et al.*, 1996). HIV infected pregnant women regardless of parity are at a greater risk of placental and clinical malaria compared to their counter parts. In addition they tend to have higher parasite densities, maternal anaemia and poor fetal outcome (ter Kuile *et al.*, 2004).

The increased susceptibility of the pregnant women to malaria infection is attributed partly to presence of a new site; the placenta which the parasites invade causing placental malaria. *Plasmodium falciparum* parasites have been thought to evade the immune system by hiding in the placenta vasculature causing the morbidities and mortalities associated with pregnancy malaria (Fried & Duffy, 1996).

World Health Organization recommends that IPTp is given to all pregnant women in addition to use of bed net and effective malaria case management for prevention of pregnancy malaria. Successful prevention of pregnancy malaria reduces the risk of severe maternal anaemia by 38%, low birth weight by 43% and perinatal mortality by 27% among primigravidae (Desai *et al.*, 2007). Despite the reported wide spread resistance to sulfadoxine/pyrimethamine (SP), it is still effective as IPTp and is the recommended antimalarial by WHO. Continued monitoring of SP IPTp effectiveness and safety of multiple doses was recommended (WHO Malaria Policy Advisory Committee and secretariat, 2012).

1.1.3 Malaria burden in Uganda

In Uganda, malaria is highly endemic and is ranked among the leading causes of morbidity and mortality affecting especially young children and pregnant women. Uganda was ranked sixth worldwide in number of malaria cases and third in number of malaria deaths (WHO, 2008). It has stable, perennial malaria transmission in 90 to 95 percent of the country. In the rest of the country, particularly in the highland areas, there is low and unstable transmission with potential for epidemics. More than 50% of population experiences high transmission levels of 50 or more infective mosquito bites per person per year. The infective bites vary from region to region ranging from four infective bites per person per year in Mubende district to more 1500 infective bites per person per year in Apac (Okello *et al.*, 2006). It has been estimated that 12 million clinical cases of malaria are managed in public health systems alone annually. Malaria is estimated to cause between 70,000 and 100,000 child deaths per year in Uganda (Lynch *et al.*, 2005). According to 2009 malaria indicator survey 42% of children below five years had peripheral parasiteamia yet only 33% of the under five years were reported to have slept under bed net the night before the survey. The household bet net ownership (at least one net) increased from 10% in 2006 to 37% in 2009 (MIS, 2009).

Despite the reported decline in the burden of malaria globally (WHO, 2005; WHO, 2011) the baseline transmission in Uganda is very high and yet malaria intervention coverage is not yet to scale (Talisuna *et al.*, 2012). The burden of malaria in Uganda has not decreased in recent years (Yeka *et al.*, 2012). Major challenges to malaria control in Uganda include very high malaria transmission intensity, inadequate health care resources, increasing resistance to antimalarials and insecticides, inappropriate case management, poor utilization of malaria preventive measures. Figure 1 shows the malaria endemicity in Uganda.

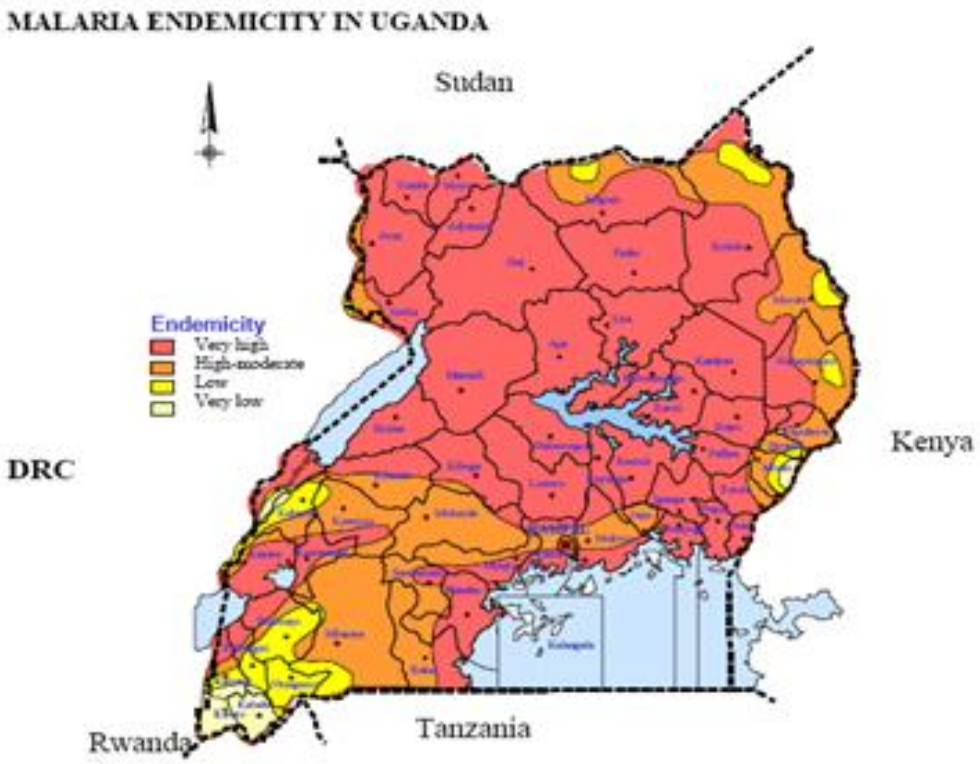
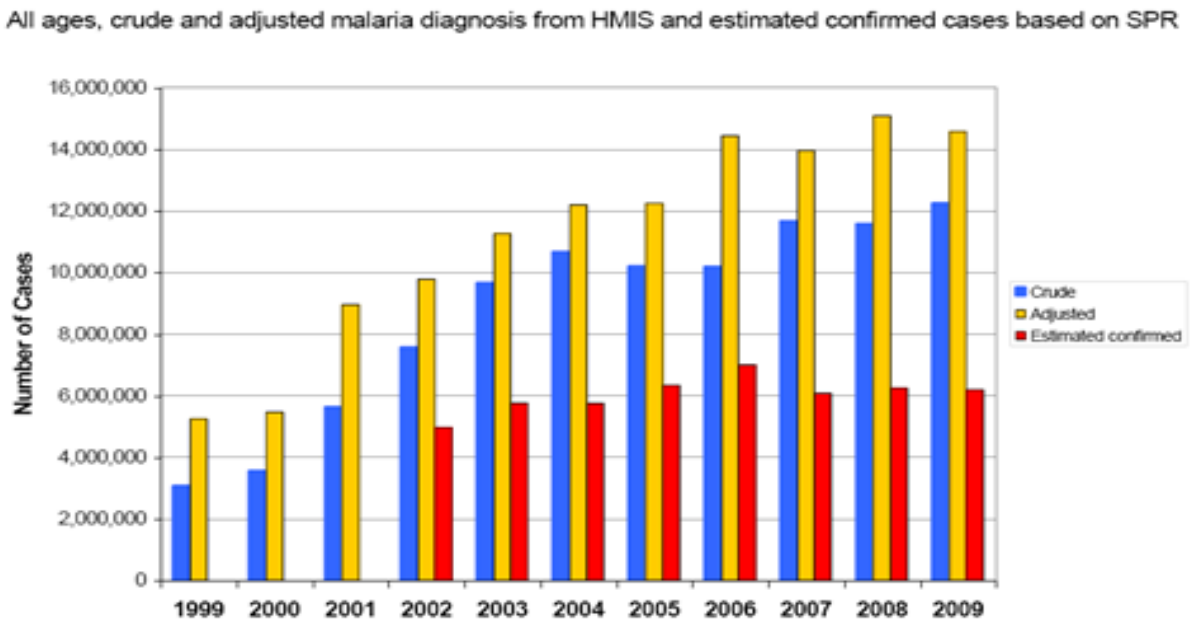


Figure 1: Malaria endemicity in Uganda

Source; Malaria Control Program, Ministry of Health, Uganda. Available at http://www.health.go.ug/mcp/distmaps.html

The estimated malaria burden in Uganda may be higher than reported since it does not put into account the patients treated at home and in private health facilities. Although a global decline in the burden of malaria since 2005 has been reported, Uganda still reports an increase in number of hospital admissions due to malaria (Okiro *et al.*, 2011) and outpatient attendance (Figure 2). One study demonstrated a decline in the prevalence of malaria in children below five years, between 2004 and

2010 in south western Uganda. Despite the reported decline in this low endemic area the burden of malaria is still unacceptably high (De Beaudrap *et al.*, 2011).



Source; Malaria Control Program, Ministry of Health, Uganda; http://www.health.go.ug/mcp/Malaria_trends_HMIS_1997-2009.pdf

Figure 2: Trends of malaria cases in Uganda

Malaria in pregnancy continues to be a serious health risk for pregnant women in Uganda and is associated with increased risk for maternal anaemia and perinatal mortality. Studies show that the prevalence of placental infection varies depending on endemicity between 6-32%. The risk is higher in the HIV positives, primigravidae women and adolescents (Kasumba *et al.*, 2000; Brahmbhatt *et al.*, 2008). In Tororo which is a very high transmission area, 29% of HIV negative mothers and 19% of HIV positive on SP IPTp and daily coltrimoxazole prophylaxis respectively had placental malaria at delivery (Newman *et al.*, 2009). According to 2009 Uganda Malaria indicator surveys 32% of pregnant women received 2 doses of IPTp during pregnancy (MIS, 2009).

In Uganda healthcare system works on a referral basis and plays a big role in delivery of IPTp. IPTp is given in the health facility by a skilled attendant during the antenatal period. The first contact for people living in rural areas is a member of the village health team. These are volunteers from the community with no basic medical training but are training to screen and handle emergency cases before referral. Antenatal care services and provision of IPTp is offered at health Centre II, III, IV and

hospitals. Despite the high antenatal attendance in Uganda where 95% of pregnant attend ANC by a skilled provider, only 25% of the pregnant mothers took the recommended two doses of IPTp (UDHS, 2011). Low IPTp coverage may be due to drug stock out and inadequate training of the health workers on the importance of IPTp (Onoka *et al.*, 2012). It has been demonstrated that frequent ANC visits do not ensure access to SP IPTp in the presence of other barriers to IPTp delivery (Ndyomugyenyi & Katamanywa, 2010). Pregnant women are more likely to take SP for IPTp if it is offered during an ANC visit than when asked to take at home (Sangare *et al.*, 2010).

Strategies to improve IPTp coverage include training the village health team, peer mothers and traditional birth attendants in administering IPTp to pregnant mothers in the community. The community-based IPTp delivery system increases access and adherence to IPTp and is generally cost-effective (Mbonye *et al.*, 2007; Mbonye *et al.*, 2008; Ndyomugyenyi *et al.*, 2009). Before community based delivery IPTp can be rolled out, there is a need to train the village health teams and the pregnant women on the importance of IPTp.

1.1.4 Malaria in infants and children

The biggest burden of malaria in endemic areas lies with the children especially those below five years (Marsh & Snow, 1999) and the pregnant women (Steketee *et al.*, 1996). Adults living in malaria endemic areas have partial protection against malaria due to time dependant exposure to malaria parasites. This immunity is not sterile but protects the adult from severe infection; in addition it requires repeated exposure to malaria parasites to be maintained. Younger children tend to have higher parasite density during an infection compared to the older ones (Beadle *et al.*, 1995). The neonates and infants below six months are less susceptible due to presence of IgG antibodies against malaria transferred from mother to fetus (King *et al.*, 2002) and fetal haemoglobin (Amaratunga *et al.*, 2011).

Studies in the sub-Saharan Africa have indicated that malaria in infants below 6 months is not rare (Afolabi *et al.*, 2001; Larru *et al.*, 2009). Factors that affect the level and transfer of immunoglobulins from mother to baby may be responsible for the reported prevalence of malaria below six months. Placental malaria and HIV

infection have been associated with a reduction in proportions of IgG antibodies transferred from mother to baby (Cumberland *et al.*, 2007).

In order to avert the adverse effects of pregnancy malaria, in April 2000 African leaders committed to halve the mortality due to malaria by 2010 during the Abuja Declaration. The target was to ensure that 60% of all the population at risk are protected/ treated from malaria (WHO, 2000). In order to have comparable data on IPTp coverage across the countries WHO recommended a standard method of collecting data on IPTp use during pregnancy. The WHO Roll Back Malaria (RBM) recommends collecting data on IPTp use in a population by estimating the proportion of all women in a given survey who had a live birth in the last year who reported having received one (or at least two where stated) doses of IPTp during their last pregnancy (Roll Back Malaria, 2004). This is self-reported data and yet self-reported data has been found to be prone to bias and its validity questioned (Hildenwall *et al.*, 2009). Self-reported data may be affected by several factors like selective recall, unawareness of the diagnosis or unwillingness to report. Data on validity of self-reported IPTp use during pregnancy is scanty.

1.1.5 Maternal and newborn immunity and Use of IPTp during pregnancy

Immunity to malaria requires repeated exposure to parasites and studies have shown a rebound susceptibility of infants to malaria after using malaria chemoprophylaxis (Greenwood *et al.*, 1995; O'Meara *et al.*, 2005). A study on pregnant women demonstrated a reduction in antibodies against placental malaria in participants who had used IPTp during pregnancy (Serra-Casas *et al.*, 2010). A study in Kenya demonstrated that using protective measures like use of insecticide treated nets during pregnancy led to reduction of anti-*P. falciparum* antibody levels in the mother (Kariuki *et al.*, 2003). More recently use of IPTp during pregnancy was associated with a reduction in antibody levels to placental malaria (Staalsoe *et al.*, 2004). Using interventions that prevent infection during pregnancy may lead to reduction in infection with consequent reduction in antibody response to *P. falciparum* which may affect antibodies transferred to the baby.

Malaria immune response involves both cell mediated and humoral immunity. Humoral immune response is important for control of blood stage malaria infection.

Several *P. falciparum* antigens have been studied and associated with clinical protection. This thesis focuses on antibody response against four *P. falciparum* blood stage antigens: Glutamine Rich protein (GLURP), Histidine Rich Protein (HRPII), Merozoite Surface Protein 3 (MSP3) and Merozoite Surface Protein 3a (MSP3a). GLURP which is expressed on the merozoite surface and liver stage schizont is present in all stages of the malaria cycle. Antibodies against GLURP have been found to be protective against clinical malaria (Dodoo et al., 2000). MSP3 a merozoite surface antigen is thought to play a role in the initial attachment of the merozoite to the erythrocyte surface. Individuals with high antibody levels against MSP3 have been found to be protected from clinical malaria compared to their counterparts. MSP3 is a target molecule for antibody dependent cellular inhibition which is a protective mechanism during P. *falciparum* infection. MSP3a peptide is formed from one of the highly conserved region on the C terminal of MSP3. MSP3a is a potential candidate for malaria vaccine. Histidine rich protein II is secreted by *P. falciparum* and is expressed on the cell membrane is thought to play a role in the polymerisation of ferriprotoporphyrin IX, a by-product of haemoglobin degradation. MSP3 and GLURLP are components of GMZ2 malaria vaccine. GMZ2 is a recombinant bivalent vaccine containing Glutamine Rich Protein and Merozoite surface protein 3 which is under development for use in malaria endemic areas (Mordmuller *et al.*, 2010).

1.2 Problem Statement

Malaria is one the leading causes of morbidity and mortality in children in Sub-Saharan Africa. The biggest burden lies mainly on children under five years of life who tend to get more frequent attacks and higher parasite density compared to their counterparts (Marsh & Snow, 1999). The infants less than six months of age are protected from severe malaria because of antibodies transferred from mother to baby in utero and presence of fetal hemoglobin (Amaratunga *et al.*, 2011).

Although studies on malaria below six months are rare, there have been increasing reports of increasing prevalence of malaria in this age group (Afolabi *et al.*, 2001; Larru *et al.*, 2009). Factors that affect transfer of anti-*P. falciparum* antibodies across the placenta and prenatal exposure of the fetus to malaria may be responsible for the increased susceptibility in children below six months. Studies have indicated that

babies with high antibody levels at delivery are more protected than their counterparts with low levels (Hogh *et al.*, 1995; Branch *et al.*, 1998). Placental malaria, HIV sero-positivity in the mother , maternal hyperglobinaemia and maternal antibody levels have been found to significantly decrease trans-placental transfer of IgG antibodies in humans (Cumberland *et al.*, 2007).

There is paucity of literature on the possible effect of using intermittent presumptive treatment with sulfadoxine/pyrimethamine in pregnancy on transfer of anti-*P. falciparum* antibodies across the placenta. Yet it has been shown that using interventions like ITNs during pregnancy led to a significant decline in antibodies against key malaria antigens at delivery (Kariuki *et al.*, 2003).

Pregnancy malaria leads to sequestration of parasites in the placenta which may lead to exposure of the fetus to malaria parasite/antigens. Congenital exposure to malaria parasites/antigens in babies born to mothers living in malaria endemic areas is not uncommon. Exposure of the fetus to key malaria antigens in utero may lead to priming of the fetal immune system. Since IgG and IgM production by the fetus starts at around 10 weeks of gestation, exposure to antigens leads to immune priming. It has been previously shown that immunologically primed newborns are more susceptible to malaria in infancy and babies born to mothers with placental malaria are more susceptible to malaria than their counterparts (Bonner *et al.*, 2005; Mutabingwa *et al.*, 2005; Malhotra *et al.*, 2009). Data on the effect of using SP IPTp during pregnancy on congenital malaria and fetal immune priming in this setting was not well documented prior to the onset of these studies

WHO recommends that in order to get uniform data across countries on use of IPTp, in cases where SP is not given as a directly observed therapy, self-reported data should be used. Some studies have questioned the validity of self-reported data since it is prone to bias and may not be reliable. The validity of self-reported use of IPTp in pregnant women delivering in Mulago hospital had not been documented.

The results of the current studies are important in informing policy on the possible effects of the present interventions of SP IPTp on the immune response of the newborn to key malaria antigens and validity of self-reported data.

1.3 Objectives of the study

1.3.1 General objective

To determine the relationship between use of SP IPTp during pregnancy and newborn and maternal *P. falciparum* infection and immunity.

1.3.2 Specific objectives

i. To establish the malaria burden in pregnancy at Mulago National Referral Hospital in Kampala, Uganda.
ii. To determine the association between use of SP IPT during pregnancy and proportions of selected anti-*Plasmodium falciparum* blood stage IgG antibodies transferred from the mother to neonate.
iii. To determine the association between use of SP IPT during pregnancy on congenital malaria and immune priming of the fetus.
iv. To validate self-reported use of SP IPTp during pregnancy.

1.3.3 Study Hypothesis

I. The use of sulfadoxine/pyrimethamine intermittent presumptive treatment does not affect the proportions of selected anti-*Plasmodium falciparum* transferred from mother to baby
II. The use of SP IPTp is not associated with protection of the newborn against congenital malaria and immune priming of the fetus.

Alternate Hypothesis

I. Using SP IPTp during pregnancy reduces the proportions of selected anti *Plasmodium falciparum* transferred from mother to baby
II. The use of SP IPTp is protective of congenital malaria and immune priming of the fetus.

1.4 Organization of the Thesis

This thesis is divided into six chapters. In chapter one of this thesis, I have presented the burden on malaria and specifically in pregnancy globally and nationally. Then stated the problem addressed by the thesis and specific objectives of the study. Critical review of the literature on the subject including immune protection of the infant, trans-placental transfer of immunoglobulins and congenital malaria is presented in chapter two of the thesis. In chapter three, I have presented methods used including the study setting, methods and data analysis tests for each specific objective of the study. Results and discussion are presented in chapter five and six respectively. General conclusion of the thesis and recommendations are presented in chapter six. The published papers and manuscripts originating from this thesis are attached.

CHAPTER TWO: LITERATURE REVIEW

2.1 Immune protection of infant against malaria

Infants under six months of age born to mothers in living in malaria endemic areas tend to be protected against episodes of clinical malaria and death from severe malaria. The possible factors responsible for the apparent protection include antimalarial antibodies transferred from the mother, unique nutrition (breast milk), and the presence of fetal haemoglobin (HbF). The protection from maternally derived antibodies has been demonstrated in many studies previously (Sehgal *et al.*, 1989; Hogh, 1995; Deloron *et al.*, 1997). Other studies have shown that maternally derived antibodies have little if any effect on measures of disease susceptibility in infants, including time to first parasite infection, density of parasites in the blood, and incidence of febrile episodes (Riley *et al.*, 2000; Riley *et al.*, 2001).

P. falciparum parasites have been shown to grow much more slowly in Red Blood Cells containing fetal haemoglobin (HbF) than in cells with adult haemoglobin (Billig & Mckenzi 2012) and yet another study demonstrated no difference (Amaratunga *et al.*, 2011). Infant susceptibility to *Plasmodium falciparum* malaria increases substantially as fetal hemoglobin (HbF) and maternal immune IgG disappear from the circulation. It's therefore difficult to determine the individual protective efficacy of either maternally derived antibodies or fetal haemoglobin. In normal children synthesis of HbF ceases shortly before birth and levels remain constant for about 15 days and 40 days for term and premature babies respectively followed by a linear decrease in both (Colombo *et al.*, 1976).

Anti-*P. falciparum* antibodies levels in maternal serum and in cord-blood or neonatal heel prick serum are highly correlated but absolute concentrations tend to be lower in babies compared to their mothers (Cot *et al.*, 2003). Infants in hyper-endemic areas tend to have a shorter duration of protection from malaria compared to those in holo-endemic areas. It has been suggested that, the duration of protection may be explained by the rate of decay of HbF which in turn is determined by the rate of turnover of RBCs (Riley *et al.*, 2001).

It has been proposed that protection against malaria in infancy involves cooperation between HbF and maternal IgG that work together to impair the cytoadherence of parasitized RBCs (Amaratunga *et al.*, 2011). This explains the conflicting results on the role of protection by the maternally derived antibodies or HbF alone in protection against malaria in the first six months of life.

Another proposed explanation for slow growth of malaria parasites in infants is that their diet may lack some of the essential nutrients for parasite growth. For example, parasites are absolutely dependent on an external source of p-amino benzoic acid (pABA) (Kicska *et al.*, 2003), levels of which are low in breast milk. It has been proposed that relative protection of infants may be due to the fact that they are kept well covered and have good stores of subcutaneous fat which make it more difficult for mosquitoes to locate a capillary from which to feed.

There is a decreased risk of developing clinical malaria in infants with high levels of anti-*P. falciparum* antibodies at the time of delivery (Branch *et al.*, 1998). Maternal and cord blood antibodies correlate at birth with levels in the mother generally lower (Deloron *et al.*, 1997). Factors that affect the levels of antibodies to P. *falciparum* in the mother during pregnancy may consequently affect the amount of antibody in the newborn and therefore immune protection in the infancy. It has been suggested that factors that affect exposure to parasites may lead to consequent reduction in antibody levels. A previous study done in Kenya, on pregnant women, found that the use of ITNs was associated with a reduction in antibody response to some *P. falciparum* antigens (Kariuki *et al.*, 2003). Intermittent presumptive treatment in infants has been found to lead to decrease in the incidence of clinical malaria however with increased susceptibility after stopping the treatment (Greenwood *et al.*, 2006). In pregnancy, using IPTp leads to reduction in levels of antibodies which protect against pregnancy associated placental malaria (Trine Staalsoe *et al.*, 2004).

2.2 Trans-placental Transfer of Immunoglobulins

Humoral immune protection of neonates during the first extra-uterine months of life is conferred through pathogen specific Immunoglobulin G (IgG) transferred from mother to fetus. The proportion of IgG antibodies transferred from mother to baby in

addition to being affected by the levels in the mother can be affected by the efficiency of transfer across the placental barrier.

The trans-placental transport of Immunoglobulin G (IgG) is an active, FcRn receptor mediated process. The transfer involves binding of IgG by Fc receptors to the surface of syncytiotrophoblasts, which is actively transported across the cell, and released into the fetal bloodstream (Simister, 2003). Fetal IgG rises from approximately 10% of the maternal concentration between 17-22 weeks of gestation to 50% at 28-32weeks of gestation. Gestational age at delivery will affect the proportion of pathogen specific antibodies transferred from mother to baby. Premature and LBW babies generally have fewer antibodies transferred from the mother compared to their counterparts (Okoko *et al.*, 2002; van den Berg *et al.*, 2011).

Trans-placental transfer of immunoglobulins generally depends on a range of factors including: placental integrity, the total IgG concentration in maternal blood, the gestational age of the fetus at delivery and the IgG subclass involved (Deloron *et al.*, 1997). In order to have efficient transfer of antibodies across the placenta, the placental barrier should be intact. Factors that lead to destruction of placental barrier have been associated with reduction of antibodies transferred. Several studies have demonstrated that placental malaria (Owens *et al.*, 2006), maternal HIV infection and maternal hypergammaglobulinemia at delivery are associated with reduction in antibodies transferred from mother to baby (Okoko *et al.*, 2001; Cumberland *et al.*, 2007). Maternal age, weight, height or parity do not affect antibody transfer across the placenta (Wesumperuma *et al.*, 1999; van den Berg *et al.*, 2011). Maternal immunization against pertussis antigen during the antenatal period has been associated with increased transfer and high levels of antibody in the first one month of life (Leuridan *et al.*, 2011). The influence of different factors on antibody transfer may vary from region to region. How the different maternal factors including IPTp use in pregnancy affects the transfer of blood stage anti-*P. falciparum* antibodies in Uganda has not been evaluated.

2.3 Placental Malaria

The red blood cells infected with *P. falciparum* parasites are sequestered in the placenta by binding to membrane proteins found especially in the endothelium. *P. falciparum* infected erythrocytes express variant proteins on their surface which interact with various endothelial proteins resulting in adherence and sequestration. Chrondrion sulphate A, non-immune immunoglobulin G and Hyaluronic acid (Beeson *et al*., 2000; Flick *et al.*, 2001; Fried *et al.*, 2006) are the proteins expressed in the placenta on to which infected Red Blood Cells (iRBCs) bind.

Since the sub population of parasites capable of binding the receptors in the placenta will not affect the non-pregnant women, primigravidae have never been primed against this subpopulation. This explains why primigravidae are more susceptible to higher morbidities and mortalities associated with pregnancy malaria. Eventually the mother develops humoral response by producing antibodies which prevent binding of the iRBCs on the placenta receptors. It has been previously demonstrated that multigravidae have higher levels of anti-adhesion antibodies compared to primigravidae (O'Neil-Dunne *et al.*, 2001). Placental malaria affects the integrity of the placental barrier and consequently the proportion of antibodies transferred from mother to baby.

Sequestration of malaria parasites in the placenta leads to alteration in the synstiotrophoblast layer. This alteration leads to exposure of the foetus to malaria parasites and/or antigens. Since the fetus is immunologically active this leads to activation of the immune system, priming of the B cells and differentiation of Ag specific lymphocytes to memory T cells.

Immunoglobulin G antibodies are the only antibodies that cross the trans-placental barrier. The presence of malaria specific antibodies (IgM and/or IgE) in cord blood has been used as evidence of *in utero* activation (priming) of B cells. Data on the prevalence of in-utero priming in malaria endemic areas is scanty. Placental malaria, cord parasiteamia and anaemia have been associated with presence of IgM in the baby at delivery (Gouling *et al.*, 2003). It has been suggested that in utero priming of the fetus may be important in immune response to the same antigen in infancy as it would be a secondary as opposed to primary response. The extent of fetal priming in

Kampala Uganda, a mesoendemic setting is not known and how this may be affected by maternal use of SP IPTp during pregnancy.

2.4 Congenital malaria

Congenital malaria, defined as malaria parasitaemia in the first week of life can be acquired transplacentally or during the time of birth. It can be diagnosed using microscopy, rapid diagnostic tests and polymerase chain reaction (PCR). PCR is important in diagnosing the submicrospoc infection. It's difficult to distinguish between transplacental malaria from that acquired during delivery. A newborn with congenital malaria presents with symptoms like poor feeding, lethargy, anaemia , hepatospleenomegally , jaundice ,irritability , drowsiness, respiratory distress and fever (Ibhanesebhor, 1995). These clinical features are similar to signs and symptoms of other neonatal illnesses like neonatal sepsis (Ekanem & Udo, 2008).

Congenital malaria in babies born to semi-immune individuals is rare due to protection conferred by the placental barrier, presence of fetal heamoglobin and maternally derived antibodies (Amaratunga *et al.*, 2011). The diagnosis is made by finding malaria parasites in peripheral or cord blood of the newborn. The prevalence is higher in the cord blood compared to peripheral blood. The prevalence of congenital malaria in Africa varies between 0-46% (Uneke, 2007; Mwaniki *et al.*, 2010; Osungbade & Oladunjoye, 2012). The prevalence may differ depending on the method used for detection of parasites. A PCR method is more sensitive with its ability to detect the parasite macromolecules and not necessarily parasites. The clinical importance of the PCR diagnosis of congenital malaria is not very clear.

The burden of Congenital Malaria is higher in babies born to mothers with low levels of IgG antibodies specific for *P. falciparum* lysate (Naniche *et al.*, 2012). Mothers with pregnancy malaria at delivery including placental and peripheral parasiteamia are at an increased risk of delivering babies with congenital malaria and the risk increases with increasing parasite density (Redd, 1996). Primigravidae and HIV infected mothers are at a higher risk of delivering babies with congenital malaria (Oduwole *et al*.,, 2011; Naniche *et al.*, 2012). Cord parasiteamia has been associated with placental malaria and high parasite density (Ouedraogo *et al.*, 2012). Placental malaria leads to destruction of the placental barrier which consequently leads to transmission of parasites to the fetus.

Congenital malaria if untreated can lead to anemia and death. Since malaria below six months of age was considered rare, they were excluded from clinical trials. Consequently there are no guidelines for treating uncomplicated malaria in this age group yet some oral antimalarials are contraindicated (D'Alessandro *et al.*, 2012). Studies to evaluate the best antimalarials and dosage for this age group are required.

IgM antibodies do not cross the placenta. Presence of these antibodies in fetal blood implies that the fetus was exposed to malaria parasites or antigens leading to stimulation of the immune system. Since the fetal immune system is active as early as 17 weeks of gestation, its exposure to antigens leads to stimulation. Priming of the fetus has been associated with increased susceptibility to infection during the first year of life.

The impact of using IPTp on congenital malaria and immune priming of the fetus is not clear. In this study the effect of using IPTp during pregnancy on intrauterine immune priming *and* congenital malaria is examined.

2.5 Adverse effects of pregnancy malaria

It is estimated that 11.4 % of neonatal deaths and 5.7 % of all infant deaths in malaria–endemic areas of Africa may be caused by malaria in pregnancy–associated low birth weight (Snow, 2004). In high transmission areas low birth weight is predominantly caused by IUGR because of the relative immunity that prevents most febrile episodes. Malaria in pregnancy in endemic areas may be responsible for up to 70% of IUGR, whereas its contribution to low birth weight is at 36% (Snow, 2004). Placental malaria has been independently associated with a decrease in antibody transfer across the placenta (Okoko , *et al.* 2001).

WHO recommends use of IPTp, INTs and effective case management for control of malaria in pregnancy .The development of 2005-2015 RBM strategic plans 80% coverage of the preventive interventions among those at risk. Estimates on IPTp coverage during pregnancy are based on self-reporting and yet studies have questioned the validity of self-reported data (Hildenwall *et al.*, 2009).

Reduction in exposure to *P. falciparum* brought about by malaria prevention strategies, such as chemoprophylaxis, might interfere with the development of malaria-specific immunity (Gosling *et al.*, 2009). Use of IPTp in infants has been associated with interference with malaria immunity and consequently making them more susceptible to malarial infection after the intervention (Greenwood, 2006). Studies assessing the effect of interventions during pregnancy on maternal immunity are scarce. It is therefore likely that a successful regimen of intermittent presumptive treatment in pregnancy with SP could decrease exposure to malaria in pregnancy and antibody titres to key malaria antigens. Since malaria antibodies are short lived, IPT could reduce synthesis, maintenance and transfer of antimalarial antibodies to the newborn thus compromising protection to the infant against malaria. These reduce the episodes of malaria in the mother during pregnancy. The presence of malaria parasites boost antibody responses and high titres of antibodies to many malarial antigens are found in pregnant women.

2.6 Prevention of pregnancy malaria

Plasmodium parasites are transmitted mostly through the bites of *Anopheles* mosquitoes. Malaria infection can be prevented by protection from mosquito bites by using Insecticide treated nets (ITNs) and/or indoor spraying with residual insecticides (IRS). In high malaria transmission, women have acquired a protective immunity prior to pregnancy and malaria infections are generally asymptomatic. During pregnancy in addition to protection from mosquito bites, the WHO recommends using intermittent preventive treatment (IPTp) with sulfadoxine/pyrimethamine (SP) at least twice, during the second and third trimesters of pregnancy (WHO, 2004). It has been previously demonstrated that use of SP as IPTp is effective in prevention of malaria in pregnancy (Parise *et al.*, 1998). A recent meta- analysis indicates IPTp with 3 or more doses of sulfadoxine/pyrimethamine was associated with a higher birth weight and lower risk of LBW than the standard 2-dose regimens in in sub-Saharan Africa (Kayentao *et al.*, 2013). A study done by Ndomugyenyi and others found no significant benefit in providing both IPTp and ITNs during routine antenatal services in an area with low endemic malaria (Ndyomugyenyi *et al.*, 2011).

Malaria parasites resistant to SP has been reported in Africa (Sendagire *et al.*, 2005), it is however still effective as IPTp during pregnancy (Feiko *et al.*, 2007).

Studies have shown increasing prevalence of resistant parasites in many countries after introducing IPTp (Mockenhaupt *et al.*, 2008; Taylor *et al.*, 2012; Lin *et al.*, 2013). It has been suggested that additional emphasis should be put on screening pregnant women for malaria parasites in areas with prevalent SP resistance even when they are already on IPTp (Lin *et al.*, 2013). One study in Muheza, Tanzania demonstrated that IPTp does not improve overall pregnancy outcomes in areas where SP-resistant parasites predominate and may increase the odds of fetal anemia. The same study suggested that increasing prevalence of parasite resistance to SP in a community may have an overall effect from net benefit to neutral or harm (Harrington *et al.*, 2011). WHO still recommends using SP IPTp despite the reported resistance and emphasizes the importance of continued surveillance as we look for an alternative drug (WHO Malaria Policy Advisory committee and secretariat, 2012).

The effectiveness of SP IPTp in Africa may be comprised as resistance to SP increases. There is therefore urgent need to find alternatives to SP IPTp in pregnant mothers living in malaria endemic areas. Studies are under way to identify a suitable alternative to SP as IPTp although none of the potential candidates have the favourable characteristics of SP when used in areas where parasites are sensitive to this drug. An alternative to failing SP IPTp is intermittent screening and treatment of women who are positive with an effective antimalarial and vector control. A study done in Ghana, in an area of moderately high malaria transmission found that intermittent screening and treatment with either SP or amodiaquine+artesunate (AQ+AS) is safe and effective strategy for the control of malaria in pregnancy (Tagbor *et al.*, 2010). This has a challenge of making available in all centers offering antenatal care facilities for screening for malaria and offering effective antimalarial drugs.

Folic acid supplementation during pregnancy is used widely, to prevent neural tube defects given in the first trimester and in the second and third trimesters, together with iron, to prevent nutritional anemia in developing countries. Since SP exerts its antimalarial activity by interfering with the folic acid metabolism of the parasite, it has been suggested that concurrent administration of folic acid with SP IPTp may interfere with SP to confer protection. High doses of folate were associated with

treatment failure with SP in pregnant mothers with uncomplicated malaria (van Eijk *et al.*, 2008) and children (Dzinjalamala *et al.*, 2005). In areas of low SP resistance however, administration of folic acid to pregnant women in a dose of 500-1,500 µg/day does not interfere with the protective effect of SP when used for IPTp (Mbaye *et al.*, 2006).

In view of the increasing resistance to SP alternative drugs are needed for IPTp. An ideal antimalarial drug or drug combination for IPTp should be safe, well tolerated, and efficacious in the clearance of malaria parasites, provide a long period of chemoprotection, available, affordable and should be given as a single dose. Mefloquine is viable alternative to SP because it is long acting and safe in pregnancy (Vanhauwere *et al.*, 1998; Schlagenhauf *et al.*, 2012). Mefloquine is a slowly eliminated 4-quinoline-methanol that acts against the asexual stages of the malaria parasite. Mefloquine was more efficacious than SP in preventing placental malaria (1.7% vs. 4.4%; P = 0.005) and clinical episodes but was associated with more side effects which included vomiting and dizziness (Briand *et al.*, 2009). Mefloquine has been found to be effective as IPTp in HIV infected pregnant women. Moderate and severe adverse reactions were more frequent when antiretrovirals were started concomitantly with a MQ intake (Denoeud-Ndam *et al.*, 2012).

Azithromycin is a slow-acting macrolide that produces delayed-death in malaria parasites by causing the progeny of exposed parasites to inherit an apicoplast incapable of protein synthesis has slow onset and is relatively weak (Retsema & Fu, 2001; Dahl & Rosenthal, 2008). Azrithromycin is considered generally safe in pregnancy (Sarkar *et al.*, 2006) and has been suggested as a candidate for IPTp. It can be given in combination with either chroloquine or SP (Chico *et al.*, 2008). In addition azithromycin has an added advantage of reducing sexually transmitted infections and preventing pneumococcol infections during pregnancy (Chico & Chandramoham, 2011). A combination of sulfadoxine/ pyrimethamine and amodiaquine has been evaluated as IPTp and was found to be effective but associated with more adverse effects like vomiting compared to SP alone (Clerk *et al.*, 2008).

The ACTs have a good safety profile in the second and third trimester of pregnancy (Manyando *et al.*, 2012). It is likely to provide little benefit as IPTp because it is short

acting, still expensive and has a high pill burden. Other potential alternatives for SP under evaluation are chlorproguanil-dapsone and piperaquine.

During pregnancy, HIV infection has been shown to impair the ability to control *Plasmodium falciparum* infection. HIV-positive pregnant women are more likely to have detectable parasitaemia, higher malaria parasite densities, develop clinical or placental malaria and malarial anaemia than HIV-negative pregnant women (ter Kuile *et al.*, 2004; Orish *et al.*, 2013). Human immunodeficiency virus was also associated with more frequent peripheral parasitemia in multigravidae and congenital malaria (Perrault *et al.*, 2009). Some studies have suggested that placental malaria could increase the risk of HIV transmission from mother to child (Brahmbhatt *et al.*, 2003; Brahmbhatt *et al.*, 2008) and not others (Msamanga *et al.,* 2009). The interaction between malaria and HIV makes the prevention of malaria in HIV-positive pregnant women a public health priority. Monthly SP IPTp is more efficacious than a 2-dose regimen in preventing placental malaria in HIV pregnant women (Filler *et al.*, 2006). HIV positive pregnant women on co-trimoxazole prophylaxis – a drug recommended for all HIV positive patients for prevention of infection cannot receive SP. There is therefore a need to identify alternative for SP in HIV positive pregnant women (Mathanga *et al.*, 2011).

2.7 Validity of self-reported data

In malaria endemic areas policy makers are faced with the task of motoring uptake of preventive measures like IPTp. It is important to have a standard way of collecting this data so that it can be comparable across countries as we struggle to achieve the millennium development goals (MDGs). The WHO recommends either recording the number of pregnant women taking IPTp from the clinic under directly observed therapy or self-reported use by the pregnant mother (Roll Back Malaria, 2004). Health workers are faced with challenges of delivering IPTp by Directly Observed Therapy (DOT) like inadequate supply of drugs, poor staffing and yet self-reported data has been suggested to be prone to bias. Studies done in Africa looking at self-reported history of taking antimalarial drugs prior to coming to hospital has been found to be inadequate (Hodel *et al.*, 2009; Hildenwall *et al.*, 2009). We report the assessment of self-reported history of use of IPTp during pregnancy in patients delivering in Mulago National Referral Hospital.

CHAPTER THREE: METHODS

3.1 Study Setting

The studies were conducted in Mulago National Refferal Hospital, located in Kampala the capital city of Uganda. In Uganda there is stable *Plasmodium falciparum* malaria in 95% of the country. The remaining 5% of the country which is mainly the highland areas has unstable malaria and are epidemic prone. According to 2009 Uganda Malaria Indicator Survey, the prevalence of malaria in Kampala (children below 59 months) was 3.4% and 7.6% by peripheral parasiteamia and Rapid Diagnostic tests respectively. This was a household survey where one hundred and eighteen children were tested. The majority of participants included from the study were residing in Kampala and Wakiso districts Table 1. Kampala district is bordered by Wakiso district to the south, west, and north and to the east as shown in Figure 3. According to the Uganda Bureau of Statistics the population of Kampala was estimated to be 1,659,600 people in 2011. Kampala is located 1,300–1,500 m above the sea level; it is close to the equator and has a tropical climate with rainfalls throughout the year. The population in the area experiences low-intermediate malaria transmission with the highest peaks toward the end of the two major rainy seasons (March to May and October to December). In 2010 Uganda Bureau of Statistics estimated the population of Wakiso to be 1,310,000.

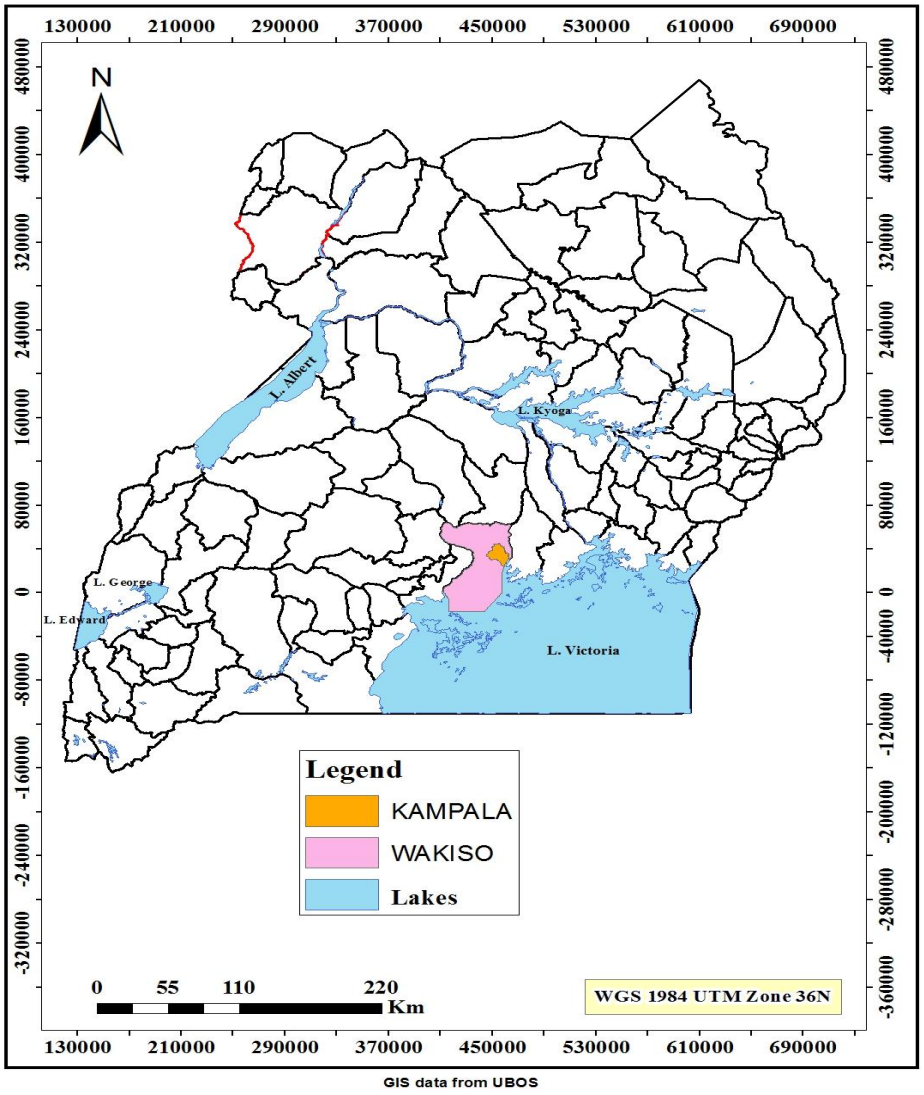


Figure 3: Location of Kampala and Wakiso districts in Uganda.

Mulago hospital serves as Uganda´s national referral hospital and is situated in the capital city, Kampala. It serves as a hospital for people around Kampala and as a National referral Hospital. Mulago Hospital department of Obstetrics and Gynaecology has two antenatal clinics and two labour wards. The upper Mulago antenatal clinic (ANC) run mainly by the senior midwives screens all pregnant mothers coming to attend ANC in the Hospital.

The antenatal clinic in Upper Mulago runs five times a week with an average of 300 patients per day. In the ANC as a routine, all pregnant mothers are tested for HIV in an effort to reduce mother to child transmission. After screening, patients with high risk pregnancies like grand multi-parity, multiple pregnancy, diabetes in pregnancy, high blood pressure are referred to Lower Mulago ANC clinic where they are

appropriately managed by the obstetricians. Upper Mulago labour ward admits only patients who have attended the respective antenatal clinic.

Lower Mulago labour ward admits in addition to patients who have attended in the respective ANC, referrals from different hospitals, Upper Mulago maternity centre labour ward and self-referrals. Between the two labour wards in Mulago hospital about 33,000 deliveries are conducted annually (Department obstetrics and Gynaecology Hospital records 2014). As part of the antenatal package all mothers are provided with provider initiated antenatal HIV testing (Opt out) as a standard of care. Routine HIV counseling and testing is done for mothers who missed the opportunity in the antenatal period. The prevalence of HIV in Uganda according to 2011 Uganda AIDS indicator survey is 7.3% in adults 15-49 years.

3.2 Substudy I (Paper I)

3.2.1 Objective:

To establish the burden in pregnancy malaria at Mulago National Referral Hospital in Kampala, Uganda.

3.2.2 Study population and data collection

In a cross-sectional study done from October 2004 to January 2005, delivering at the Mulago Hospital labour suite, aged >=15 years and >=28 weeks of gestation, were recruited in to this study. Patients with cardiac disease, chronic hypertension, renal disease, clinical AIDS, or diabetes and those with obstetric complications during the present pregnancy, such as preeclampsia, eclampsia, antepartum haemorrhage, and chorioamnionititis, were excluded from the study. On average five to seven participants were recruited consecutively per day, from 8.00 am to 5.00 pm excluding weekends and public holidays.

Using a pre-coded, standardized questionnaire data on pregnancy history, clinical examination outcome, and pregnancy outcome for each study subject were recorded. Some key aspects covered included area of residence, age, marital status, occupation, education, parity, visits to antenatal clinic (ANC), bednet use, use of intermittent preventive antimalarial treatment (IPT), iron and folic acid

supplementation, gestational age, birth status (live or stillbirth) and birthweight. The information on use of IPT, iron and folic acid supplementation was obtained from interview and /or antenatal card.

3.2.3 Sample collection and laboratory studies

Venous blood was collected aseptically within a few hours (2-4 hrs) prior to delivery for peripheral blood diagnosis of malaria and for haemoglobin estimation.. After delivery, the placentas were collected in 0.9% NaCl for smear and histological assessment of malaria. A small incision was made paracentric on the maternal facing side of the placentas to prepare blood films. Thick and thin blood films of peripheral and placental blood were stained by Giemsa and malaria diagnosis was assessed by microscopy following standard procedures. A small biopsy of the maternal-facing surface of each collected placenta was also removed and preserved in 10% neutral buffered formalin. The biopsies were paraffin embedded and stained with haematoxylin and eosin for histological evaluation of placental malaria infection. The slides were examined by a pathologist blinded to other patient data. Re-examination was performed in all cases where histology and blood films were in disagreement. For quality control 10% of the samples were re-examined another experienced pathologist blinded to the patients' data.

3.2.3 Definitions

After histological assessment of the placenta for malaria ,the results were classified according to the following criteria (Bulmer *et al.*, 1993a; Bulmer *et al.*, 1993b): a) *active acute infection*: parasites present in maternal erythrocytes, b) *active chronic infection*: presence of parasites and a significant amount of pigment deposition in fibrin or monocytes within fibrin, c) *past infection:* presence of pigment within fibrin only, no parasites, d) *not infected:* no evidence of parasites or pigment. Low birthweight is defined as weight <2500 g. Anaemia is defined as haemoglobin (Hb) level <11 g/dl and severe anaemia as Hb <7 g/dl. Preterm delivery is regarded as deliveries occurring prior to 37 weeks of gestation.

3.3 Substudy II (Paper II)

3.3.1Objective

To validate self-reported use of Sulfadoxine/pyrimethamine IPTp during pregnancy.

3.3.2 Study population and data collection

Two hundred and four pregnant women admitted at Mulago National Referral Hospital labour suite were enrolled into a cross–sectional study after informed oral and written consent. Data on pregnancy history, socio-economic indicators and pregnancy outcome was collected using a pre-coded standardized questionnaire. Key aspects recorded included area of residence, age, marital status, occupation, education, parity, visits to antenatal clinic (ANC) and bed net use. Birth weight of baby was taken after delivery. In addition, information on use of IPT for prevention of malaria during that pregnancy, the drug administered, number of IPTp doses taken, history of taking sulpha-containing drugs such as co-trimoxazole, history of fever during pregnancy, and use of antimalarial drugs was recorded. The date on which the IPTp was taken was noted in the questionnaire. This information was collected from the participants' self-report and antenatal card where available. In cases where the patient was not able to state the dates with certainty, it was then recorded it as the 15^{th} day of that particular month. This information was used to estimate the gestation age corresponding to when the IPTp was taken.

3.3.3 Sample collection and laboratory studies

Before delivery of baby, mother's venous blood was collected for microscopy to detect parasites, for haemoglobin estimation and sulfadoxine (SDX) detection. Blood was collected in EDTA anticoagulant containing tubes, centrifuged, plasma separated and stored at $-70^{o}C$ until drug assays.

Thick blood smears were made from the maternal venous blood and cord blood. These were stained using the Geimsa stain and examined using standard procedures. At least 200 HPF were examined before confirming that the slide was negative. This was done by two independent microscopist and in case of discrepancy, a tie breaker was used.

3.3.4 Serum Sulfadoxine determination

The validity of self-reported SP IPTp use during pregnancy was assessed by determining the SP level in maternal sera. Sulfadoxine/pyrimethamine in the maternal sera was determined using High Performance Liquid Chromatography (HPLC). Sulfadoxine (SDX) was used as a proxy for SP. The regimen is given as a combined dose comprising 1500 mg sulfadoxine and 75mg pyrimethamine. The analysis was done according to the method described by Bergqvist *et al*., 1987 the limit of quantification for SDX was 15 µM. Basing on the average Cmax for sulfadoxine of 260 (Green *et al.*, 2007; Nyunt *et al.*, 2010) and assuming a $T_{1/2}$ for Sulfadoxine of five to nine days, participants who reported as having taken IPTp before 20 weeks of their pregnancy were excluded from the analysis for self-report validation. All plasma specimens were analyzed twice along with calibration standards and quality controls. To prevent bias, the HPLC analysts were blinded to the data of self-reported IPTp uptake and composition of quality control samples.

3.4 Substudy III (Paper III)

3.4.1 Objective

To determine the association between use of SP IPT during pregnancy and proportions of selected anti- *P. falciparum* blood stage IgG antibodies transferred from the mother to neonate.

3.4.2 Study population and data collection

Pregnant mothers admitted in Mulago Hospital labour ward were screened. Mothers admitted in Mulago Hospital labour suite (Upper Mulago Labour suite) aged 15 years and above were recruited after oral and written informed consent in a cross sectional study. Pregnant mothers below 28 weeks of gestation and with obstetric complications during the present pregnancy. Mothers with preeclampsia, eclampsia, Diabetes in pregnancy and chorioamnionitis were excluded from the study. These were excluded because of the known associated placental pathology that may affect transplacental transfer of immunoglobulin.

A pre-coded; standardized questionnaire was used to record pregnancy history, clinical examination findings, and pregnancy outcome for each study participants. Some key aspects covered included place of residence, age, marital status, occupation, education, parity, visits to antenatal clinic (ANC), bed net use, use of intermittent preventive antimalarial treatment (IPT), number of IPTp doses taken, HIV status was noted, gestational age, birth status (live or stillbirth) and birth weight of the baby.

The mothers were asked whether they took IPT for prevention of malaria during that pregnancy, history of fever during pregnancy and any antimalarial drugs used. The date on which the IPTp was taken was noted in the questionnaire. In cases where the patient was not able to state the dates with certainty we estimated assuming it as 15^{th} day of that particular month. This information was used to estimate the gestation age when the IPTp was taken.

Maternal venous blood was collected within four hours prior to delivery for peripheral blood diagnosis of malaria and determining antibody levels. After delivery the baby's cord blood was taken aspetically for thick smear preparation and determination of anti-*P. falciparum* antibodies. The maternal and cord serum was kept at -70^{o}C till the analysis for antibodies and sulfadoxine was done.

3.4.3 Anti-P. *falciparum* antibody determination

The antibody response to four synthetic *P. falciparum* blood antigens were determined in the maternal and cord sera by indirect enzyme linked immunorsobent assay (ELISA).The blood stage antigens included merozoite surface protein (MSP); MSP3 and MSP3a, glutamate-rich protein (GLURP) and Histidine Rich Protein II (HRPII). These antigens are expressed during the asexual blood stage *P. falciparum* infection. The antigens were chosen because studies have shown that they are important in the immune protection during the blood stage infection and are therefore potential vaccine candidates.

Merozoite surface proteins (MSP) are named Msp1-19 according to the order of discovery. Antibodies against merozoite surface proteins have been found to be

protective against clinical malaria and it has been suggested as a potential malaria vaccine candidate.

Glutamate-Rich Protein (GLURP) is present in all the developmental stages of *Plasmodium falciparum* in humans, including on the surface of newly released merozoites (Borre *et al.*, 1991) and it's a potential for development of the malaria vaccine. GLURP-specific antibodies have been found to be associated with protection against clinical disease and against high levels of parasitemia (King *et al.*, 1989; Dodoo *et al.*, 2000). GLURP antigen is therefore of interest as a target for protective immune responses against *P. falciparum*.

Histidine rich protein-2 is secreted by *P. falciparum* into the host erythrocyte cytosol and is expressed on the cell membrane or associated with the cytoskeleton of infected erythrocytes. Histidine rich protein-2 is thought to polymerise free haem to form haemozoin. It is also thought to play a role in the polymerisation of ferriprotoporphyrin IX, a by-product of haemoglobin degradation.

Synthetic antigens

The amino acid sequence of synthetic peptides representing *P. falciparum asexual* blood stage *proteins* included;

i. GLURP :(NH2)CGDKNEKGQHEIVEVEEILPEGC(CONH2),
ii. HRP II: (NH2)GCAHHAADAHHAADAHHAADAHHAADGC(CONH2),
iii. MSP3a :(NH2)TLAGLIKGNNQIDSTLKDLV(CONH2),
iv. MSP3: (NH2)AKEASSYDYILGWEFGGGVPEHKKEEN(CONH2).

These synthetic antigens were prepared as described (Borre, 1991; Theisen, 1998; Soe, 2004). Plasma antibody determinations in the maternal and cord sera were done by indirect ELISA, as described by (Theisen *et al.*, 1998). In brief, microtiter plates (Nunc, Roskilde, Denmark) were coated with recombinant protein 100micrograms per well, incubated overnight at 4^0C, for at least 12 hours and blocked with 5% skimmed milk for one hour at room temperature. Plasma samples diluted 1: 200 were added in duplicate and incubated at room temperature for one hour. Plasma sample of the mother and the corresponding neonate (mother/baby pairs) were run on the same plate in all cases. Plates were washed with washing buffer four times waiting for one minute before discarding between steps. The plates

were washed after coating with the antigens before blocking and then between blocking and putting a primary antibody (serum). Plates were developed by Peroxidase conjugated goat anti-human IgG (secondary antibody). Bound secondary antibody was quantified by colouring with ready to use TMB (*3,3', 5,5'-Tetramethylbenzidine*) substrate. Optical density (OD) was read at 450 nm with a reference at 620nm in a plate reader. The mean OD of the sample was determined from the duplicate wells. A value of two standard deviations above the mean absorbance of the samples from unexposed control donors was used as the negative cut-off. The antibody level in the sample was considered to be proportionate to the absorbance determined by ELISA.

3.5 Substudy IV (Paper IV)

3.5.1 Objective

To determine the association between use of SP IPT during pregnancy on congenital malaria and immune priming of the fetus.

3.5.2 Study population and data collection

A total of 150 mother baby pairs analysed in this Substudy were part of those recruited in Substudy III above. Consecutive sampling was done taking into account availability of sera for laboratory analysis.

Mothers admitted in labour at 28 weeks of gestation and above were recruited into the study. Excluded mothers with pre eclampsia, diabetes in pregnancy, cardiac disease in pregnancy and multiple pregnancy. At enrollment data on demographic characteristics antenatal care, use of IPTp and use of bed net was documented using an interviewer administered questionnaire.

3.5.3 Anti-P. *falciparum* antibody determination

IgM antibody levels in maternal and cord sera against four P. *falciparum* blood stage antigens GLURP, HRPII, MSP3 and MSP3a were determined using Indirect ELISA as described in Substudy III above. The plates in this case were developed by anti-human IgM as the secondary antibody. Bound secondary antibody was quantified by

colouring with ready to use TMB (*3,3', 5,5'-Tetramethylbenzidine*) substrate. Optical density (OD) was read at 450 nm with a reference at 620nm in a plate reader. In all cases corresponding maternal and cord sera were run on the same plate in duplicates .Mean value of the negative control plus 2 standard deviations was considered as cut off for negative IgM.

3.6 Statistical Analysis

3.6.1 Substudy 1

Data was cleaned and entered using Epi Info version 6.1 and exported to SPSS version 12.0 for further analysis. Placental *P. falciparum* infection, birth weight and maternal aneamia were taken as principal outcomes. The potential confounders included place of residence, age, literacy, use of bed net, iron and folic acid supplementation and use of IPTp during pregnancy. Using logistic regression, adjusted odds ratios were obtained .The significance of each variable was obtained was reconsidered by backward stepwise elimination. Variables with a P-value < 0.1 were included in the final model. Proportions were compared using chi-square test and significance was considered at 5% level.

3.6.2 Substudy II

Data was cleaned coded and entered into Microsoft Acess 2007 and exported to SPSS for analysis .Summary statistics, graphs of residual sulfadoxine in maternal blood were done using SPSS version 9. Kappa statistics was used to determine the level of agreement between self-reports and presence of sulfadoxine in blood.

Validity of self-reported use of IPTp was assessed using presence of sulfadoxine in blood as a gold standard. Agreement or disagreement between self-report and HPLC results on actual detection of sulfadoxine in blood at delivery was determined by calculation of Kappa coefficients (Sim *et al.*, 2005). A kappa value of 0.1 to 0.40 was considered poor-to-fair agreement, a kappa value of 0.41 to 0.60 was considered moderate agreement, and a kappa value of 0.61 to 0.80 was considered substantial agreement, while a kappa value of 0.81 to 1.00 was considered excellent agreement.

3.6.3 Substudy III

Data was cleaned, coded and double entered using Microsoft Acess 2007 and exported to STATA version 9 for analysis. The principal outcome variable was transfer of any antibody level from mother to baby against the respective antigens and proportion of antibody transferred.

The relationship between proportions of antibodies transferred with maternal age, birth weight, gravidity, use of IPTp, number of IPTp doses, and presence of sulfadoxine (HPLC) in blood at delivery affected proportions with different blood stage antigens tested using linear regression. Using transfer of any amount of IgG antibody as main outcome variable logistic regression analysis was done to establish factors related to transfer of antibodies from mother to baby. Variables assed included gravidity, birthweight, and use of IPTp and presence of sulfadoxine in blood.

Antibody levels were categorized into negative and positive. A cut off value for the positive ELISA OD was put at the mean OD for a pool of negative control plasma plus 2 standard deviations from the mean (cut off = mean OD negative control plus two standard deviations). The antibody levels maternal blood was categorized as high and low using the median of the positives as a cutoff point. This was used in determining the association between antibody levels in the mother and proportions transferred the antibody levels were analysed as continuous variables.

All variables tested with p value less than 0.25 at bivariate analysis were included in the multivariate analysis model. In the multivariate analysis for all antigens IPTp use and sulfadoxine in blood (HPLC) at delivery were included in all models. The outliers on proportions of antibody transferred for different antibodies were excluded before fitting the final model.

3.6.4 Substudy IV

Data was cleaned, coded and entered into Microsoft Acess 2007 and exported to STATA (version 9) for analysis. The main outcome variables were Immunoglobulin M (IgM) antibody sero-positivity against respective *P. falciparum* blood stage antigens in cord and /or maternal blood at delivery

Logistic regression was done to determine factors associated with immune priming (cord IgM sero-positivity). Variables assessed at bivariate included; use of IPTp and the number of doses taken, gestational age, presence of sulfadoxine in maternal sera, parity of the mother, HIV sero-status and maternal age. Variables with *P*-value of < 0.25 were included in multivariate analysis.

Mann Whitney Rank Sum test, Kruskal–Wallis one-way analysis of variance were employed to determine difference between antibody levels (Mann, 1947). Differences with *P*-value less than 0.05 were taken as statistically significant.

3.7 Ethical Considerations

All ethical aspects of the study were approved by the Makerere University Medical School Research and Ethics Committee and the Uganda National Council for Science and Technology .Informed consent (or assent for those <18 years of age) was obtained from all the participants. No patient was denied any care for refusing to take part in the study. The participants were identified by numbers and the information collected was treated as highly confidential. In cases where the participants had infection or were anaemic the results were used in their management.

3.8 Methodological considerations

The primary objective of this study was to determine the effect of using this intervention of anti-P*. falciparum* immunity in the mother and newborn and yet using SP IPTp for prevention of malaria in pregnancy in malaria endemic areas is standard of care. In order to answer this objective we had to compare to groups of mother/baby pairs on basis of maternal use of SP IPTp during pregnancy. We used cross-sectional studies to achieve the objectives. Cross-sectional studies have many potential confounders and can only determine association but not cause effect relationship. Data on the potential confounders was collected and multivariate analysis done.

Data on SP IPTp use was based on self-report since it is common to have drug stock out and therefore patients asked to buy the medicine out of the hospital. In addition,

often the SP IPTp is not given under directly observed therapy because of lack of drugs and supplies. We decided to use self-reported use of SP IPTp as a measure of IPTp use.

Since self-reports are prone to bias, and we then determined the validity of self-reported use of IPTp. This was done using a validation study where having or not having SP (sulfadoxine) in blood at the time when it should be detectable was taken as a gold standard. In analyzing for the effect of using SP IPTp, we corrected for possible low validity of self-report using information on presence of absence of sulfadoxine in blood at delivery.

Once we finalized with the design, we had to select a study site where we would enroll mothers and follow them up to delivery and accessibility of services for handling the samples. Mulago National Refferal Hospital was selected as a study site. This selection introduced a limitation of generalizability of the results since this is in a low malaria endemic region. The burden of malaria in pregnancy in this setting was not documented prior to the onset of these studies. The first objective was then to determine the burden of malaria in pregnancy in Mulago National Referral Hospital; this was done using a cross-sectional survey.

CHAPTER FOUR: RESULTS

4.1 Malaria burden at Mulago National Refferal Hospital

Three hundred and ninety nine participants were recruited into this substudy after informed verbal and written consent. Most of the participants were residing in Kampala (68.6%) and the neighbouring Wakiso districts (22.9%). The majority had primary (46.4%) or secondary (42.3%) level of education; 66.3% were housewives or unemployed; 74% were married. Most women (96.8%) had attended an antenatal clinic at least once during the present pregnancy. One hundred and sixty five (41.5%) had received intermittent preventive antimalarial treatment (IPT); of these, the majority had received one dose (74.5%), whereas 20.5% had received the two recommended doses. Sulfadoxine/pyrimethamine (SP) was the drug of choice (89.3%). Most women reported taking iron (79.3%) and folic acid (70.4%) supplementation during pregnancy. The age of participants ranged from 15 to 44 years with a median of 20yrs and (IQR: 18–25). The general characteristics of study participants are shown in Table 1.

Prevalence *of Malaria*

Nine percent *(35/391)* of the women had malaria by peripheral smear, 11.3% (44/389) by placental smear and 13.9% (53/282) by placental histology. Out of 53 women with histological evidence of infection, 34 (64.1%) were classified as acute, 2 (3.8%) as chronic, and 17 (32.1%) as past infection. *P. falciparum* was the sole species found in all cases. All participants diagnosed as acute or chronic infection by histology or as *P. falciparum* positive by placental blood film examination were regarded as active infection. Based on this case definition, a total of 15.5% (59/380) and 4.5% (17/380) had active versus past placental infection, respectively.

High maternal age and use of bed net were protective of placental malaria while district of residence, educational level and use of IPTp had no association. Multigravida women were more protected at crude analysis however this was lost in the adjusted model (Table 2).

Table 1: General characteristics of the participants Substudy 1

District of residence (%)	
Kampala	68.6
Wakiso	22.9
Other	8.5
Ethnic group (%)	
Ganda	62.8
Nyankole	8.0
Soga	6.0
Rwandese	5.3
Other	17.9
Median age (years)	20 (IQR: 18–25)
Education (%)	
Illiterate	6.9
Primary	46.4
Secondary	42.3
Higher	4.3
Marital status (%)	
Married	74.0
Single	20.4
Cohabitant	5.6
Occupation (%)	
Housewife	55.8
Peasant	2.9
Student	9.7
Casual worker	18.2
Professional job	2.9
Unemployed	10.5
Visit to ANC (%)	96.8
Received IPT (%)	41.5
Use ofbednet (%)	67.3
Use ofITN (%)	32.0
Median Hblevel (g/dl)	12.3 (IQR: 10.9–13.4)
Hb <11 g/dl(%)	22.0
Folic acid supplementation (%)	70.4
Iron supplementation (%)	79.3
Median birthweight (g)	3100 (IQR: 2800–3500)
LBW(%)	12.2
Preterm delivery(%)	3.1
Stillbirth (%)	2.8
Caesarean (%)	17.6
Peripheral malaria (%)	9.0
Placental malaria (%)	
Histology	13.9
Blood smear	11.3

ANC- antenatal clinic, at least one visit during the present pregnancy, IPT; Intermittent presumptive antimalarial treatment at least one dose, ITN; insecticide –treated net, LBW; low birthweight

Table 2: Factors associated with placental malaria

Factor level	Risk factor	Crude OR	Adjusted OR	(95% CI)	*P-value*
Background	*Age (continuous per year)*	*0.95∗*	*0.95*	*(0.89–1.00)*	*.066*
	District of residence				
	Kampala	1.0	1.0		
	Wakiso	1.18	1.4	(0.74–2.60)	.31
	Other	1.80	1.9	(0.79–4.74)	.15
	Education (continuous per level)	0.79	0.75	(0.48–1.17)	.21
Intermediate	Gravidity				
	G1	1.0	1.0		
	G2-3	0.81	1.0	(0.54–2.07)	.88
	≥G4	0.38∗	0.72	(0.21–2.50)	.61
Proximate	Received IPT				
	None	1.0	1.0		
	1dose of SP	0.93	1.11	(0.60–2.07)	.73
	2doses of SP	0.55	0.49	(0.16–1.49)	.21
	Used bednet	*0.56∗*	*0.56*	*(0.31–0.99)*	*.046*

∗Significant associations (P<.05).

4.2 Low validity of self-reported IPTp use during pregnancy

A total of 204 women were recruited in a cross-sectional study to determine the validity of self-reported use of IPTp during pregnancy. Majority (98.5%) of the participants attended antenatal clinic at least once during pregnancy as evidenced by self-report and presence of an antenatal clinic card. All the participants who attended antenatal had a card and since SP IPTp was not given as directly observed therapy, self-report was used as a measure for use. Approximately fifty nine percent of participants (n = 204) reported using IPTp during pregnancy, with 90% taking one dose of SP while 17.2% reported using an insecticide spray for controlling mosquito bites. From the self-reports on when the last SP dose closest to delivery was taken, the median reported interval between SP intake and baby delivery was computed as 12 weeks (IQR:8–18.8);. Frequency distribution of the calculated interval between reported date of SP intake and baby delivery for the mothers who reported having used IPTp is shown in Figure 4 (histogram B). The frequency distribution of the same interval for mothers (n = 35) who were found to have detectable SDX in blood (Figure 4D) and those (n = 85) whose blood was negative for SDX (Fig 4C) are also shown. It can be seen that SDX was detected in blood of mothers whose self-reports

indicated SP intake before 9 weeks to baby delivery (Figure 4D) , a result suggesting that the reported dates of the IPT dose was incorrect since SDX would be undetectable by HPLC beyond two months after administration. On the other hand, the blood of more than 15 mothers who reported to have taken SP within nine weeks preceding baby delivery lacked any detectable SDX (Figure 4 C), likewise suggesting that the reported dates for when the SP doses were taken are inaccurate. Thus, the results suggest that the self- reports were unreliable for finding out whether the patients used IPTp or not and determining when the SP doses were taken Figure 4.

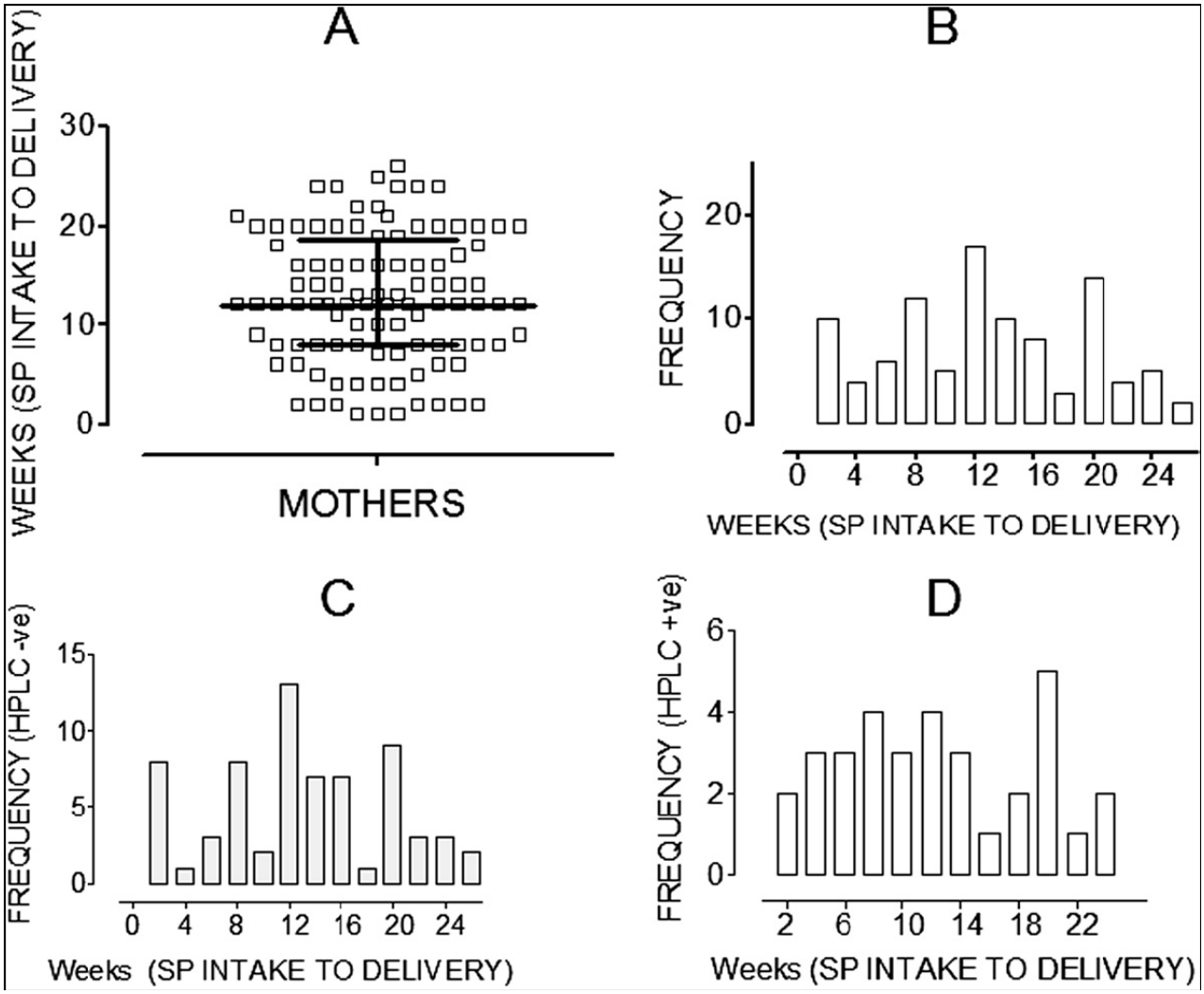


Figure 4: Reported time interval between SP administration and delivery

The Figure 4 shows: A. Variability in interval (weeks) between reported date of SP intake and baby delivery (median: 12 weeks, IQR: 8–18.8 weeks, see whiskers), B. Frequency distribution of the reported interval (SP input to delivery) for the mothers whose blood was analyzed for SDX. Histogram of reported interval (SP intake to delivery) for HPLC negative (C) and HPLC positive (D) mothers.

Table 3: Self–reported IPTp use during pregnancy and demographic characteristics Substudy 2

Variable	Used IPTp	Not used IPTp	P Value	(OR) 95%CI
Bed net use				
Always	101	64		1
Sometimes	8	9	0.25	1.7(0.6-4.8)
Never Education level	11	11	0.31	1.5(0.60-3.80)
Up to primary	38	45		1
Post-primary	82	39	<0.01	0.4 (0.22-0.72
Age group				
Up to 20yrs	88	64		
Above 20years	32	20	0.070	1.1 (0.6-2.2)
Iron supplement				
Yes	98	57		1
no	22	27	0.03	2.1 (1.1-4.0)
Use of insecticide spray				
Yes	25	10		1
no	95	74	0.10	1.9 (0.88-4.3)
Maternal parasitemia				
Negative Positive	111 9	76 8	0.60	1 1.2 (0.47-3.51)

The more educated mothers (P = < 0.01 95% CI: 0.2-0.7) and those who took iron supplementation during their pregnancy (P = 0.03 95% CI: 1.1-4.0) were more likely to report using IPTp. The other factors were not statistically different in the group that reported IPTp use and the cluster that reported IPTp non-use Table 3. None of the maternal demographic characteristics was associated with presence of sulfadoxine in maternal serum at delivery.

Kappa statistic on self-reported IPTp use and sulfadoxine in blood at delivery

Of 120 study participants who self-reported to have used IPTp, 35 (29.2%) tested positive by HPLC while 63 (75%) of 84 patients who reported not to have used IPTp tested negative for SDX. On the other hand, 85 (70.8%) patients who reported to have used IPTp tested negative for blood SDX by HPLC. Yet, 21 (25%) of patients who self- reported not to have taken SP were found to have detectable SDX in blood

Table 4. The result of the analysis indicates Kappa statistics equal to 0.037. This level of kappa signifies a very slight (poor-to-fair) agreement between reported IPTp use and sulfadoxine in blood at delivery (Sim *et al.*, 2005; Viera *et al.*, 2005).

Table 4: Self-reported IPTp use and presence of sulfadoxine

		Reported use of IPTp		Total
		Yes	**No**	
Sulfadoxine	Positive	35	21	**56 (m1)**
	Negative	85	63	**148 (m0)**
	Total	**120(n1)**	**84 (n0)**	**204 (n)**

4.3 Mother to infant transfer of anti-P*. falciparum* antibodies IgG

4.3.1 Demographic characteristics of the study participants

To determine the effect of using IPTp during pregnancy on the proportions of IgG antibodies transferred from mother to baby 290 mother/baby pairs were recruited into a cross-sectional study. Majority of the participants were residing in Kampala and Wakiso districts with 79% and 19% respectively. Over 98% (286/290) of study participants attended the Antenatal clinic at least once during pregnancy with 95% (272/286) of them having ANC card as evidence. Majority of the participants who attended ANC (56%) had the first visit in the second trimester of pregnancy, 37% in the third trimester with only 7% in the first trimester. The mean gestation age for the first ANC visit was 24 weeks with 75% of the coming before 28 weeks of amenorrhea.

Fifty nine percent (171/290) of the participants reported taking at least one dose of IPTp during pregnancy with 79%, 16% and 5% of these taking one, two and three or more doses, respectively. Primigravidae women were the most predominant at 33.4% (97/290) followed by gravidae 2 at 23.8% (69/290) of the study participants. Bed net use in this population was reported at 82.4% for strict bed net users and with 42% of them aware that the bed nets were insecticide treated. Fifty eight (20%) of

the participants were using insecticide sprays for controlling mosquito bites. The range of participants' age varied between 16 years and 40 years with a mean of 24 years and 24.5% (71/290) were less than 20 years (Table 5). All the participants were asymptomatic with temperature below 37.5^{o}C and 30.7% (79/290) reported having had at least one fever episode during pregnancy.

Table 5: Demographic characteristics of the study participants Substudy 3

Variable	Frequency (n = 290)	Percentage
Age of mothers (years):		
<20	71	24.48
≥20	219	75.52
Birth weight of babies (kg):		
< 2.5	6	2.1
≥2.5	284	97.9
Gravidity:		
1	97	33.4
2	69	23.8
3	67	23.3
4	30	10.3
5	21	7.2
≥6	6	2.1
WOA*:		
<37	12	4.1
≥37	260	89.7
Don't know	18	6.2
Use of IPTp:		
No	119	41
Yes	171	59
No of IPTp doses		
1	135	78.9
2	28	16.4
≥3	8	4.7
Use of mosquito bed-nets:		
Always	239	82.4
Sometimes	20	6.9
Never	31	10.7
Was bed-net treated:		
Yes	109	42.1
No	108	41.7
Don't Know	42	16.2
Maternal peripheral parasiteamia		
Negative	270	93.1
Positive	20	6.9
Cord parasiteamia		
Negative	286	98.6
Positive	4	1.4

WOA* weeks of amenorrhea

4.3.2 Factors associated with Use of IPTp

Mothers who had iron and folic acid supplementation during pregnancy were more likely to have used IPTp during that pregnancy. The partcipants who had attained post primary education were more likely to have taken IPTp during pregnancy than their counterparts. Those who had IPTp during pregnancy were more likely to use insectcide spray during pregnancy Table 6.

Table 6: Socio-demographic characteristics and self-reported use of IPTp

Variable	Used IPTp (n = 170)	No IPTp (n = 120)	OR(95% CI)	P value
Age of mothers (years):				
<20	29	17	1	
≥20	141	103	1.25(0.65-2.39)	0.506
Gravidity:				
1	60	37		
2	75	61	1.32(0.78- 2.24)	0.307
≥3	35	22	1.02(0.52-2.00)	0.956
Bed Net use				
Always	145	94	1	
Sometimes	9	11	1.89(0.75-4.72)	0.170
Never	16	15	1.45(0.68-3.06)	0.333
Insectcide spray				
Yes	41	17	1	
No	129	103	1.93(1.03-3.59)	**0.037**
ANC attendance				
No	0	4		
Yes	170	116		
Iron supplement				
Yes	143	77	1	
No	27	43	2.96(1.70-5.15)	**<0.001**
Folic acid supplement				
yes	138	76	1	
No	32	44	2.5(1.46-4.26)	**0.001**
Education level mother				
Up to primary	56	60	1	
Post primary	114	60	0.49(0.30-0.79)	**0.004**

4.3.3 Maternal IgG sero-positivity higher than newborn

The proportions of participants whose serum was sero-positive to different *P. falciparum* antigens were generally higher in the maternal sera compared to the

cord sera. The IgG antibody sero-prevalance in maternal sera was highest against HRPII antigen (92%) Table 7.

Table 7: IgG antibody sero-positivity in mothers and babies at delivery

Variable	Frequency N=290	Percentage (%)
Anti –GLURP IgGmcat**		
Positive	211	72.8
Negative	79	27.2
Anti –GLURP IgGbcat*		
Positive	152	52.4
Negative	138	47.6
Anti –HRPII IgGmcat**		
Positive	268	92.4
Negative	22	7.6
Anti –HRPII IgGbcat*		
Positive	224	77.2
Negative	66	22.8
Anti -MSP3a IgGmcat**		
Positive	206	71.0
Negative	84	29.0
Anti -MSP3a IgGbcat*		
Positive	107	36.9
Negative	183	63.1
Anti -MSP3 IgGmcat**		
Positive	243	83.8
Negative	47	16.2
Anti -MSP3 IgG bcat*		
Positive	183	63.1
Negative	107	36.9

mcat****Immunoglobulin G Maternal serum, *****; bcat**** *Immunoglobulin G cord serum*

4.3.4 Anti- *P. falciparum* sero-reactivity in maternal sera is not affected by IPTp use

Participants who reported IPTp intake during pregnancy were more likely to be sero-positive for IgG antibodies against MSP3a (P=0.025, OR =1.79) Table 8. Antibody sero-positivity against other antigens were not affected by IPT use during pregnancy at bivariate analysis. Factors adjusted for at multivariate included HIV status, weeks of pregnancy, maternal age, number of IPTp doses taken, birth-weight and gravidity. After multivariate analysis participants who used IPTp were more likely to be sero-reactive against MSP3a (P=0.06) and protective against HRPII antibodies (P=0.09) although none of the factors reached statistically significant levels. Primigravidae

were more likely to be sero-positive (IgG) to MSP3a compared to their counterparts after controlling for all confounders (P=0.01). This effect was not demonstrated for antibodies against the other three blood stage antigens (GLURP, HRPII and MSP3a).

Table 8: IgG Sero-reactivity in maternal blood and IPTp use during pregnancy

Variable	**Sero-positivity**		Odds Ratio (95% CI)	*P*-value
	Positive	Negative		
Anti –GLURP IgG				
Used IPTp: Yes	121	50	1	
No	90	29	0.78 (0.46, 1.34)	0.340
Anti –HRPII IgG				
Used IPTp: Yes	154	17	1	
No	114	5	0.40 (0.14, 1.11)	**0.069**
Anti -MSP3a IgG				
Used IPTp: Yes	130	41	1	
No	76	43	1.79 (1.07, 3.0)	**0. 025**
Anti -MSP3 IgG				
Used IPTp: Yes	145	26	1	
No	98	21	1.20 (0.64, 2.24)	0. 579

4.3.5 Anti-*P. falciparum* IgG levels and IPTp use, maternal age and gravidity

Maternal self-reported use of IPTp during pregnancy did not affect the anti-*P. falciparum* antibody levels in the mothers and babies at delivery. Antimalarial antibodies in mothers and babies at delivery were not affected by using IPTp during pregnancy when we compared median antibody levels GRULP (Mother P=0.17 and new-born P=0.27) Figure 5 .

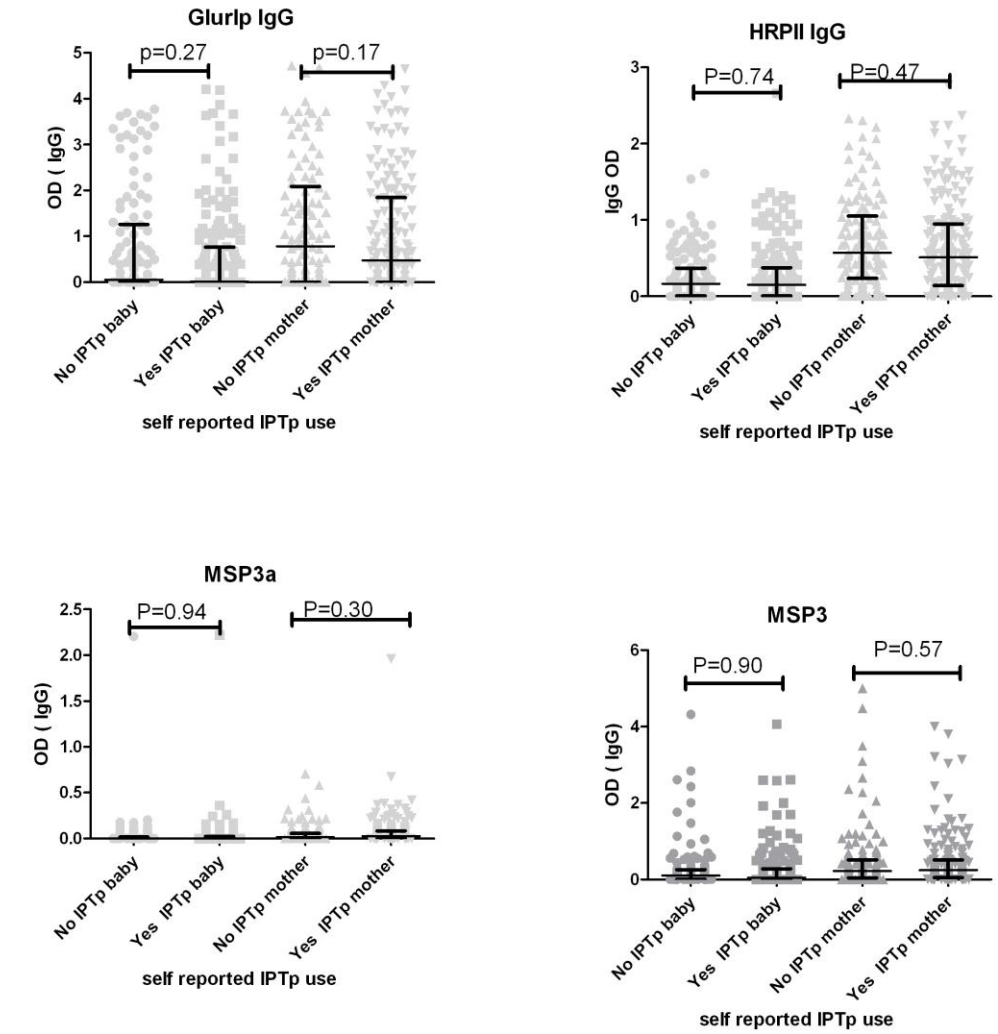


Figure 5: Use of IPTp during pregnancy on the antibody levels in serum

The median antibody levels in the mothers and newborn in relation to IPTp use during pregnancy was compared using Mann-Whitney rank sum test which showed no significant difference against all the tested peptides (Figure 5). Anti-*P. falciparum* IgG, antibodies against GLURP and HRPII were higher in participants below 20 years although this did not reach statistically significant levels (Figure 6).

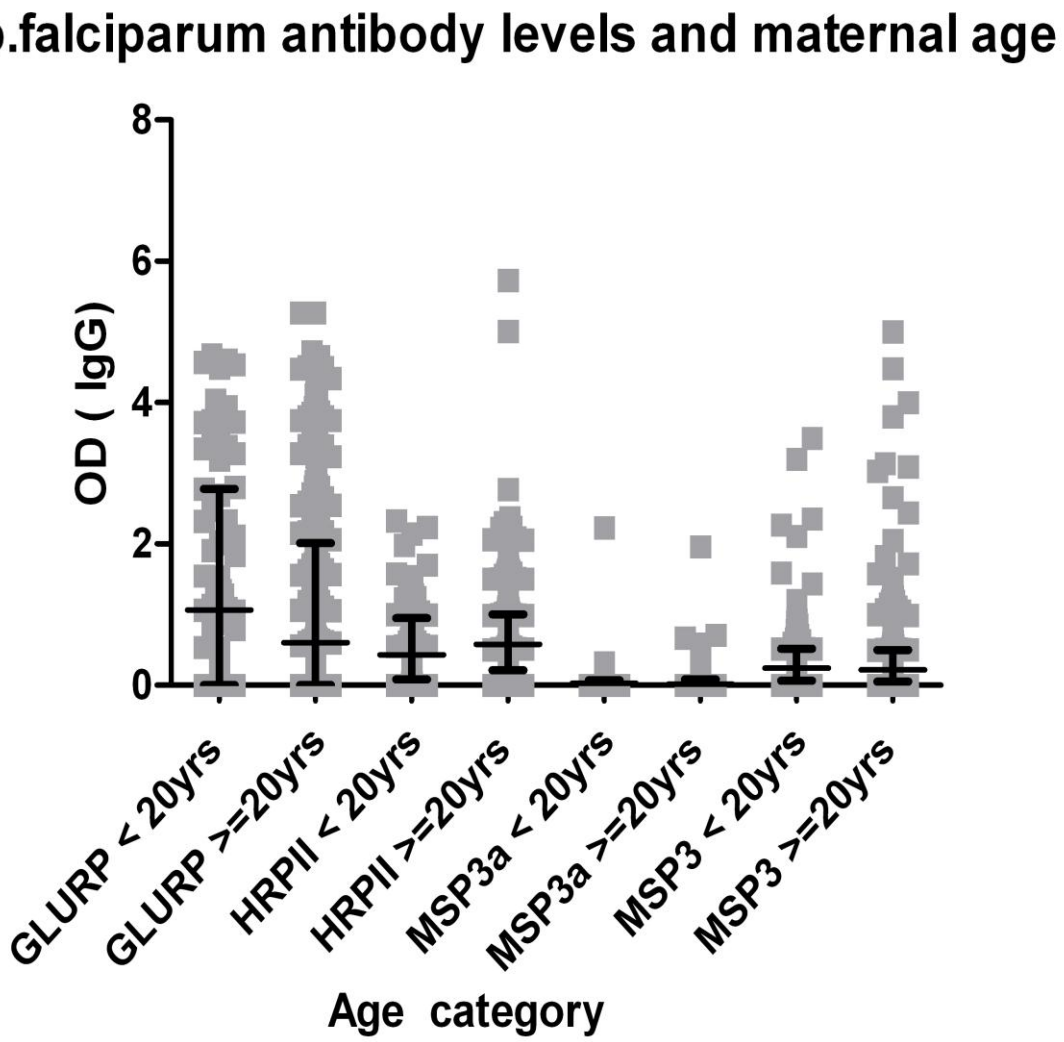


Figure 6: Maternal age and antibody levels in mothers.

GLURP P=0.25 , HRPII mothers P=0.54 , MSP3a P=0.26 , MSP3 mothers P=0.93 (Mann-Whitney rank sum test).

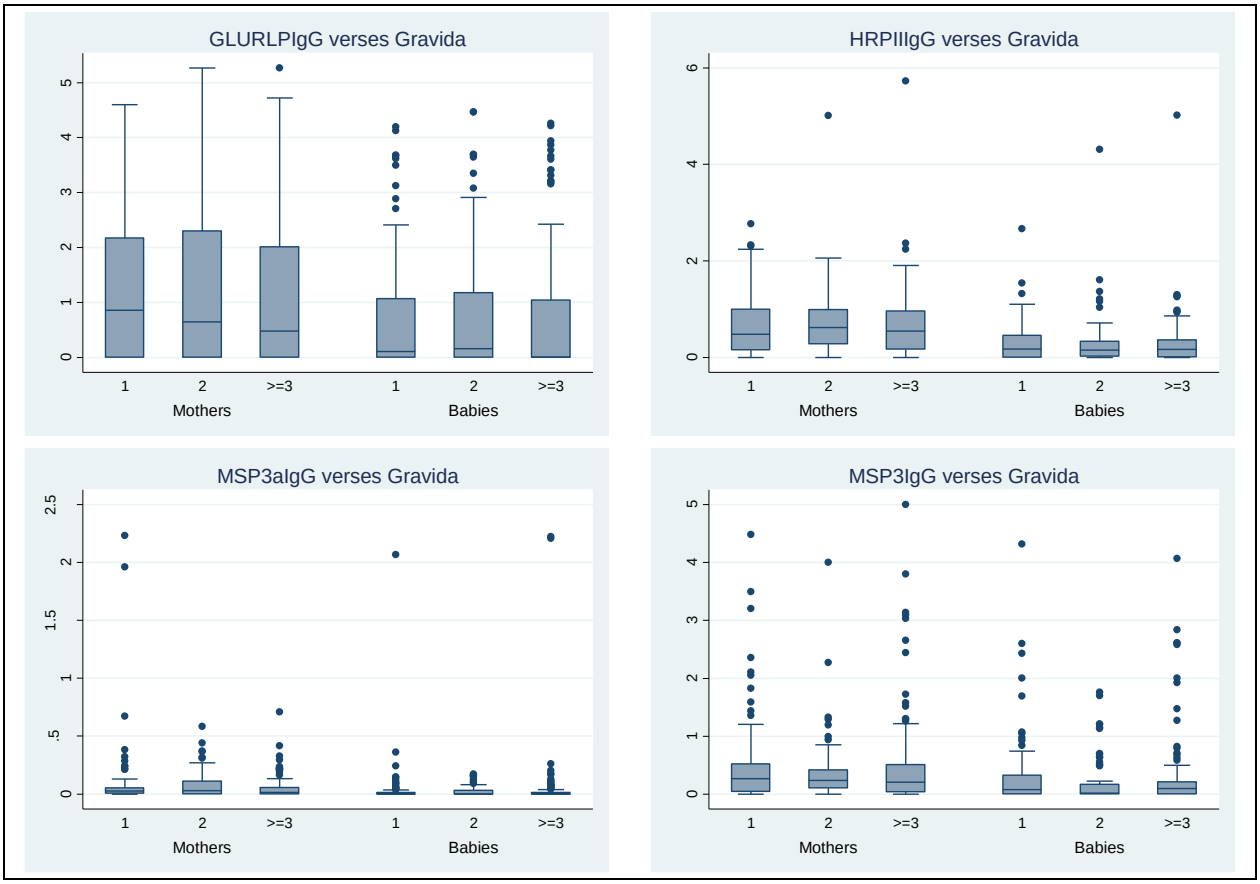


Figure 7: Parity and antibody levels in the mothers and babies

Parity of the mother did not affect antibody levels in mothers or babies assessed using Kruskal Wallis test comparing median levels.

Primigravidae women generally had higher IgG antibodies levels against GLURP compared to their counterparts. However the difference did not reach statistically significant level Figure 7. The number of pregnancies the mother had before the current pregnancy did not have any effect on antibody levels in the mother and baby.

4.3.6 Anti-*P. falciparum* antibody Transfer from mother to baby

There was a linear relationship between antibody levels in the mother and corresponding babies Figure 8. Proportions of anti-*P. falciparum* antibodies transferred from mother to baby were determined as a fraction of the antibody level in the baby to the corresponding mother. In cases where the baby was negative for IgG to particular antigen proportion was recorded as zero. Maternal age did not affect the proportions of antibodies transferred from mother to baby Figure 6.

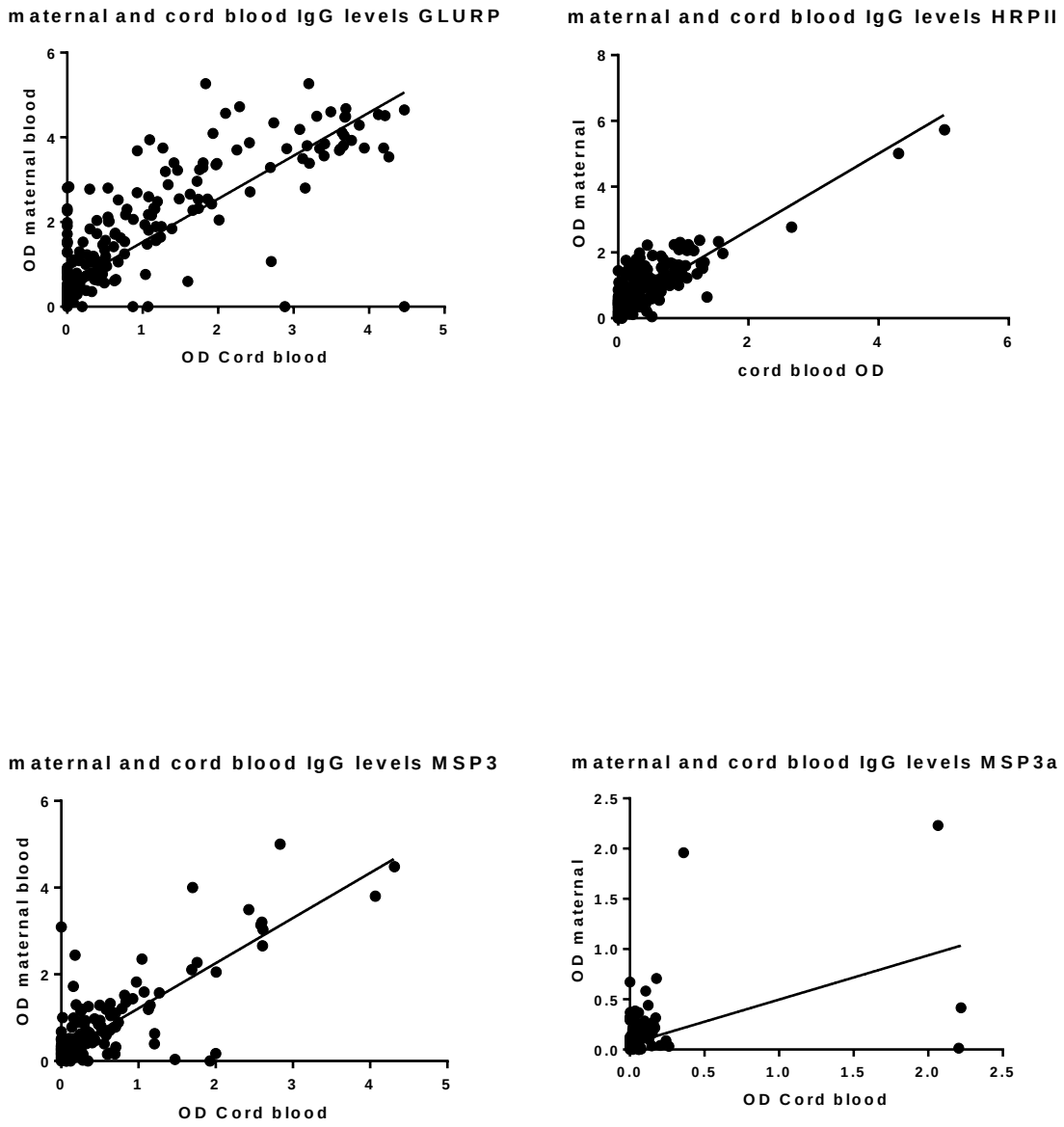


Figure 8: Relationship between maternal and cord blood antibody levels

Linear relationship between maternal and cord blood antigen levels for all antibodies GlURP R^2 = 0.68, HRPII R^2 =0.72, MSP3a R^2 =0.24, MSP3 R^2 =0.67

4.3.7 Anti-*P. falciparum* antibody levels and transfer to newborn

Mothers with *P. falciparum* parasiteamia at delivery were more likely to transfer antibodies against MSP3a compared to their counter parts after multivariate logistic regression (P=0.043). Mothers who had sulfadoxine in blood at delivery were more likely to transfer IgG antibodies against MSP3 Table 9.

Table 9: Maternal characteristics affecting transfer of IgG from mother to baby

Variable	OR	Se	P value	95%CI
GLURP				
Used IPTp	1.070	0.513	0.886	0.41-2.73
HPLC	0.613	0.305	0.327	0.23-1.63
IgG category1*	7.491	3.897	**<0.001**	2.70-20.76
HRPII				
Used IPTp	1.335	0.747	0.605	0.44-3.99
HPLC	1.018	0.657	0.977	0.28-3.61
IgG category1*	1.742	5.177	**0.002**	2.08-28.71
MSP3a				
Used IPTp	0.759	1.015	0.837	0.05-10.43
HPLC	0.219	0.174	0.057	0.04-1.04
BS mother	0.069	0.091	**0.043**	0.01-0.91
IgG category1*	20.952	13.699	**<0.001**	5.81-75.47
MSP3				
HPLC	8.313	6.965	**0.011**	1.60-42.95
Gravidae1	0.432	0.291	0.214	0.11-1.62
Gravidae2	0.303	0.196	0.066	0.08-1.08
IgG Category1*	9.520	5.608	**<0.001**	3.00-30.20

IgG Category1* *category with antibody level above 0 and below median. Median antibody levels for antibodies against different antigens; GLURP IgG=1.319, HRPII IgG= 0.587, MSP3a IgG= 0.041, MSP3 IgG= 0.296; These were used as the levels up to median and then above median* . IgGCat1=antibody level below median, BS mother = presence of maternal P. *falciparum* parasiteamia at delivery, HPLC= presence of sulfadoxine in maternal blood at delivery IgG= Immunoglobulin G antibody levels Category 1*= antibody level in a particular category above zero and below median

4.3.8 Proportions of antibodies transferred from mother to baby

At bivariate analysis the proportion of antibodies transferred from mother to baby were significantly affected by the antibody levels in the mother. The mothers with high antibody levels tended to transfer a less proportion compared to their counterparts Table 10.

Table 10: Bivariate analysis on proportions of IgG antibodies from mother to baby

Out come	Variable	Estimate (s.e)	*P*-value
GLURP			
	Used IPTp	-0.213 (.143)	0.139
	HPLC pos	0.193(.240)	0.424
	BS negative	0.381 (0.260)	0.147
	GLURPCat1	-0.500(0.14)	>0.013*
HRPII			
	Used IPTp	0.107 (0.123)	0.385
	HPLC Positive	0.261 (0.182)	0.155
	BS negative	0.090 (0.247)	0.714
	HRPII IgGCat1	0.088(0.163)	0.502
MSP3a			
	Used IPTP	-0.439 (0.263)	0.099
	HPLC Positive	0.227 (0.520)	0.664
	BS Negative	0.773 (1.213)	0.525
	MSP3a IgGCat1	1.406(0.313)	<0.015*
MSP3			
	Used IPTp	0.044 (0.146)	0.763
	HPLC Positive	0.218(.206)	0.291
	Gravidae2	-0.390 (0.187)	0.039
	BS Negative	-0.010 (0.301)	0.972
	MSP3 IgGcat1**	0.245(0.151)	0.202*

*Cat1** category with antibody level above 0 and below median. Median antibody levels for antibodies against different antigens; GLURP IgG=1.319, HRPII IgG= 0.587, MSP3a IgG= 0.041, MSP3 IgG= 0.296; These were used as the levels up to median and then above median* . IgGCat1=antibody level below median, BS Negative =Malaria Blood Slide, HPLC=High performance liquid chromatography (Sulfadoxine in maternal blood). * Factors included in the multivariate analysis model.

4.3.9 Proportion of IgG transferred to the newborn and IPTp use

After excluding the outliers, maternal parasiteamia at delivery was no longer significantly associated with proportions of antibody transferred (p=0.33) and it was dropped from the model. Participants who had lower antibody levels were more likely to transfer a higher proportion of antibodies against GLURP and MSP3a to the babies than their counterparts. Using IPTp during pregnancy did not significantly affect the proportions transferred from mother to baby for all the antigens Table 10.

After multivariate analysis, mothers with high antibody (against GLURP) level tended to transfer a less proportion compared to their counterparts and use of IPTp did not affect the proportion of antibody transferred in all cases Table 11.

Table 11: Proportions of antibodies transferred not affected by use of IPTp

Variable	Coefficient	se	*P*-value	
GLURP				
Used IPTp	-0.292	0.197	0.065	
HPLC	0.170	0.225	0.451	
BS mother	0.241	0.289	0.408	R^2= 0.1271
IgG Category1	0.608	0.207	**0.004**	N=77
Constant	-1.161	0.221	**0.000**	
HRPII				
Used IPTp	0.083	0.161	0.606	
HPLC	0.271	0.184	0.144	R^2= 0.023
IgG Category1	0.117	0.165	0.482	N=120
Constant	-1.433	0.171	0.000	
MSP3a				
Used IPTp	-0.465	0.311	0.141	
HPLC	0.514	0.524	0.331	R^2= 0.095
IgG Category1	-0.841	0.469	**0.079**	N= 55
Constant	-0.296	0.486		
MSP3				
Used IPTp	0.031	0.194	0.870	
HPLC	0.214	0.206	0.303	R^2=0.047
Gravidae1	0.231	0.237	0.334	N= 93
Constant	-1.125	0.187	0.000	

MSP3aIgGcat=1 if MSP3aIgGM >0 and <= median(0.041) else MSP3aIgGcat=2 if MSP3aIgGM>median GLURPIgGcat =1 if GLURPIgGm>0 and <= median (0.041) else GLURPIgGcat =2 if GLURPIgGm>0 and >median (IgGm =Immunoglobulin G in maternal serum), HPLC= presence of sulfadoxine in maternal blood at delivery,Used IPTp= self-reported using at least on dose of IPTp

4.4 IgM sero-positivity in maternal and cord sera

4.4.1 Anti-P. *falciparum* IgM in mothers and babies

Immunoglobulin M antibodies in the mothers and babies indicate recent exposure to the malaria antigen since it has a short half-life. Presence of these antibodies in the cord sera indicates recent exposure of the fetus to malaria antigens since it does not cross the placenta. The anti-P. *falciparum* IgM sero-positivity in maternal sera

against GLURP, HRPII, MSP3a and MSP3 was 89.9%, 86.3%, 57.5% and 79.9% respectively. The IgM levels in the babies were lower at 5.0%, 10%, 2.9% and 33% against GLURP, HRPII, MSP3a and MSP3 respectively (Figure 9). The participants who reported IPTp use during pregnancy had a significantly higher proportion of sero-positivity to HRPII.

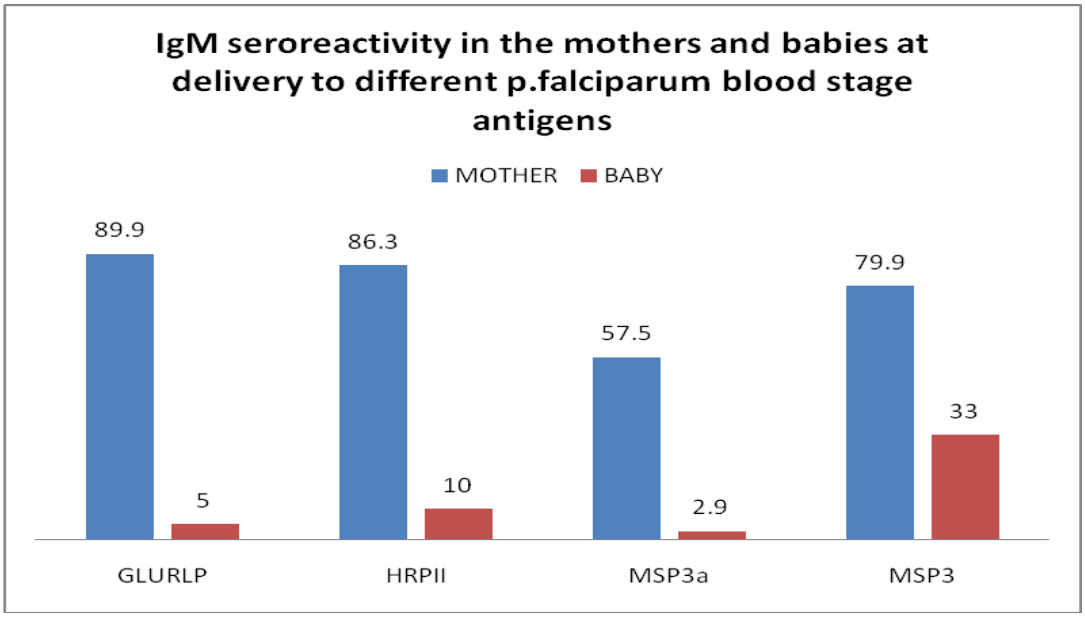


Figure 9: *P. falciparum* IgM sero-reactivity in the mothers and babies

4.4.2 IgM Sero-positivity of maternal serum and IPTp use

Table 12: Demographic characteristics of participants involved IgM studies Substudy 4

Variable	Number (n)	Percentage
IPTp Use		
Yes	61	43.88
No	78	56.12
IPTp doses		
1	69	88.46
= >2	9	11.54
HPLC		
Positive	35	25.18
Negative	104	74.82
Age group		
< 20yrs	31	22.30
= > 20 yrs	108	77.70

Sixity percent of participants analysed for IgM reported to have used IPTp during pregnancy Table 12. Majority of participants received one dose of IPTp (88.5%) and 25.2% of them had sulfadoxine in blood at delivery.

All mothers who had malaria parasites in blood at delivery and those who were HIV positive had IgM antibodies against GLURP. All HIV positive participants had antibodies (IgM) against MSP3, and pariticipants who had sulfadoxine in blood at delivery were less likely to have IgM antibodies at delivery Table 13. Presence of IgM antibodies against blood stage antigens in the maternal sera was not affected by parity or age. Participants who reported taking IPTp during pregnancy and those with sulfadoxine were less likely to have evidence of recent exposure (IgM). Primigravidae tended have more evidence of recent infection (IgM against HRPII). Antibody seropositivity against MSP3a antigen however was not affected by IPTp use or presence of sulfadoxine in maternal blood at delivery or malaria parasites at Delivery (Table 13).

Table 13: IgM sero-positivity in maternal sera and demographic characteristics

Outcome	Effect	OR	s.e	P value	95% CI
GLURP					
	Used IPTp	0.477	0.295	**0.231**	**0.14-1.60**
	HPLC positive	0.685	0.460	0.574	0.18-2.55
	Less than 20years	4.105	4.346	**0.182**	**0.51-32.69**
	Gravidae 1	1.037	0.792	0.961	0.23-4.63
	Gravidae 2	0.613	0.393	0.446	0.17-2.15
	<37 WOA	0.693	0.174	**0.147**	**0.42-1.13**
HRPII					
	Used IPTp	8.888	5.838	**0.001**	**2.45-32.20**
	HPLC positive	11.333	6.160	**>0.001**	**3.90-32.89**
	Gravidae 1	7.291	7.817	**0.064**	**0.89-59.62**
	Gravidae 2	0.963	0.503	**0.943**	**0.34-2.68**
	<20years	1.088	0.657	0.888	0.33-3.55
	BS positive	0.304	0.200	**0.072**	**0.08-1.11**
	HIV positive	1.486	1.611	0.715	0.17-12.44
	<37WOA	1.221	0.255	0.340	0.81-1.84
MSP3a					
	Used IPTp	0.625	0.218	**0.179**	**0.31-1.24**
	HPLC positive	1.010	0.397	0.978	0.46-2.18
	BS positive	1.742	1.093	0.376	0.50-5.95
	<37WOA	0.835	0.123	**0.225**	**0.50-5.95**
MSP3					
	Used IPTp	1.361	0.577	0.466	0.59-3.12
	HPLC positive	0.173	0.131	**0.021**	**0.03-0.76**
	BS positive	1.430	1.143	0.655	0.29-6.85

All variable where the P value was less than 0,025(*) were included in multivariate logistic regression model.
HPLC positive= presence of sulfadoxine in blood at delivery, BS Positive= maternal P. *falciparum* parasiteamia, Used IPTp= self-reported using at least one dose of IPTp, WOA= weeks of ammenorrhea

4.4.3 Maternal IgM sero-positivity and IPT use in pregnancy

Variables which were fitting perfectly for a particular antigen were not included in the model. After multivariate analysis ,participants who reported to IPTp use during pregnancy were less likely to have IgM at delivery Table 14.

Table 14: Effect of IPTP use and IgM sero-positivity in maternal sera

Outcome	Effect	OR	s.e	*P*-value	95% CI
GLURP					
	Used IPTp	0.481	0.297	0.237	0.14-1.61
	HPLC Positive	0.826	0.567	0.781	0.21- 3.17
HRPII					
	Used IPTp	13.107	9.738	**0.001**	**3.05-56.22**
	HPLC Positive	18.497	12.079	**0.000**	**5.14-66.52**
MSP3a					
	Used IPTp	0.623	0.218	0.178	0.31-1.23
	HPLC Positive	1.053	0.419	0.895	0.48-2.30
MSP3					
	Used IPTp	1.469	0.639	0.376	0.62-3.44
	HPLC Positive	1.176	0.134	**0.023**	**0.03-0.78**

4.4.4 Maternal IgM sero-positivity predicts cord blood recent exposure

IgM seropositivity in the cord blood to different *P. falciparum* antigens was used as the main outcome variable. Bivariate analysis was performed to assess factors affecting IgM sero-positivity in cord blood in relation to reported use of IPTp during pregnancy number of doses taken, presence of IgM antibodies in the maternal sera and other demographic characteristics were assessed on all representative antigens.

All the babies whose cord blood had IgM antibodies against GLURP were born to mothers who reported not to have Used IPTp during pregnancy and presence of IgM in the mothers was generally protective to the babies against all the antigens Table 15. All babies whose mothers reported using IPTp and who had sulfadoxine in blood at delivery had no IgM antibodies against MSP3a.

Table 15: IgM sero-positivity in the babies at delivery

Outcome	Effect	OR	s.e	*P*-value	95%CI
GLURP					
	HPLC positive	0.970	0.815	0.972	0.18-5.04
	Mother IgM positive	0.177	0.139	**0.028**	0.03-0.82
	<20years	2.785	2.208	0.196	0.58-13.17
	HIV Positive	2.240	2.541	0.477	0.24-20.68
	WOA	0.961	0.309	0.903	0.51-1.80
HRPII					
	Used IPTp	2.095	1.295	0.231	0.62-7.03
	HPLC Positive	1.333	0.902	0.671	0.35-5.02
	HIV Positive	2.395	2.028	0.302	0.45-12.59
	<37WOA	1.441	0.363	0.147	0.87-2.36
	Mother IgM positive	0.612	0.426	0.481	0.51-2.39
MSP3a					
	IgM mother positive	0.734	0.612	0.711	0.14-3.76
	HIV positive	4.592	5.534	0.206	0.43-48.74
MSP3					
	IgM mother positive	0.164	0.073	**<0.001**	0.06-0.39
	HPLC positive	1.632	0.711	0.260	0.69-3.83
	Used IPTp	2.022	0.762	**0.062**	0.96-4.23
	<20 years	0.516	0.244	0.163	0.20-1.30
	HIV positive	0.471	0.383	0.355	0.09-2.31

Anti-*P. falciparum* IgM seropositivity in cord blood in relation with reported use of IPTp by the mother during pregnancy and presence of sulfadoxine in maternal blood at delivery (HPLC).

4.4.5 Use of IPTp and prenatal immune priming to malaria

Factors which fitted perfectly were not included in the multivariate model. Since in Bivariate analysis, all main factors; IPTp and sulfadoxine in blood fitted perfectly and yet none of the other factors was significant no model was fitted for sero-positivity against MSP3a. After multivariate analysis, babies born to mother who were exposed to malaria toward delivery were more likely to have evidence of IgM to malaria antigens at delivery GLURP P=0.028 (Table 16).

Table 16: Factors affecting *P. falciparum* IgM sero-positivity in the babies

Outcome	Effect	OR	s.e	Pvalue	95% CI
GLURP					
	IgM mother positive	0.175	0.138	**0.028**	0.03-0.82
	HPLC positive	0.873	0.751	0.875	0.16-4.71
HRPII					
	IgM mother positive	0.510	0.484	0.479	0.07-3.28
	Used IPTp	2.405	1.601	0.187	0.65-8.86
	HPLC Positive	1.453	1.08	0.617	0.33-6.28
MSP3					
	IgM mother positive	0.128	0.064	**>0.001**	0.048-0.34
	Used IPTp	2.633	1.120	**0.023**	1.14-6.06
	HPLC positive	1.010	0.482	0.982	0.39-2.57

CHAPTER FIVE: DISCUSSION

This thesis discusses the *P. falciparum* infection and immunity to blood stage infection in the mother and baby at delivery and its relation with sulfadoxine/ pyrimethamine intermittent presumptive treatment during pregnancy. It further describes the association between use of IPTp during pregnancy and congenital malaria , fetal immune priming and proportions of IgG antibodies against selected P. *falciparum* antigens transferred from mother to baby. We found that 15% of the mothers had active placental malaria at delivery in Mulago Hospital. Only 1.4% of the babies delivered had evidence of active *P. falciparum* infection although up to 33% had evidence of recent exposure to *P. falciparum* parasites /antigens at delivery. Transplacental transfer of IgG antibodies from the mother to baby was not affected by using IPTp during pregnancy. We however found that self-reported use of sulfadoxine pyrimethamine as IPTp during pregnancy may not be valid for determining IPTp use in Mulago National Referral Hospital.

5.1 Burden of infection

Using placenta histology and blood film examination, a high proportion of the study population was found to have active placental infection (15.5% in total). In addition, histological examination identified a number of women with past placental infection (4.5%). Adverse clinical outcomes associated with malaria in pregnancy are linked to pathological changes in the parasite-burdened placentas (Rogerson *et al.,* 2003), and histology is the only method that provides insight on pathological changes as well as timing of infection (acute, chronic, past). Past infections would have been missed had we relied solely on blood film examination. Of note, a minor proportion of women with no evidence of placental infection (1.3%) were found to have peripheral parasites. Individuals in endemic areas can, however, harbor circulating parasites asymptomatically. Hence, the mere presence of peripheral parasites coinciding with pregnancy is not a proof of their involvement in placental sequestration and adverse clinical outcomes. Placenta infection was thus used as a reliable measure of pregnancy associated malaria burden in the present study.

The majority of the placental malaria cases were concentrated among gravidae one through three. Observed pattern may be explained by the development of protective antibodies in successive pregnancies. Acquired antibodies recognize an antigenically and functionally distinct subpopulation of parasitized Red Blood Cells (pRBCs), which is clonally expanded owing to the new niche of growth provided by the placenta (Fried *et al.*, 1998; Ricke *et al.,* 2000). The functional distinction between pregnancy-associated parasites and parasites of non-pregnant individuals has partly been explained by the CSA-adhesion ability of the former (Fried & Duffy, 1996; Beeson *et al.*, 1999). Accordingly, in another study (Rasti *et al.*, 2006), we found CSA-adhesion to constitute a prominent functional feature of Ugandan placental pRBCs. The parity dependency found here in, was however not as marked as previously reported from high transmission areas (Desai *et al.*, 2007). This shift may be a reflection of the lower transmission level in the area but may also reflect the presence of other confounding factors such as HIV. Interestingly, placental parasites from this region were found to interact with several placental receptors (Rasti *et al.*, 2006); whereas exclusive CSA-adhesion has been reported from highly endemic areas. Of note, parity dependency of placental infection was only significant in the crude analysis. Although a trend of higher infection rate in younger primigravidae was observed, the effect of gravidity could not be separated from age. The analysis was hampered by sample size limitations, in particular the absence of higher parities (≥G4) in the younger age group.

5.2 Low validity of self-reported use of IPTp during pregnancy

This study explored the validity of self-reported sulfadoxine/pyrimethamine IPTp by testing for presence of SDX in maternal blood at delivery using HPLC. Two main findings of this study are that self-report on sulfadoxine/pyrimethamine IPTp use is unreliable not only for knowing whether the pregnant patient took the SP or not but also for finding out when the patient took the drug. Several patients who reported not having taken SP were found to have the drug metabolites in their blood. Further, some patients who reported having taken the drug before nine weeks preceding baby delivery (when SDX would be too low to be detected in blood by HPLC) were also found to have the drug in the blood. On the other hand, some patients claiming to have taken SP within nine weeks before delivery (when blood SDX would be

detected by HPLC) actually did not have detectable SDX blood levels. Interestingly, participants who self-reported IPTp use during their present pregnancy were more likely to have SDX in their circulation at delivery, although the level of agreement was only slight as assessed by kappa statistics. Although only 29.2% of participants who reported IPTp use actually had SDX in their blood at the time of delivery, 25% of participants who reported not taking IPTp had SDX in blood at delivery. This finding questions the validity of self-reported data in estimating the IPTp coverage. The findings of this study concur with a previous study in Uganda which found low validity of caretakers' report on use of antimalarials and antibiotics (Hildenwall *et al.*, 2009).

Despite over 95% of participants attending antenatal care at least once during the current pregnancy only about 60% self-reported to have received at least one dose of IPTp during pregnancy. Older mothers and the more educated were more likely to use IPTp during pregnancy. It is likely that the more educated pregnant women are aware of the importance of IPTp and are more likely to demand for it and use it during pregnancy.

The high antenatal coverage and the fact that more than 75% of the participants had the first ANC visit before 28 weeks of gestation did not improve the IPTp coverage in this population. This is in agreement with a previous study in Uganda which demonstrated that frequent antenatal visits did not have an influence on the uptake of IPTp during pregnancy. Pregnant women not receiving drugs in the ANC clinic for any reason and drug stock out in the clinic has been found previously to be the main reason for not taking (Ndyomugyenyi *et al.*, 2010). It has been proposed that a community based approach on delivery of IPTp may be effective in improving IPTp uptake and adherence (Mbonye *et al.*, 2008). This approach however is more expensive to establish compared to improving the services in the existing health care delivery systems. Studies to identify facility related factors hindering IPTp use and how these can be solved in Uganda are recommended.

Participants who reported SP IPT use during pregnancy were more likely to have the drug in their circulation at delivery. There was a slight agreement between reported use and finding the drug in the maternal blood. Although only 28.9% of participants who reported IPTp use actually had the drug at the time of delivery, 25% of the participants who denied taking IPTp had the drug in circulation at delivery. This

finding questions the validity of self-reported data in estimating the IPTp coverage. A previous study in Uganda indicated low validity of care takers' report on use of antimalarials and antibiotics before coming to hospital (Hodel *et al.*, 2009; Hildenwall *et al.*, 2009).

In our results participants who reported use of iron sulphate and folic acid during pregnancy were more likely to report use of IPTp during pregnancy. Interestingly, we found that reporting having used IPT in the current pregnancy was associated with having post primary education in women although no independent factors were associated with finding sulfadoxine in blood at delivery.

In another study in Kenya which looked at antimalarial drugs before initiating treatment in participants who reported no use of drug in 28 days prior to enrolment, it was found that the proportion of participants with residual antimalarials was high and self-report on drug intake was unreliable (Weinhardt, 1998). This is in keeping with the findings in this study where we found that self-report is only in slight agreement with finding the drug in blood at delivery. In this study however the agreement was weak. A previous study has suggested that the validity of self-reported data may be improved by using focus group discussions, in the language which the respondents are very familiar and with direct open ended questions sequenced from the least to the most threatening

Although self-reported use of SP IPTp use during pregnancy has low validity, blood sampling is not practical in performing population surveys. We recommend that in addition to the questions given in survey the participants should be shown the samples of medicines being asked.

5.3 Association between the use of SP IPTp and IgG antibody transfer

Acquisition of protective immunity to malaria is slow and requires repeated parasite exposure to be maintained .The malaria immunity is strain specific and *P. falciparum* parasite stage specific. In malaria endemic areas, children born to immune mothers are protected against disease during their first half year of life by maternal antibodies transferred in-utero (Sehgal *et al.*, 1995) and presence of fetal haemoglobin which

does not favour growth of the parasites. The aim of this Substudy was describe the association between antimalarial IgG antibody levels to selected P. *falciparum* parasite blood stage antigens in the mother/ baby pairs and proportion transfered is affected by use of IPTp during pregnancy. Antibodies to malaria blood stage antigens have been previously been found to be important in protection against clinical malaria (Dodoo *et al.*, 2000).

The proportions of selected anti-*P. falciparum* blood stage antibodies transferred from the mother to the newborns were not affected by use of IPTp (at least one dose) during pregnancy. This was not influenced by the number of doses of IPTp taken. The mothers with high levels of antibodies however generally transferred less proportion to the corresponding babies for all antigens tested on bivariate analysis Table 10. After multivariate analysis only proportions of anti-GLURP and anti-MSP3a transferred from the mother to baby remained significantly affected by the levels of antibodies in the mother where mothers with less anti-*P. falciparum* antibodies transferring a higher proportion compared to their counter parts Table 11.

Previous studies have indicated that mothers with higher antibody levels tend to transfer less to the corresponding neonates (Hood *et al.*, 1994; Palmeira *et al.*, 2012). The amount of IgG transferred depends on the amount of cell surface receptors available, because unbound IgG molecules are digested by lysosomal enzymes inside the vesicles (Saji *et al.*, 1994). Previously it has been shown that IPTp use during pregnancy led to reduction in antibodies against placental malaria in some studies (Staalsoe *et al.*, 2004) and not others (Serra-Casas *et al.*, 2010).

In the study for proportions transferred, 12 mothers delivered premature babies Table 5. Prematurity however did not affect the proportions transferred as previously reported (Saji *et al.*, 1999). This could have been due to other confounding factors like placental integrity influencing the effect of prematurity on transfer, this was possibly due to the fact that even the premature babies were born between 36 and 37 weeks of gestation.

The absolute antibody levels in the mothers and their corresponding babies were not affected by use of IPTp during pregnancy. Using IPTp being a short intervention may not affect the antimalarial antibodies in the mothers and their babies. Maternal age

and parity did not influence the levels of antibodies to all blood stage antigens tested. Yet it is well known that the young women and primigravidae are more susceptible to malaria during pregnancy (Saute *et al.*, 2002). This is in line with previous studies which demonstrated that maternal age, parity, weight and height do not affect the amount of antibodies transferred from mother to baby (Wesumperuma *et al.*, 1999; van den Berg *et al.*, 2011).

Congenital exposure of the fetus to malaria parasites/antigens may have affected the levels of antibodies in cord blood. Children born to mothers in malaria endemic areas may get congenital malaria (Uneke, 2007). The IgG antibody levels in the newborn may be attributed to both trans-placental transfer and fetal exposure to malaria antigens. All cases where the antibody levels were higher in the cord blood compared to the corresponding mother were eliminated from the analysis for proportions transferred.

These findings imply that with no significant effect on the proportions of antibodies transferred with use of IPTp, its use should be advocated with no anticipated adverse effects on newborn immunity. Having sulfadoxine in blood at delivery as evidence of using IPTp during pregnancy did not affect the proportion of IgG antibodies transferred from mother to baby. This may imply that even in cases where self-report may be inadequate we can still confirm that IPTp use does not affect antibody transfer. We recommend more studies to assess the effect in different transmission intensity and with at least two doses.

5.4 Association between IPTp on congenital malaria and immune priming of the fetus

Cord blood *falciparum* parasiteamia in the participants by microscopy was 1.4% although 2-33% had IgM in cord blood as evidence of recent exposure to malaria parasites/antigens. This is lower than what was reported in Hoima district in Uganda of 47% and this was not affected by using Chloroquine chemoprophylaxis (Ndyomugyenyi & Magnussen, 2000). This was done before the IPTp policy was implemented in Uganda. A study done in malaria endemic area in Burkina Faso found prevalance of cord parasiteamia of 1.4% which is comparable to our findings in this study (Ouedraogo *et al.*, 2012) which was significantly associated with

parasite density in the maternal and cord blood. Generally transplacental transmission of *P. falciparum* appears to be low in malaria endemic areas ranging from 1-5% (Uneke, 2007).

The effect of exposure of the fetus to malaria parasites/antigens in utero on the immune response during early infancy is not very clear. Some studies have shown that babies born to mothers with placental malaria are more susceptible to malaria infection in infancy (Schwarz *et al.*, 2008; Malhotra *et al.*, 2009). Trans-placental passage of parasite-derived antigens may leads to, tolerance of the fetal immune system. Another study demonstrated no effect of placental malaria on neonatal immunity (Soulard *et al.*, 2011). Universal use of malaria preventive measures of all the mothers at risk to prevent the adverse effects in the fetus should be encouraged

Self-reported Use of IPTp (at least one dose) by the mother during pregnancy and presence of sulfadoxine in maternal blood at the time of delivery were protective of congenital exposure to *P. falciparum*. The protective effect was observed for all *P. falciparum* blood stage antigens tested (GLURP, MSP MSP3a) except HRPII antigens. This finding implies that using IPTp during pregnancy is effective in protecting the fetus against congenital exposure, this may be through control of placental parasiteamia. Placental malaria and maternal anemia have been associated with in utero priming to *P. falciparum* antigens (Gouling *et al.*, 2003). It has been postulated that presence of parasites for an extended period may alter the fetal maternal barrier leading to congenital malaria. The presence of IgM in cord blood indicates that the fetus was exposed in utero since it does not cross the placenta.

The prevalence of cord blood parasitemia was 1.4% and IgM to different *P. falciparum* antigens ranged from 2-33%. A proportion of newborns had IgM in cord blood in absence of parasiteamia. Malaria parasites do not usually cross the placental barrier and such sensitization is most probably caused by trans-placental passage of soluble *P. falciparum* antigens or cross-reactive antigens from other pathogens leading to fetal T and B cell activation (Metenou *et al.*, 2007). Finding IgM in cord blood was significantly associated with having IgM in maternal blood which confirms that the source of fetal infection was maternal.

Pregnant women in malaria endemic areas are often infected with malaria parasites and expose the fetus to malaria antigens. The trans-placental transmission of malaria from the mother to the fetus called congenital malaria has been well documented (King *et al.*, 2002; Uneke, 2007). The mechanisms underlying the trans-placental transfer are not clear but malaria parasites have been detected in cord blood (Gouling *et al.*, 2003). *P. falciparum* antigens and possibly cross reactive from the other parasite cross the placenta and activate fetal T and B cells in utero.

It has been postulated that infants born with primed cells may produce secondary response upon exposure to that antigen whereas those who did not produce primary response. This is important in terms of infant immune response and consequently on severity to the acute infections. Others studies have suggested increased susceptibility in infants who had intrauterine exposure to malaria (Bonner *et al*., 2005; Mutabingwa *et al*., 2005).

Presence of malaria parasites in maternal blood was significantly associated with detecting IgM against all tested blood stage antigens in maternal blood. Since IgM is the first antibody to be produced after its presence in sera indicates recent exposure to malaria parasite/antigens. All HIV positive participants had IgM antibodies against GLURP and were more likely to have IgM antibodies against MSP. There was however there was no association with HRPII and MSP3a .Since IgM is evidence of recent infection it is clear that HIV infection was highly associated with having malaria infection in the mother towards delivery as shown in previous studies (Nkhoma *et al.* , 2012) .

Participants who had sulfadoxine in blood were less likely to have IgM in maternal sera for MSP3 and HRPII although no effect with GLURP and MSP3a. Reporting IPTp use during pregnancy protected the mothers against infection towards delivery (IgM). These findings indicate that the sulfadoxine is still effective in controlling parasiteamia in the during pregnancy in this area with reported resistance to SP.

5.5 Effectiveness of SP IPTp

The recent recommendation by WHO to give four doses of IPTp and it can be given up to the time of delivery (WHO, 2012). Although malaria parasite resistance to SP has compromised its use in case management of symptomatic children, it is effective

as IPTp possibly because of the pre-existing immunity in the adult. One retrospective study in Tanzania with 36% prevalence of 581dhfr mutation indicated that use of SP IPTp was harmful to the mother and led to fetal anaemia (Harrington *et al*., 2011). In a cross-sectional study done in Uganda (Tororo), where 92% of mothers took at least one dose of SP IPTp during pregnancy, found that using two or more doses of SP IPTP was not associated with protection against the adverse effects of pregnancy malaria (Arinaitwe *et al*., 2013). In a randomized clinical trial done in Mozambique in an area with high level of quintuple mutation, using SP IPTp was associated with reduction in neonatal mortality but not with parasite density or any malaria related morbidity (Menéndez *et al*., 2010). In Malawi which is one of the countries where SP IPTp was first implemented in 1993, a recent study shows that it is effective in prevention of adverse effects of pregnancy malaria (Gutman *et al.,* 2013). A meta-analysis across 32 countries in Africa, where the prevalence of SP resistant parasites is high have indicated use of SP IPTp leads to reduction in neonatal mortality (Eisele *et al*., 2012). These however, were observational studies which limited the ability to control for potential confounders. Using SP is still beneficial as IPTp in areas with high levels of resistance to SP in case management of children. It is important to monitor its effectiveness and the challenge is that, there are no standard methods of doing this.

5.6 Study limitations

The studies done in this thesis were largely cross-sectional and described associations which could have limited the ability to control for confounders. This is because using SP IPTp was standard of care for all pregnant mothers and therefore no option of doing randomized trials. Data on the potential confounders was collected. Multivariate analysis done minimized the effect caused by confounders on association. In the studies, association between use intervention like ITN and IPTp during pregnancy was assessed using reported data from the participants. This may be prone to bias since some women may have been aware of what is required during pregnancy and therefore reported falsely. Self-reported use of SP IPTp was corrected for by presence or absence of sulfadoxine in blood at delivery. For babies with congenital malaria and IgM antibodies at delivery, we were not able delineate the infection acquired in-utero and that acquired during delivery. In addition

congenital exposure to malaria antigens may have affected the levels of antibody in the newborn and therefore the apparent proportion transferred. The number of newborn with evidence of intrauterine exposure was small and therefore effect may have been small.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. Up to 15.5% of mothers delivering in Mulago National Refferal Hospital have active placental malaria at delivery which is associated with maternal anaemia and low birthweight.

2. Self-reported data on use of IPTp during pregnancy in Mulago has low validity when determining IPTp use.

3. IPTp does not affect antibody levels in the mothers and proportions transferred from mother to baby.

4. The prevalence of cord blood parasiteamia in Mulago Hospital is 1.4% and IPTp use is protective against congenital malaria and immune priming of the fetus

6.2 Recommendations

1. Intermittent presumptive treatment with sulfadoxine/pyrimethamine should be given under directly observed therapy to ensure better estimates and compliance.

2. Importance of IPTp during pregnancy should be emphasised to the pregnant mothers in the antenatal clinic. This may increase the compliance of the pregnant mother to IPTp and prevent the adverse effects of pregnancy malaria.

3. More studies aiming at improving reliability of self-reported data on IPTp use during pregnancy should be done.

4. More studies should be done to establish factors that affect transfer of immunoglobulins from the mother to baby and the effect of in-utero priming on development of immunity in the infant.

5. Studies to assess the effect of IPTp to immunity in different malaria endemic settings are recommended.

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PAPER I

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Research Article

Malaria Burden in Pregnancy at Mulago National Referral Hospital in Kampala, Uganda

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Pregnancy-associated malaria is a major global health concern. To assess the *Plasmodium falciparum* burden in pregnancy we conducted a cross-sectional study at Mulago Hospital in Kampala, Uganda. Malaria prevalence by each of three measures—peripheral smear, placental smear, and placental histology was 9% (35/391), 11.3% (44/389), and 13.9% (53/382) respectively. Together, smear and histology data yielded an infection rate of 15.5% (59/380) of active infections and 4.5% (17/380) of past infections; hence 20% had been or were infected when giving birth. A crude parity dependency was observed with main burden being concentrated in gravidae 1 through gravidae 3. Twenty-two percent were afflicted by anaemia and 12.2% delivered low birthweight babies. Active placental infection and anaemia showed strong association (OR = 2.8) whereas parity and placental infection had an interactive effect on mean birthweight ($P = .036$). Primigravidae with active infection and multigravidae with past infection delivered on average lighter babies. Use of bednet protected significantly against infection (OR = 0.56) whilst increased haemoglobin level protected against low birthweight (OR = 0.83) irrespective of infection status. Albeit a high attendance at antenatal clinics (96.8%), there was a poor coverage of insecticide-treated nets (32%) and intermittent preventive antimalarial treatment (41.5%).

1. Introduction

Malaria is a major public health problem affecting between 300–500 million people annually. *Plasmodium falciparum* is responsible for the main disease burden afflicting primarily sub-Saharan Africa. In areas with stable malaria transmission, due to protracted exposure to infectious bites, partial protective immunity to clinical malaria is gradually acquired with increasing age. Severe *P. falciparum* malaria is thus predominantly a childhood disease. There is however one exception to this general rule: pregnancy-associated malaria (PAM). Despite their semi-immune status, women become more susceptible to malaria upon pregnancy. In endemic areas, approximately 25 million pregnancies are at risk of *P. falciparum* infection every year, and 25% of these women have evidence of placental infection at the time of delivery [1–3].

Clinical features of infection during pregnancy vary with the degree of preexistingimmunity and thus the epidemiological setting. In high-transmission areas, maternal anaemia and low birthweight (LBW), as a result of prematurity and/or intrauterine growth restriction (IUGR), are the main adverse outcomes of placental infection and tend to be more severe in first pregnancies and in younger mothers [2, 4–8]. These effects are less marked by gravidity in low-transmission areas [9]. Moreover, LBW babies are in general at increased risk of death during infancy. Each year between 100 000 to 300 000 infant deaths may be attributable to maternal malaria in Africa [10, 11].

The pathophysiological processes preceding adverse outcomes in PAM are initiated by the accumulation of *P. falciparum*-infected red blood cells (pRBCs) in placental intervillous spaces, causing inflammatory responses and deposition of fibrinoid material. Adhesive interactions between parasite-encoded erythrocyte surface antigens and intervillous host receptors such as chondroitin sulphate A (CSA), hyaluronic acid (HA), and nonimmune immunoglobulins (Igs) are believed to be involved in the sequestration process [12].

The exact details of how sequestration causes LBW are unknown. Local inflammatory immune responses in the infected placenta may induce early labour [13]. IUGR appears to be related to reduced nutrient transport to the foetus due to high parasite and inflammatory cell density [13, 14]. Maternal anaemia may also independently contribute to IUGR, most likely via a reduction in oxygen transport to the foetus [13].

In Uganda, the overall burden of malaria is high and its adverse outcomes to the infected mother and the unborn child are widespread. There is growing awareness that pregnancy-associated malaria is also of importance in areas of low and seasonal transmission worldwide. Although Uganda is regarded as being a malaria-endemic region, the transmission level varies considerably across the country [15]. Similar to studies from other countries, data on malaria burden are mainly available from areas of high transmission. In light of this, we conducted a cross-sectional study to assess the PAM burden in a periurban/urban setting with low, seasonal malaria transmission. Moreover, this is the first study providing baseline data on the burden of PAM and its possible adverse outcomes (anaemia, LBW) at Uganda's National Referral Hospital at Mulago.

2. Patients and Methods

2.1. Study Site. Mulago Hospital serves as Uganda's National Referral Hospital and is situated in the capital city of Kampala. In Uganda, there is stable *P. falciparum* transmission in 95% of the country. The remaining 5% of the country, mainly the highland areas with altitudes >1,600 m, are subject to low and unstable malaria transmission. Kampala is located 1,300–1,500 m above the sea level close to the equator and experiences a tropical climate with rainfalls throughout the year. The population in the area experiences low-intermediate malaria transmission with the highest peaks toward the end of the two major rainy seasons (March to May and October to December). This study was conducted from October 2004 to January 2005. The rainfall patterns in Kampala were typical, with two peaks, during 2004. There was an average of 146.7 mm of rainfall between October and December 2004 and 40 mm in January 2005, a level comparable to the corresponding seasons in previous years. Since the city is built on hills and valleys, the entomological infection rates (EIR) vary considerably depending on the residential/occupational area. Water usually collects in the valley floors resulting in breeding sites for the anopheline mosquitoes. But generally speaking the EIR is low (<10 bites per person per year). Except for the main commercial centre, the city and the surrounding areas are essentially rural.

Mulago Hospital has 33,000 antenatal attendances and 23,000 deliveries per year, a maternal mortality ratio of 505 deaths per 100,000 live births, a stillbirth rate of 5%, and an HIV prevalence of about 11% among pregnant women. The current national policy for prevention of malaria in pregnancy in Uganda is the use of insecticide-treated bednet and intermittent preventive treatment with two doses of sulfadoxine-pyrimethamine. In Uganda, pregnant women are also given iron and folic acid supplementation and antihelminth drugs to prevent anaemia and hookworm infestation, respectively.

2.2. Study Population and Data Collection. From October 2004 to January 2005, women delivering at the Mulago Hospital labour suite, aging ≥15 years and ≥28 weeks of gestation, were recruited to the study. Patients with cardiac disease, chronic hypertension, renal disease, clinical AIDS, or diabetes and those with obstetric complications during the present pregnancy, such as preeclampsia, eclampsia, antepartum haemorrhage, and chorioamnionitis were excluded from the study. Full informed consent (or assent for those <18 years of age) was obtained from all the participants. On average, five to seven participants were recruited consecutively per day, from 8.00 am to 5.00 pm excluding weekends and public holidays. All ethical aspects of the study were granted by the Makerere University Medical School Research and Ethics Committee and Uganda National Council for Science and Technology (permit No. MV922), and the ethical committee at Karolinska Institutet, Sweden (permit No. 04-533/2). A precoded, standardized questionnaire was used to record pregnancy history, clinical examination outcome, and pregnancy outcome for each study subject. Some key aspects covered included area of residence, age, marital status, occupation, education, parity, visits to antenatal clinic (ANC), bednet use, use of intermittent preventive antimalarial treatment (IPT), iron and folic acid supplementation, gestational age, birth status (live or stillbirth), and birthweight. The information on use of IPT, iron and folic acid supplementation was obtained from interview and/or antenatal card.

2.3. Sample Collection and Laboratory Studies. Venous blood was collected within a few hours (2–4 hours) prior to delivery for peripheral blood diagnosis of malaria and for haemoglobin testing. After delivery, the placentas were collected in 0.9% NaCl for smear and histological assessment of malaria. A small incision was made paracentric on the maternal-facing side of the placentas to prepare blood films. Thick and thin blood films of peripheral and placental blood were stained by Giemsa, and malaria diagnosis was assessed by microscopy following standard procedures. A small biopsy of the maternal-facing surface of each collected placenta was also removed and preserved in 10% neutral buffered formalin. The biopsies were paraffin embedded and stained with haematoxylin and eosin for histological evaluation of placental malaria infection. The slides were examined by a pathologist blinded to other patient data. Reexamination was performed by two different pathologists in all cases where histology and blood films were in disagreement.

2.4. Definitions. Upon histological assessment, placental biopsies were classified according to the following criteria [16, 17]: (a) *active acute infection*: parasites present in maternal erythrocytes, (b) *active chronic infection*: presence of parasites and a significant amount of pigment deposition in fibrin or monocytes within fibrin, (c) *past infection:*

presence of pigment within fibrin only, no parasites, and (d) *not infected:* no evidence of parasites or pigment. Low birthweight is defined as weight <2500 g. Anaemia is defined as haemoglobin (Hb) level <11 g/dl and severe anaemia as Hb <7 g/dl. Preterm delivery is regarded as deliveries occurring prior to 37 weeks of gestation. Bednet users refer to individuals using net of any category (untreated as well as insecticide-treated nets). ITN users refer to those using insecticide-treated nets only.

2.5. Statistical Analysis. Data were entered and verified by two independent individuals using Epi Info version 6.1 and exported to SPSS version 12.0 for further analysis. Placental *P. falciparum* infection, anaemia, and birthweight were regarded as principal outcomes. Potential risk factors or confounders considered were area of residence, literacy, age, use of bednet, IPT, and iron and folic acid supplementation. Following the assessments of the crude effect of each risk factor, the list of interesting risk factors was organised into three blocks: background, intermediate and proximate risk factors. Background risk factors were adjusted for each other: intermediate for background and intermediate, and proximate for all other risk factors. Adjusted odds ratios were obtained using logistic regression. The significance of each variable was reconsidered by backward stepwise elimination. Variables with a P-value of <.1 were included in the final model. Due to the presence of missing values in the dataset, missing value analysis was performed prior to logistic regression modelling in order to assess the total number and randomness of missing values.

Proportions were compared using χ^2 tests and validated at a 5% significance level. Multiway univariate analysis of variance (ANOVA) was used to study the effects of placental infection, gravidity and age, and combinations thereof, on haemoglobin level and birthweight.

3. Results

3.1. General Description. A total of 399 women who consented to take part in the study were recruited between October 2004 and January 2005. The age of the participants ranged from 15 to 44 years, median 20 (IQR: 18–25). Most participants were residents of Kampala (68.6%) and Wakiso (22.9%) districts. The majority had primary (46.4%) or secondary (42.3%) level of education; 66.3% were housewives or unemployed; 74% were married. Most women (96.8%) had attended an antenatal clinic at least once during the present pregnancy. One hundred and sixty five (41.5%) had received intermittent preventive antimalarial treatment (IPT); of these, the majority had received one dose (74.5%), whereas 20.5% had received the two recommended doses. Sulfadoxine-pyrimethamine (SP) was the drug of choice (89.3%). Other preventive measures taken during pregnancy consisted of the use of mosquito bednets: two thirds (267/397; 67.3%) utilized nets of any sort: 32% (127/397) were strict insecticide-treated net (ITN) users. Most women had also received iron (79.3%) and folic acid (70.4%) supplementation during the present pregnancy (see Table 1 for general characteristics). The study population consisted of 196 (49.4%) gravidae 1 (G1) or primigravidae, 142 (35.8%) gravidae 2-3 (G2-3), and 59 (14.9%) gravidae 4 or above (≥G4). A number of factors showed crude associations with gravidity. Primigravidae tended to be younger ($P < .0001$) and more literate ($P = .001$), delivered more low-birthweight babies ($P = .06$), used less IPT ($P < .0001$), and were more afflicted by placental malaria infection ($P = .035$) as compared to multigravidae (G2 and above; Table 2).

3.2. Prevalence of Malaria. The prevalence of malaria by each of the three measures peripheral smear, placental smear and placental histology, was 9% (35/391), 11.3% (44/389) and 13.9% (53/382), respectively. Out of 53 women with histological evidence of infection, 34 (64.1%) were classified as acute, 2 (3.8%) as chronic, and 17 (32.1%) as past infection. *P. falciparum* was the sole species found in all cases. A total of 380 cases, where placental histology and the corresponding blood film data were available, were used for further associative analysis. In order to avoid loss of data, all cases diagnosed as being acute or chronic infection by histology or as *P. falciparum* positive by placental blood film examination were regarded as active infection. Based on the new case definition criteria, a total of 15.5% (59/380) and 4.5% (17/380) had active versus past placental infection, respectively. Peripheral parasites were present in 50.9% (28/55) and 5.9% (1/17) of cases with active versus past infection. In patients with no evidence of active or past placental infection, only 1.3% (4/304) had peripheral parasitaemia. Placental infection could thus be used as a reliable measure of malaria burden in the remainder of the analysis.

3.3. Prevalence of Anaemia and Low Birthweight. Twenty-two percent of the women were anaemic (Hb <11 g/dl) prior to delivery. Severe anaemia (Hb <7 g/dl) was however uncommon (3/389; 0.8%). The mean haemoglobin level was 12.3 g/dl (IQR = 10.9–13.4) (Table 1). The overall prevalence of stillbirths was 2.8% (11/389) and preterm deliveries 3.1% (12/389). Among live-born babies, 12.2% (46/378) were of low birthweight and the mean birthweight was 3100 g (IQR = 2800–3500) (Table 1).

3.4. Risk Factors Associated with Placental Malaria. Table 3 illustrates crude and adjusted odds ratios for factors associated with placental *P. falciparum* infection. Whilst higher age and use of bednet were found protective, district of residence, educational level, and use of IPT showed no association. Being multigravid (≥G4) was protective in the crude analysis (OR = 0.38; CI =0.14–0.88; $P < .05$) (Figure 1(a)); the effect was, however, lost in the adjusted model (Table 3). As gravidity and age are highly correlated variables, an age-stratified analysis was performed to separate out the effect of the two. Gravidity groups were stratified by two age groups (15–19 years versus ≥20 years). A trend of higher infection rate, although not significant, was observed in young primigravidae (G1: 33/126 = 23.1% versus ≥G2: 4/28 = 14.3%; OR = 2.1; CI = 0.69–6.6; $P = .18$). The analysis was,

Table 1: General characteristics.

District (%)	
Kampala	68.6
Wakiso	22.9
Other	8.5
Ethnic group (%)	
Ganda	62.8
Nyankole	8.0
Soga	6.0
Rwandese	5.3
Other	17.9
Median age (years)	20 (IQR: 18–25)
Education (%)	
Illiterate	6.9
Primary	46.4
Secondary	42.3
Higher	4.3
Marital status (%)	
Married	74.0
Single	20.4
Cohabitant	5.6
Occupation (%)	
Housewife	55.8
Peasant	2.9
Student	9.7
Casual worker	18.2
Professional job	2.9
Unemployed	10.5
Visit to ANC (%)	96.8
Received IPT (%)	41.5
Use of bednet (%)	67.3
Use of ITN (%)	32.0
Median Hb level (g/dl)	12.3 (IQR: 10.9–13.4)
Hb <11 g/dl (%)	22.0
Folic acid supplementation (%)	70.4
Iron supplementation (%)	79.3
Median birthweight (g)	3100 (IQR: 2800–3500)
LBW (%)	12.2
Preterm delivery (%)	3.1
Stillbirth (%)	2.8
Caesarean (%)	17.6
Peripheral malaria (%)	9.0
Placental malaria (%)	
Histology	13.9
Blood smear	11.3

ANC: antenatal clinic, at least one visit during the present pregnancy.
IPT: intermittent preventive antimalarial treatment, at least one dose.
ITN: insecticide-treated net. LBW: low birthweight.

however, limited by the absence of higher parities (≥G4) in the younger age group.

3.5. Risk Factors Associated with Anaemia. The crude analysis and the adjusted final model, both, identified a strong association between active placental malaria infection and anaemia (OR = 2.76; CI = 1.4–5.5; $P = .003$) (Table 4, Figure 1(b)). The mean haemoglobin level was 11.6 g/dl (CI: 11.1–12.3) versus 12.6 g/dl (CI: 12.3–12.9) in patients with active infection or no infection, respectively, ($P = .02$). No associations were observed with any of the other considered risk factors. Of note, use of bednet showed a trend towards a protective effect (Table 4).

3.6. RiskFactors Associated with Low Birthweight. Factors associated with low birthweight are outlined in Table 5. Increased haemoglobin level and to some extent age showed protective association with low birthweight. Increased gravidity (≥G4) showed a crude but not adjusted protective association. No overall associations were found with district of residence, education, placental malaria infection, use of bednet, IPT, and iron and folic acid supplementation. A significant association was, however, found when interactions between gravidity and placenta infection groups and their effect on mean birthweight were analyzed (Table 6; $P = .036$). However, interpretation of the effect size of gravidity and placental infection on mean birthweight becomes complicated in light of the significant covariation of gravidity by age ($P = .034$; see Table 6 and Supplementary table in supplementary material available online at doi: 10.406/2010/913857). To analyze the pattern of the identified interaction, the age parameter was thus fixed at a mean value in all subgroups (Tables 7 and 8). The birthweight was on average lower in primigravidae irrespective of infection status. The smallest mean birthweight in primigravidae were found in mothers with active infection, whereas in multigravidae it was observed in mothers with past infection (Tables 7 and 8).

4. Discussion

Data on the burden of malaria in pregnancy, in particular from areas of low transmission, are scarce in Uganda as well as in other countries. A cross-sectional study was thus conducted from October 2004 to January 2005 in the periurban/urban setting of Kampala where the population experiences relatively low and seasonal malaria transmission. Exploiting placenta histology and blood film examination, a high proportion of the study population was found burdened with active placental infection (15.5% in total). In addition, histological examination identified a number of women with past placental infection (4.5%). Adverse clinical outcomes associated with malaria in pregnancy are linked to pathological changes in the parasite-burdened placentas [18], and histology is the only method that provides insight on pathological changes as well as timing of infection (acute, chronic, and past). Past infections would have been missed had we relied solely on blood film examination.

Table 2: General characteristics by gravidity.

Characteristics	Primigravidae (%)	Multigravidae (%)		*P*-value for gravidity change[a]
	G1 (*n* 196)	G2-3 (*n* 142)	≥G4 (*n* 59)	
District of residence				
Kampala	70.2	67.4	66.7	.97
Wakiso	22.0	23.2	25.0	
Other	7.9	9.4	8.3	
Age (years)				
15–19	68.9	14.9		<.0001
≥20	31.1	85.1		
Education				
Illiterate	4.4	7.1	12.5	.001
Primary	38.3	52.0	60.7	
Higher education	57.2	40.9	26.8	
Visit to ANC	97.2	95.6	98.2	.61
Recieved IPT	31.6	49.3	55.7	<.0001
Bednet (of any kind)	65.8	65.9	75.0	.38
Stillbirth	3.7	2.0		.31
LBW (<2500 g)	15.2	9.0		.06
Anaemia (<11 g/dL)	23.2	22.6	18.3	.73
Placental malaria				
Active infection	19.6	12.5	10.3	.035
Past infection	3.8	7.4	0	

ANC: antenatal clinic, at least one visit during the present pregnancy.
IPT: intermittent preventive antimalarial treatment, at least one dose.
[a]Chi-square test used. Age, stillbirth, and LBW were analysed using multigravidae as a pooled group (≥G2).

Table 3: Risk factors associated with placental malaria.

Factor level	Risk factor	Crude OR	Adjusted OR	(95% CI)	*P*-value
Background	*Age (continuous per year)*	*0.95**	*0.95*	*(0.89–1.00)*	*.066*
	District of residence				
	Kampala	1.0	1.0		
	Wakiso	1.18	1.4	(0.74–2.60)	.31
	Other	1.80	1.9	(0.79–4.74)	.15
	Education (continuous per level)	0.79	0.75	(0.48–1.17)	.21
Intermediate	Gravidity				
	G1	1.0	1.0		
	G2-3	0.81	1.0	(0.54–2.07)	.88
	≥G4	0.38*	0.72	(0.21–2.50)	.61
Proximate	Received IPT				
	None	1.0	1.0		
	1 dose of SP	0.93	1.11	(0.60–2.07)	.73
	2 doses of SP	0.55	0.49	(0.16–1.49)	.21
	Used bednet	*0.56**	*0.56*	*(0.31–0.99)*	*.046*

*Significant associations ($P < .05$).

Of note, a minor proportion of women with no evidence of placental infection (1.3%) were afflicted by peripheral parasites. Individuals in endemic areas can, however, harbor circulating parasites asymptomatically. Hence, the mere presence of peripheral parasites coinciding with pregnancy is not a proof of their involvement in placental sequestration and adverse clinical outcomes. Placental infection was thus used as a reliable measure of PAM burden in this study.

The majority of the placental malaria cases was concentrated among gravidae 1 through 3. Observed pattern may be explained by the development of protective antibodies by successive pregnancies. Acquired antibodies recognize

Table 4: Risk factors associated with anaemia (<11 g/dl).

Factor level	Risk factor	Crude OR	Adjusted OR	(95% CI)	*P*-value
Background	Age (continuous per year)	1.0	0.98	(0.93–1.04)	.51
	District of residence				
	Kampala	1.0	1.0		
	Wakiso	1.15	1.11	(0.60–2.07)	.74
	Other	0.40	0.44	(0.13–1.52)	.20
	Education (continuous per level)	1.14	1.14	(0.73–1.77)	.57
Intermediate	Gravidity				
	G1	1.0	1.0		
	G2-3	1.04	1.0	(0.56–1.80)	.98
	≥G4	0.86	0.70	(0.30–1.63)	.51
	Placental malaria (active + past)	*2.31**	*2.35*	*(1.27–4.35)*	*.006*
	Active infection	*2.66**	*2.76*	*(1.40–5.45)*	*.003*
	Past infection	1.37	1.53	(0.44–4.66)	.48
Proximate	Used bednet	0.73	0.67	(0.37–1.20)	.18
	Received IPT				
	None	1.0	1.0		
	1 dose of SP	1.05	1.17	(0.64-2.13)	.62
	2 doses of SP	1.46	1.17	(0.47-2.86)	.74
	Iron supplementation	1.51	1.96	(0.67-5.71)	.22
	Folic acid supplementation	1.26	0.70	(0.29-1.71)	.43

*Significant associations ($P < .05$).

Table 5: Risk factors associated with low birthweight (<2500 g).

Factor level	Risk factor	Crude OR	Adjusted OR	(95% CI)	*P*-value
Background	*Age (continuous per year)*	*0.91**	*0.93*	*(0.86–1.0)*	*.068*
	District of residence				
	Kampala	1.0	1.0		
	Wakiso	0.86	0.98	(0.45–2.13)	.97
	Other	0.21	0.21	(0.01–1.06)	.14
	Education (continuous per level)	1.1	1.05	(0.61–1.81)	.87
Intermediate	Gravidity				
	G1	1.0	1.0		
	G2-3	0.69	1.22	(0.54–2.77)	.63
	≥G4	0.30*	0.70	(0.14–3.51)	.67
	Placental malaria (active + past)	1.48	0.72	(0.29–1.80)	.48
	Active infection	1.61	0.69	(0.24–1.96)	.49
	Past infection	1.05	0.77	(0.16–3.77)	.75
	Haemoglobin level	0.83*	*0.83*	*(0.70–0.99)*	*.033*
Proximate	Use of bednet	1.33	1.65	(0.72–3.79)	.24
	Received IPT				
	None	1.0	1.0		
	1 dose of SP	1.20	1.21	(0.56–2.63)	.63
	2 doses of SP	0.84	0.96	(0.30–3.09)	.94
	Iron supplementation	0.74	0.19	(0.02–1.58)	.12
	Folic acid supplementation	1.0	4.48	(0.57–34.9)	.15

*Significant associations ($P < .05$).

Table 6: Multiway ANOVA—birthweight as dependent variable.

	Type III sum of squares	Df	Mean square	*F*	*P*-value	Partial Eta Squared
Corrected model	*9.063 E6*	*7*	*1294773.8*	*4.45*	*.000*	*0.078*
Intercept	*6.878 E7*	*1*	*6.878E7*	*236.21*	*.000*	*0.39*
Gravidity	*1832523.0*	*1*	*1832523.0*	*6.29*	*.013*	*0.017*
Placental malaria	420890.8	2	210445.4	0.72	.49	0.004
Age	*1055645.5*	*1*	*1055645.5*	*3.63*	*.058*	*0.010*
Gravidity age*	*1314216.3*	*1*	*1314216.3*	*4.51*	*.034*	*0.012*
Gravidity placental malaria*	*1957844.9*	*2*	*978922.4*	*3.36*	*.036*	*0.018*
Error	1.074 E8	369	291178.3			
Total	3.858 E9	377				
Corrected total	1.165 E8	376				

[a]R-Squared = 0.078 (Adjusted R-Squared = 0.060).

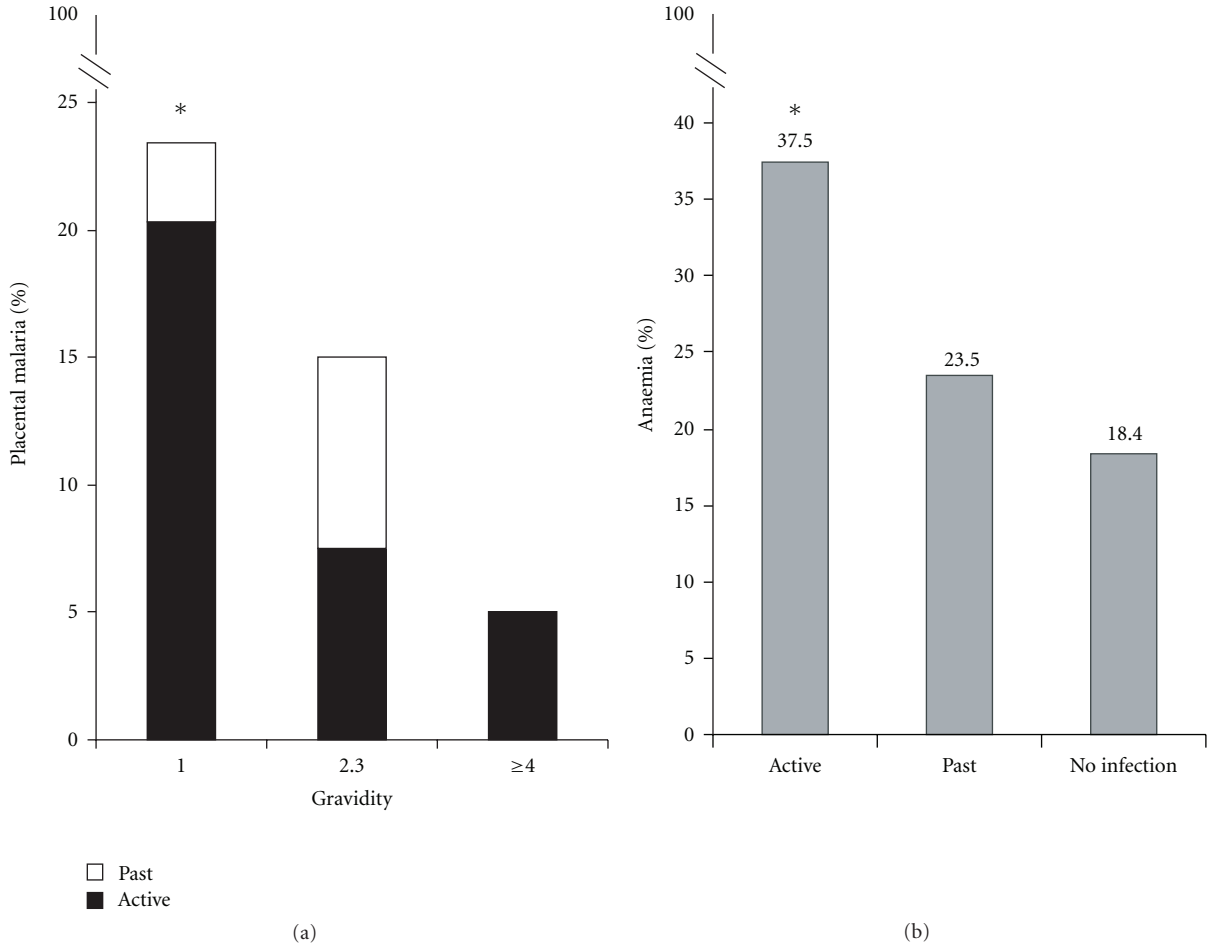

Figure 1: (a) Prevalence of placental malaria by gravidity. $^*P < .05$ for crude comparison between primigravidae and multigravidae. (b) Prevalence of anaemia by placental malaria infection status: active, past, or no infection. $^*P < .005$ for comparison between active versus no infection groups.

an antigenically and functionally distinct subpopulation of pRBCs, which is clonally expanded owing to the new niche of growth provided by the placenta [19, 20]. The functional distinction between pregnancy-associated parasites and parasites of nonpregnant individuals has partly been explained by the CSA-adhesion ability of the former [21, 22]. Accordingly, in a related study [23], we found CSA-adhesion to constitute a prominent functional feature of Ugandan placental pRBCs. The parity dependency, found herein, was however not as marked as previously reported from high-transmission areas [24]. This shift may be a reflection of the lower transmission level in the area but may also reflect

Table 7: Effect of gravidity and placental malaria infection on mean birthweight.

	Placental malaria	Mean birthweight	Std. error	(95% CI)
Primigravidae	Active infection	3015	105.8	(2810–3220)
	Past infection	3180	207.5	(2775–3590)
	No infection	3180	65.7	(3050–3310)
Multigravidae	Active infection	3440	114.0	(3220–3665)
	Past infection	2930	168.9	(2600–3265)
	No infection	3260	48.0	(3170–3355)

The table depicts the marginal mean birthweights estimated from the following model: (Intercept) gravidity, placental malaria, age, gravidity*age, gravidity*placental malaria. Covariates appearing in the model are fixed at the following values: age = 21.91.

Table 8: Pairwise comparisons of birthweight means across gravidity versus placental malaria subgroups.

Pair 1 versus Pair 2		Difference in mean birthweight	Std. error	df	*P*-value	95% CI Lower	95% CI Upper
G1* active	≥G2* active	−427.6	155.5	1	.006	−732.5	−122.8
	≥G2* no infection	−247.6	116.2	1	.033	−475.3	−19.9
G1* no infection	≥G2* active	−265.3	131.5	1	.044	−523.1	−7.5
≥G2* active	≥G2* past	508.1	204.2	1	.013	107.9	908.3
≥G2* past	≥G2* no infection	−328.0	176.0	1	.062	−673.1	17.0

Pairwise comparison of estimated birthweight marginal means. The mean difference is significant at the 0.05 level. Comparisons were made for all possible subgroup combinations but only significant combinations are included in the table. G1 = primigravidae; ≥G2 = multigravidae.

the presence of other confounding factors such as HIV. Interestingly, placental parasites from this region were found to interact with several placental receptors [23]; whereas exclusive CSA adhesion has been reported from highly endemic areas. Of note, parity dependency of placental infection was only significant in the crude analysis. Although a trend of higher infection rate in younger primigravidae was observed, the effect of gravidity could not be separated from age. The analysis was hampered by sample size limitations, in particular the absence of higher parities (≥G4) in the younger age group.

Anaemia, one of the main adverse outcomes of placental infection, was present in 22% of the study participants. The women were, however, mainly affected by moderate level of anaemia. A median Hb level of 12.3 g/dl and the low prevalence of severe anaemia (0.8%) may reflect a good general health status but is also comparable with levels reported previously from low- and intermediate-transmission areas in Africa [4]. A majority of the study participants had received iron and folic acid supplementation during pregnancy (79.3% and 70.4%, respectively; Table 1), which may in part explain the moderate level of anaemia observed in the study population.

Active placental infection constituted the only significant risk factor for anaemia in women of all parities (OR = 2.8). In the tropics, anaemia is multifactorial and may be caused by iron deficiency, parasitic infections, and haemoglobinopathies. Available data also suggest that severe anaemia is more common in women coinfected by malaria and HIV, which probably is a consequence of the increased level of parasitaemia observed in HIV-infected pregnant women [25, 26]. The potential role of infections other than malaria was not assessed in this study. In a recent study from Western Uganda, both hookworm infections and malaria were reported to be considerably associated with anaemia during pregnancy [27].

Whilst stillbirths and preterm deliveries were uncommon at Mulago's labour suite, delivery of LBW babies was more prevalent (12.2%). No direct associations were found between placental infection and LBW or mean birthweight (Tables 5 and 6). Parity and placental infection were, however, found to have an interactive effect on mean birthweight ($P = .036$). Birthweight reduction was most obvious in primigravidae with active infection and multigravidae with past infection (Tables 7 and 8). It is however difficult to assess the significance of the observed pattern as the role of other confounding factors could not be assessed due to sample size limitation.

The worst birthweight outcomes, for example, LBW, (<2500 g) have previously mainly been reported to be associated with chronic infections [28, 29]. A recent longitudinal study has also reported that the risk of LBW is higher with increased frequency of malaria episodes and with infections occurring in the second than in the third trimester or at delivery [30]. The majority of the cases in our study was afflicted by acute infection suggesting that infection was contracted close to delivery, which may explain the lack of association between placental infection and LBW. Moreover,

the low transmission level in the area in combination with better access of preventive measures in urban settings probably decreases the number of malaria episodes and prevents the establishment of stubborn chronic infections, hence affecting the risk of LBW.

Increased haemoglobin level was interestingly identified as the only factor with a strong protective effect against LBW (OR = 0.83; Table 5). According to previous reports, maternal anaemia may on its own, independent of placental malaria infection, contribute to intrauterine growth retardation and consequently LBW, most likely via a reduction in oxygen transport to the foetus [13].

Antenatal clinics constitute an important channel for administration of IPT and for passing on information on malaria prevention in general. A great majority of the women in this study attended ANC during their pregnancy (96.8%), which is comparable to the national levels (ANC attendance = 94%) reported in Uganda Demographic Health Survey of year 2000-2001. Still, relative to this, the coverage of IPT was low (41.5%), with a majority receiving only one dose of SP. Uganda's national policy for IPT administration during pregnancy is 2 doses of SP. Of note, significantly fewer primigravidae used IPT as compared to multigravidae (Table 2; G1 = 31.6%; G2-3 = 49.3%; ≥ G4 = 55.7%). The low coverage may be explained by inadequate knowledge on how to offer IPT by the health workers and/or scarcity of the drugs. It is pivotal to reach first-time mothers in time, especially since they are more prone to the most adverse outcomes of PAM. The use of bednet was widespread (67.3%) but only 32% were strict ITN users, a number that is unacceptably low. Using bednet significantly protected against placental malaria (OR = 0.56; Table 3). A trend of protective effect, although not significant, was also observed with anaemia. Considering their ease of use and reported benefits to both mother and the newborn baby [31], increased accessibility and use of low-cost ITNs should be more promoted.

This study was limited by the small sample size, the lack of HIV, and helminth infection data, which may have weakened observed associations. The HIV seroprevalence in pregnant women at Mulago has been around 11% during the past recent years, a level that is higher than that of the national estimates (6.4%) but comparable to the estimates in other major urban hospitals in Uganda. Hence, although we excluded AIDS patients, a proportion of HIV-positive mothers are most probably present in the study population. Moreover, the study may be biased by the time of patient recruitment which was limited to weekdays between 8:00. a.m and 5:00 p.m. Despite these limitations, our study provides a first glimpse of the burden of malaria in pregnancy at Uganda's National Referral Hospital in an urban area of lower transmission. It may also have bearings for the future design of larger studies and the development of public health policies to prevent PAM, maternal anaemia, and LBW.

Ultimately, to gain a better insight on the actual clinical burden of malaria in pregnancy versus transmission level and to identify additional high-risk subpopulations, data from multi-site longitudinal cohort studies are required. However, such studies are logistically demanding, thus very infrequent but would, for example, provide insights on disease kinetics and enable the optimal targeting of preventive interventions, such as IPT, during pregnancy.

Authors' Contributions

F. Namusoke was in charge of patient enrollment, management and care, specimen collection and helped in data analysis and manuscript writing. N. Rasti co-supervised laboratory-related activities, analyzed the data, drafted and revised the manuscript and assisted in manuscript submission. F. Kironde co-supervised laboratory-related activities, helped in data analysis and manuscript writing. M. Wahlgren helped in manuscript writing and coordinated manuscript submission. F. Mirembe was in charge of hospital-related activities and helped in manuscript writing. All authors conceptualized and designed the study together, read and approved the final version of the manuscript.

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PAPER II

MALARIA JOURNAL

RESEARCH Open Access

Validity of self-reported use of sulphadoxine-pyrimethamine intermittent presumptive treatment during pregnancy (IPTp): a cross-sectional study

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Abstract

Background: Malaria in pregnancy is a major health problem that can cause maternal anaemia, stillbirth, spontaneous abortion, low birth weight and intra-uterine stunting. The WHO recommends use of sulphadoxine-pyrimethamine (SP) for intermittent preventive treatment of malaria during pregnancy (IPTp) in endemic areas. Towards monitoring and assessing IPTp coverage in the population, the Roll Back Malaria Partnership recommends the use of self-reported data. The aim of this study was to assess the validity of self-reported IPTp by testing for sulphadoxine in maternal blood at delivery.

Methods: Two hundred and four pregnant women were consented and enrolled in a cross-sectional study in Mulago National Referral Hospital in Kampala Uganda. - Participants who reported a history of taking sulpha-containing drugs like co-trimoxazole , those who were not sure of dates relating to last menstrual period or who took IPTp within the first 20 weeks of gestation were excluded from the study. Data on demographic characteristics, obstetric history, and delivery outcome were collected. At birth, maternal venous blood was taken off aseptically and used to make thick blood smears for malaria parasites and plasma for determining sulphadoxine using high performance liquid chromatography (HPLC).

Results: Of 120 participants who self reported to have used IPTp, 35 (29.2%) tested positive for sulphadoxine by HPLC, while 63 (75%) of 84 patients who reported not having used IPTp tested negative for sulphadoxine. Participants possessing post-primary education were more likely to have reported using IPTp. The low agreement (kappa coefficient = 0.037) between self-report and actual presence of the drug in the blood casts doubt on the validity of self-reported data in estimating IPTp coverage.

Conclusions: The results of this study question the accuracy of self-reported data in estimating IPTp coverage in the population. More studies on validity of self reported data are recommended. Since the validity of IPTp self reports is vital for guiding policy on malaria control in pregnancy, ways should be sought to improve accuracy of the information from such reports.

Keywords: Pregnancy malaria, Intermittent presumptive treatment, Self-reported data

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Background

Malaria in pregnancy is a major health problem affecting both mother and unborn child [1]. Worldwide, malaria affects 300–500 million people and causes nearly one million deaths annually, mostly in children and pregnant women in sub-Saharan Africa [2]. Pregnancy malaria is associated with abortion, stillbirth, low birth weight, and intra-uterine foetal retardation [3]. The WHO Roll Back Malaria Partnership (RBM) recommends reducing the burden of pregnancy malaria by three established interventions: prompt management of malaria cases, intermittent preventive treatment of malaria in pregnancy (IPTp), and extensive use of insecticide-treated bed nets (ITNs) in endemic countries [4].

The use of IPTp regimen consists of giving two or three curative doses of SP (each dose comprising 1500 mg sulphadoxine plus 75 mg pyrimethamine) during pregnancy [5]. While, SP has a good safety profile in pregnancy, it is not normally administered in the first trimester and after 36 weeks of amenorrhoea or gestation because of fear of congenital abnormalities in early pregnancy and kernicterus later. In Uganda, according to the Uganda Demographic Health survey 2011 [6], the IPTp coverage is 67.5% while in Mulago Hospital, it was 41% in 2005 despite a high coverage (98%) in the antenatal care unit of the same hospital [7]. Sulphadoxine/pyrimethamine-resistant *Plasmodium falciparum* strains have been widely reported in Uganda and SP is now largely reserved for use as IPTp [8]. This is so because in semi-immune individuals, anti-malarial drugs with partial parasite resistance such as SP are still effective for intermittent presumptive treatment [9].

The Roll Back Malaria Partnership recommends using self reported data to determine IPTp coverage in a population. For this, self-reported data is collected from women who have had delivery of a baby in a year or more prior to the survey [10]. However, self-reported information on drug use has been found to be prone to bias and its validity questioned [11,12]. Doubts about the validity of self-reported drug use arise from several factors which adversely affect the accuracy of patients' reporting, including selective recall, unawareness of the diagnosis or unwillingness to report [13]. Yet, accurate data on IPTp coverage is key to the design and implementation of effective control measures against the harmful effects of malaria to pregnant women and the newborn. The aim of this study was to assess the validity of self-reports on IPTp use by detecting sulphadoxine in maternal blood at the time of delivery.

Methods

Study site

The study was carried out in Mulago Hospital, which serves as Uganda's National Referral Hospital and is located in the capital city of Kampala. Situated at 1,300–1,500 m above sea level close to the Equator, Kampala has a tropical climate with rainfalls throughout the year. There is stable *P. falciparum* transmission in 95% of Uganda. The remaining 5% of the country, mainly the highland areas with altitudes >1,600 m, experiences low and unstable malaria transmission. Kampala has low to intermediate malaria transmission with frequency peaks toward the end of the two major rain seasons (March to May and August to November). The national treatment guidelines recommend that pregnant women should receive at least two doses of SP to prevent malaria and its effects. At time of this study, HIV prevalence in the Ugandan population aged 15 to 49 years was 6.4% and prevalence among admitted patients at Mulago Hospital was 10%. Pregnant mothers with known HIV infection are expected to follow national guidelines of weekly trimethoprim-sulphamethoxazole (co-trimoxazole) prophylaxis to prevent opportunistic infections.

Study population and data collection

Two hundred and four pregnant women admitted at Mulago National Referral Hospital labour suite were enrolled into a cross–sectional study after informed oral and written consent. Data on pregnancy history, socio-economic indicators and pregnancy outcomes was collected using a pre-coded standardized questionnaire. Key aspects recorded included area of residence, age, marital status, occupation, education, parity, visits to antenatal clinic (ANC) and bed net use. Birth weight of baby was determined after delivery. In addition, information on use of IPT for prevention of malaria during that pregnancy, the drug administered, number of SP doses taken, history of taking sulpha-containing drugs such as co-trimoxazole , history of fever during pregnancy, and use of anti-malarial drugs was recorded. The date on which the SP was taken was noted in the questionnaire. In cases where the patient was not able to state the dates with certainty, it was then recorded as the 15^{th} day of that particular month. This information was used to estimate the gestation age corresponding to when the SP was taken.

All ethical aspects of the study were approved by the Makerere University Faculty of Medicine Research and Ethics Committee and the Uganda National Council of Science and Technology (UNCST).

Sample collection and laboratory analysis

Before delivery of baby, mother's venous blood was collected for microscopy to detect parasites, for haemoglobin estimation and sulphadoxine (SDX) detection. Blood was collected in EDTA anticoagulant containing tubes, centrifuged, plasma separated and stored at −70°C until drug assays.

Malaria parasite detection

Thick blood smears were made from the maternal venous blood and the cord blood. These were then stained with Giemsa and examined microscopically by two trained workers. In case of discrepancy, a third microscopist examined the smears.

HPLC analysis

Plasma drug levels were assayed using the high performance liquid chromatography (HPLC) facility at the Department of Pharmacology and Therapeutics, College of Health Sciences, Makerere University, Kampala, Uganda. Sulphadoxine was used as a proxy for SP. The HPLC analysis (UV) was carried out according to the method described by Bergqvist *et al.* [14]. Sulphamethaxazole was used as the internal standard. The limit of quantification for SDX was 15 μM. Basing on average Cmax for SDX of 260 μM and assuming a half life ($T_{1/2}$) for SDX of 6 to 9 days [15,16], it is calculated that SDX is detectable in blood from few hours after intake of SP until 7 to 9 weeks. Therefore, participants who reported as having taken IPTp before 20 weeks of their pregnancy were excluded from HPLC analysis. In addition, HIV-positive participants who reported being on co-trimoxazole prophylaxis were excluded since this antifolate combination is similar to SP and is a sulpha-containing drug that can interfere with HPLC detection of SDX. Further, as it was important to know the time interval between IPTp intake and blood sampling at delivery, the blood specimens of individuals who were unsure of the month of their last menstrual period (period of amenorrhea) were excluded. All plasma specimens were analysed twice along with calibration standards and quality controls. To prevent bias, the HPLC analysts were blinded to the data of self-reported IPTp uptake and composition of quality control samples.

Data analysis

Data was cleaned, coded and entered into Microsoft Access 2007. Summary statistics, Chi-square tests, multivariate analysis and graphs of residual plasma concentrations of SDX were carried out using SPSS. Agreement or disagreement between self-report and HPLC results on actual detection of SDX in blood at delivery was determined by calculating kappa coefficients [17]. A kappa value of 0.1 to 0.40 was considered poor-to-fair agreement, a kappa value of 0.41 to 0.60 was considered moderate agreement, and a kappa value of 0.61 to 0.80 was considered substantial agreement, while a kappa value of 0.81 to 1.00 was considered excellent agreement.

Results

In a study to assess the validity of self-reported data on the use of anti-malarial IPTp, 284 pregnant participants were screened between September 2008 and July 2009 (Figure 1).

Majority (98.5%) of the participants attended antenatal clinic at least once during pregnancy as evidenced by self-report and presence of an antenatal clinic card. Approximately fifty nine percent of participants (n = 204) reported using IPTp during pregnancy, with 90% taking one dose of SP while 17.2% reported using an insecticide spray for controlling mosquito bites. From the self-reports on when the last SP dose closest to delivery was taken, the median reported interval between SP intake and baby delivery was computed as 12 weeks (IQR: 8–18.8); see Figure 2. Frequency distribution of the calculated interval between reported date of SP intake and baby delivery for the mothers who reported having used IPTp is shown in Figure 2 (histogram B). The frequency distribution of the same interval for mothers (n = 35) who were found to have detectable SDX in blood (Figure 2D) and those (n = 85) whose blood was negative for SDX (Fig 2C) are also shown. It can be seen that SDX was detected in blood of mothers whose self reports indicated SP intake before 9 weeks to baby delivery (Figure 2D) , a result suggesting that the reported dates of the IPT dose was incorrect since SDX would be undetectable by HPLC beyond two months after administration. On the other hand, the blood of more than 15 mothers who reported to have taken SP within 9 weeks preceding baby delivery lacked any detectable SDX (Figure 2C), likewise suggesting that the reported dates for when the SP doses were taken are inaccurate. Thus, the results suggest that the self reports were unreliable for finding out whether the patients used IPTp or not (Table 1) and for determining when the SP doses were taken (Figure 2). It is unlikely that HPLC assay errors

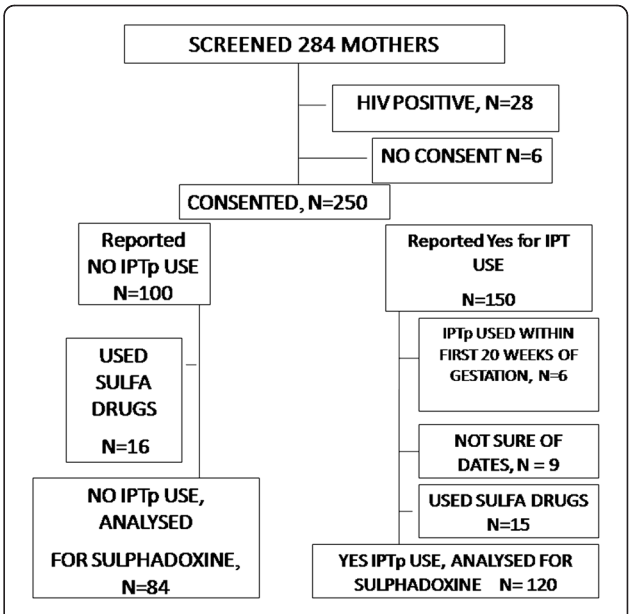

Figure 1 Participant flow chart.

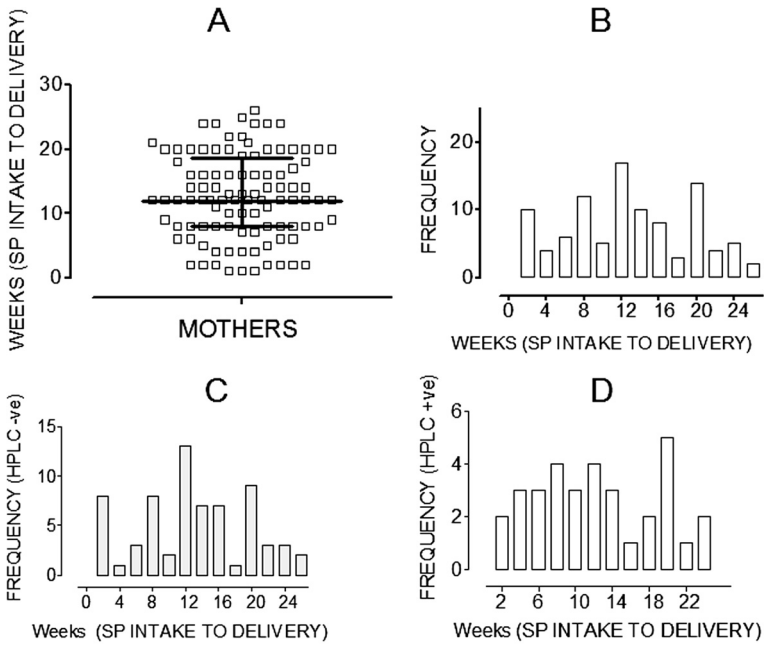

Figure 2 Reported time interval between SP administration and delivery. At baby delivery, women donated blood to measure sulphadoxine in their blood by HPLC (UV) and were interviewed to self-report on whether and when they received pyrimethamine-sulphadoxine (SP) during the pregnancy. Agreement between the HPLC result and self report was calculated by kappa analysis. The Figure shows: **A**. Variability in interval (weeks) between reported date of SP intake and baby delivery (median: 12 weeks, IQR: 8–18.8 weeks, see whiskers), **B**. Frequency distribution of the reported interval (SP input to delivery) for the mothers whose blood was analyzed for SDX. Histogram of reported interval (SP intake to delivery) for HPLC negative (**C**) and HPLC positive (**D**) mothers.

are responsible for discrepancy with self-reports because SDX was detected both in blood of many patients reporting to have taken SP within two months before delivery and in specimens of others reporting the IPT dose intake to have occurred more than two months before delivery. If the self reports were accurate and the HPLC assay falsely detected SDX in blood specimens, then the vast majority of patients reporting use of IPT particularly within two months before delivery would have been SDX positive unlike what the results show (Table 1 and Figure 2C).

One hundred and sixty five (80.9%, n = 204) of the participants were resident in Kampala district, 18.1% were from nearby (approximately 10 to 20 km) Wakiso district, with the remainder from further away (approximately 20 to 40 km) in Mukono and Luwero districts. Most (64.2%) participants had some form of employment as businesswomen or self-employed individuals. Skilled workers, including professionals were 11.3% while 13.2% had no formal employment. The median age of the participants was 23 years (interquartile range: 20–27). The other details of the demographic characteristics of the study population are shown in Table 2.

Table 1 Self-reported IPTp use and presence of sulphadoxine in blood

Blood Sulphadoxine	Reported use of IPTp		Total
	Yes	No	
Positive	35	21	**56 (m1)**
Negative	85	63	**148 (m0)**
Total	**120 (n1)**	**84 (n0)**	**204 (n)**

Of the study participants, 2.5% (n = 204) delivered low birth weight babies (< 2.5 kg), 5.9% delivered before 37 weeks of gestation while 91.6% delivered at term. Prevalence of *P. falciparum* parasitaemia (peripheral blood) among the mothers at delivery was 8.3% while 2.0% of the newborns had cord-blood parasitaemia. The majority (73.5%) of participants reported using iron sulphate and folic acid supplements during pregnancy.

The relationship between self-reported IPTp use and the general characteristics of the population are shown in Table 3. The more educated mothers (P = < 0.01 95% CI: 0.2-0.7) and those who took iron supplementation during their pregnancy (P = 0.03 95% CI:1.1-4.0) were more likely to report using IPTp. The other factors were not statistically different in the group that reported IPTp use and the cluster that reported IPTp non-use. None of the maternal demographic characteristics was associated with presence of sulphadoxine in mothers' blood at delivery.

Table 2 Demographic characteristics of study participants

Variable	*Frequency*	*(%) Percentage*
IPTp use		
Yes	120	58.8
No	84	41.2
ANC attendance		
Yes	201	98.5
No	3	1.5
IPTp doses taken		
1	108	90
2	10	8.3
3	2	1.7
Bed net use		
Always	165	80.9
Sometimes	17	8.3
Never	22	10.8
Bed net		
Insecticide-treated	72	35.3
Not treated	70	34.3
Don't know	38	18.6
Folic acid use		
yes	150	73.5
no	54	26
Iron sulphate use		
Yes	155	76
No	49	24
Birth weight		
<2.5 kg	5	2.5
> = 2.5 kg	199	97.5
Gravidity		
Primigravidae	68	33.3
Gravid −2	48	23.5
Gravid 3 and above	88	43.1
Maternal age group		
Up to 20 yrs	52	25.5
Above 20 yrs	152	74.5
Education mother		
Up to primary	83	40.7
Post-primary	121	59.3

Kappa statistic on self-reported IPTp use and sulphadoxine in blood at delivery

Of 120 study participants who self-reported to have used IPTp, 35 (29.2%) tested positive by HPLC while 63 (75%) of 84 patients who reported not to have used IPTp tested negative for SDX (see Table 1). On the other hand, 85 (70.8%) patients who reported to have used IPTp tested negative for blood SDX by HPLC. Yet, 21 (25%) of patients who self reported not to have taken SP were found to have detectable SDX in peripheral blood at delivery.

To determine agreement between self-report and HPLC detection of the drug in blood, Kappa analysis was used. The kappa statistic gives a numerical assessment of the degree at which two ratings or observers would actually agree compared to how much they would be in agreement just by chance. Assuming probability of observed agreement where P(a) is the percentage of agreement for self-reported IPTp use and SDX in blood, then by calculation, the probability of observed agreement, $P(a) = (35 + 63)/204 = 0.48$. The probability of expected agreement, P(e) is given by the formula: $P(e) = [(n1/n) \times (m1/n)] + [(n0/n) \times (m0/n)$.

By substitution, $P(e) = [\{120/204\}x\{56/204\}] + [\{84/204\} x\{148/204\}] = 0.46$.

Since Kappa, $K = [p(a)-p(e)]/[1-p(e)]$, then by substitution, $K = \{0.48-0.46\}/\{1-0.46\} = 0.037$. This result ($K = 0.037$) signifies a very slight (poor-to-fair) agreement between reported IPTp use and SDX in blood at delivery [18,17].

Discussion

This study explored the validity of self-reported sulfadoxine-pyrimethamine IPTp by testing for presence of SDX in maternal blood at delivery using HPLC. Two main findings of this study are that self-report on sulphadoxine-pyrimethamine IPTp use is unreliable not only for knowing whether the pregnant patient took the SP or not but also for finding out when the patient took the drug. Several patients who reported not having taken SP were found to have the drug derivatives in their blood. Further, some patients who reported having taken the drug before 9 weeks preceding baby delivery (when SDX would be too low to be detected in blood by HPLC) were also found to have the drug in the blood. On the other hand, some patients claiming to have taken SP within 9 weeks before delivery (when blood SDX would be detected by HPLC) actually did not have detectable SDX blood levels.

Interestingly, participants who self-reported IPTp use during their present pregnancy were more likely to have SDX in their circulation at delivery, although the level of agreement was only slight as assessed by kappa statistics. Although only 29.2% of participants who reported IPTp use actually had SDX in their blood at the time of delivery, 25% of participants who reported not taking IPTp had SDX in blood at delivery. This finding questions the validity of self-reported data in estimating the IPTp coverage. The findings of this study concur with a previous study in Uganda which found low validity of caretakers' report on use of anti-malarials and antibiotics [11]. Okura *et al.* found that the young and more

Table 3 Self-reported IPTp use during pregnancy and other demographic characteristics

Variable	Used IPTp	Not used IPTp	P Value	(OR) 95%CI
Bed net use				
Always	101	64		1
Sometimes	8	9	0.25	1.7(0.6-4.8)
Never	11	11	0.31	1.5(0.60-3.80)
Education level				
Up to primary	38	45		1
Post-primary	82	39	**<0.01**	0.4 (0.22-0.72
Age group				
Up to 20yrs	88	64	0.070	1.1 (0.6-2.2)
Above 20years	32	20		
Iron supplement				
Yes	98	57		1
no	22	27	**0.03**	2.1 (1.1-4.0)
Use of insecticide spray				
Yes	25	10		1
no	95	74	0.10	1.9 (0.88-4.3)
Maternal parasitaemia				
Negative	111	76		1
Positive	9	8	0.60	1.2 (0.47-3.51)

educated were more likely to report correctly on the prevalence of diseases such as hypertension, diabetes and history of myocardial infarction [19]. Another study found a good agreement of self report with diagnosis of diabetes and hypertension [13]. In another study in Kenya, which looked at anti-malarial drugs before initiating treatment in participants who reported no use of drug in 28 days prior to enrolment, it was found that the proportion of participants with residual anti-malarials was high and self-report on drug intake was unreliable [12]. Yet another study found that self reported compliance in use of antibiotics among sexually transmitted disease patients was also unreliable [20]. Thus, several previous studies concur with the findings of the present study where self-report data on IPTp use is only in slight agreement (kappa = 0.03) with results of HPLC detection of the drug ingredients in blood. In view of the demonstrable weakness of self reports, a previous study has suggested increasing the validity of self-reported data through focus group discussions, using language with which the respondents are very familiar, sequencing the questions from the least to the most threatening, using open-ended and direct questions [21]. In a review on validity of self reported data, Brener *et al.* [22] noted that validity can be improved when the patients understand the questions and are able to recall, their answers are anonymous and there is no fear of reprisals [22]. Using focus group discussions may reduce the fear of reprisals and increase anonymity, which improve validity of self-report. In contrast to household surveys, the present study was undertaken in a hospital setting, which may have led to selective recall bias for fear of possible repercussions. A previous review indicated that the validity of self-reported data may be affected by cognitive issues including clarity of the questions, memory needed to answer the questions and influence of the survey settings [22].

IPTp use and other characteristics

Participants who reported IPT use during pregnancy tended to be younger in age, more educated, and reported having received iron supplementation during pregnancy (Table 3). In other words, the more educated participants were more likely to have reported IPTp intake than the less educated. There was a high ITN coverage (89.2%) in the participants, an encouraging finding which is important for prevention of malaria in pregnancy. This high bed net use could be due to the relatively high socio-economic status of the participants as the study population was largely urban and had access to contemporary distribution of ITNs to pregnant mothers free of charge. A recent study in Tanzania found that timely uptake of IPTp depends more on practices of health workers at the health units than individual characteristics of pregnant women and that early ANC attendance did not influence IPTp use [23]. Although 98.5% of the participants reported having attended the antenatal clinic at least once during that pregnancy, only 58.8% reported IPTp use during the current pregnancy.

This lower than expected use of IPTp could reflect suboptimal care at the antenatal clinics, lack of drugs in the health units and inadequate sensitization of the health workers which have been found to affect uptake [24]. Significantly, in areas with high transmission, pregnancy malaria still causes considerable morbidity and mortality in spite of high bed net use [25]. This emphasizes the importance of improving IPTp coverage to reduce the incidence and effects of pregnancy malaria efficiently.

Of the participants who reported non-use of IPT during pregnancy, 25% (n = 84) actually had the drug in circulation at the time of delivery. This finding suggests false reporting by the participants, which could be due to recall bias or the possibility that participants were not informed about the drugs given during the pregnancy, since the participants who reported using any sulpha drug during their pregnancy were excluded from the study.

Conclusion

Current policy for control of pregnancy malaria emphasizes IPTp and use of insecticide-treated bed nets. To assess compliance with these recommendations and to estimate intervention coverage, RBM recommends the simpler and affordable approach of self-report in determining IPTp coverage. Blood drug levels are too costly for such population-based assessment. But, as shown by the present study, assessment of IPTp coverage by self-report is unreliable. Therefore, towards obtaining dependable data on IPTp coverage, the need to device ways of improving the accuracy of IPTp self-reports and the records that capture the data is very important.

Limitations of the study

The study lacked precise information on the weight of the participants prevailing at the time of taking IPTp and relied on estimations basing on the weight of the mothers at delivery. Consequently, the C_{max} (SDX) for each patient could have been slightly overestimated for heavier mothers and *vice versa*. For a few participants (estimated at <5%), the time of taking the drug could not be accurately determined because of inexact recall of dates and incomplete case records. To the best of our knowledge, there are no previous similar studies of the validity of IPTp self-reports.

Abbreviations

ANC: Antenatal Clinic; EDTA: Ethylene diamine tetra-acetic acid; HIV: Human Immune Deficiency Virus; HPLC: High Performance Liquid Chromatography; IPTp: Intermittent Presumptive Treatment; ITNs: Insectcide-treated Nets; RBM: Roll Back Malaria; SDX: Sulphadoxine; SP: Sulphadoxine + Pyrimethamine; UDHS: Uganda Demographic Health Survey; WHO: World Health Organization.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FN collected the clinical data, carried out data analysis and drafted the manuscript. MN carried out the high performance liquid chromatography and participated in design of the study. FM participated in the design of the study and performed the statistical analysis. MW and FK conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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PAPER III

Maternal-infant transfer of Anti-*P. falciparum* IgG antibodies not affected by use of Intermittent Presumptive Treatment but antibody levels in the mother .

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Abstract

It has been well established that infants below six months born to mothers in endemic areas are protected from malaria. This protection has been attributed to presence of foetal haemoglobin and anti-*P. falciparum* antibodies (IgG) transferred from mother to baby. Inadequate transfer of IgG antibodies may lead to increased susceptibility of the infants to malaria. The aim of the study was to determine the effect of using sulfadoxine/pyrimethamine intermittent presumptive treatment during pregnancy (IPTp) on proportions of anti-P.*falciparum* IgG antibodies to selected P.*falciparum* blood stage antigens transferred from mother to baby. We recruited 290 mother/baby pairs in a cross-sectional study at delivery and anti-*P.falciparum* antibodies to four different blood stage malaria antigens were measured; Glutamine Rich Protein (GLURP), Histidine Rich Protein II (HRP II), Merozoite Surface Protein 3 (MSP3) and Merozoite Surface Protein3a (MSP3a). The levels of IgG antibodies in the mother were not affected by maternal age, parity or use of IPTp during pregnancy. The proportion of antibody transferred from mother to baby was determined as a fraction of antibody levels in the mother compared to the corresponding mother. The results indicate that the proportion of anti-*P. falciparum* IgG antibodies transferred from mother to baby depends on the amount of antibody in the mother but not use of IPTp . From these results we recommend continued use of IPTp as recommended by WHO without fear of interfering with newborn immunity.

Key words: Intermittent presumptive Treatment, P. falciparum, antibody transfer

1.0 Introduction

Malaria still remains a serious global health burden in Africa, with an annual incidence of 247 million cases and nearly one million deaths mostly children [1] . Children and Pregnant mothers with naive and compromised immune systems respectively are particularly vulnerable to malaria and carry the highest risk for malaria related deaths. Approximately 25 million pregnant women are currently are at risk for malaria annually Sub-Saharan Africa [2, 3].

In these regions, malaria in pregnancy is predominantly asymptomatic and yet is a major cause of severe maternal anaemia and low birth weight babies. It is estimated that the malaria infections result in 75,000–200,000 low birth weight (LBW) babies each year [4], as a result of preterm delivery and foetal growth restriction. The effects of pregnancy malaria on miscarriage and stillbirth are unknown, but adequate malaria control alone has been estimated to prevent 3–8% of infant deaths.

In malaria endemic areas WHO recommends control of adverse effects of pregnancy malaria by using three strategies; adequate case management of malaria illness, use of insecticide treated nets, and Intermittent presumptive treatment (IPTp) with sulfadoxine/pyrimethamine [5]. Despite the increasing levels of p.*falciparum* resistance to sulfadoxine /pyrimethamine [6], it is still effective as IPTp in malaria endemic areas [7, 8].

Pregnant women who are previously semi-immune before pregnancy become more susceptible during pregnancy. This is due to presence of the placenta, which is a new site that favours sequestration of parasites. This is more pronounced in the primigravidae than in the multigravidae [9]. The sequestration of parasites in the placenta is favoured by presence of receptors in placenta, which have been well characterized; chrondroin sulphate A (CSA) [10] and hyaluronic acid [11]. Placental malaria leads to LBW babies [9], and increase the risk of mother to child transmission of HIV [12]. Placental malaria has also been reported to affect transplacental transfer of immunoglobulins from mother to the foetus [13].

Infants born to mothers living in malaria endemic areas are thought to be immune to malaria in the first six months of life. This immunity is due to presence of foetal haemoglobin which does not favour growth of the parasites, and presence of anti-P.*falciparum* antibodies transferred from the mother to baby. The antibody transfer from mother to foetus is an active process involving Fc receptors on the surface of the synciotrophoblast. Gestational age [14], placental malaria, maternal hyperglobinmia [13], maternal HIV sero-positivity [15] and levels of antibodies in the maternal blood have been reported to affect transplacental transfer of Immunoglobulin G antibodies .

Despite the reduced burden of malaria in infants below six months, malaria in this age group in endemic areas is not uncommon. Infants with high anti-P. *falciparum* IgG antibodies at delivery are more protected from malaria than their counterparts. Factors that affect antibody transfer from mother to baby may affect the susceptibility of the infant to malaria. A study in Kenya indicated that use of insecticide treated nets during pregnancy lead to a reduction in anti-P.*falciparum* antibodies [16]. Another study has recently indicated that use of IPTp during pregnancy was associated with reduction in antibodies to placental malaria [17] .It has been proposed that adequate control of the immunising mosquito bites may lead to increased susceptibility to infection [18]. Data on the effect of using protective measures like IPTp during pregnancy on levels of anti-P.*falciparum* blood stage antibodies is scarce.

The aim of this study was to determine the effect of using IPTp during pregnancy on the levels of anti-*P. falciparum* antibodies against selected blood stage antigens and proportions of antibodies transferred to the baby.

2.0 Methods

2.1 Study setting

The study was carried out in Mulago National Referral Hospital in Kampala (details of the site are described elsewhere [19]).

In a cross sectional study we recruited mothers at delivery after informed oral and written consent. The study enrolled mothers at 28 weeks of gestation and above

admitted in labour.Mothers with ante-partum haemorrhage, severe pre-eclampsia and babies with confirmed gross congenital abnormalities and still births were excluded. All ethical aspects of the study were granted by the Ethics Committee at Makerere University College of Health Sciences Research, and at the Uganda National Council for Science and Technology. Data on demographic characteristics, obstetric history, order of pregnancy, use of intermittent presumptive treatment during pregnancy and number of doses taken and gestation age at which it was taken was recorded on an interviewer administered questionnaire. Use of bed net during pregnancy and after delivery, as well as pregnancy outcome, were recorded.

2.2 Sample collection and laboratory studies

Venous blood was drawn asceptically from the study mother at recruitment, for a thick blood smear for malaria examination. A sample was sent to the laboratory where it was separated using a centrifuge at 3000G for 30 minutes and serum kept at -70degrees till analysis . After delivery 2-4mls of cord blood was taken asceptically for malaria parasite examination and serum kept at -70 degrees. Thick blood films of peripheral and cord blood were stained by Giemsa and malaria diagnosis was assessed by microscopy following standard procedures.

Synthetic antigens

The synthetic peptides representing p.*falciparum* blood stage *proteins* which included GLURP :(NH2)CGDKNEKGQHEIVEVEEILPEGC(CONH2),

HRP II: (NH2)GCAHHAADAHHAADAHHAADAHHAADGC(CONH2),

MSP3a :(NH2)TLAGLIKGNNQIDSTLKDLV(CONH2),

MSP3: (NH2)AKEASSYDYILGWEFGGGVPEHKKEEN(CONH2) } were used to determine antibody levels .These synthetic antigens were prepared as described [20-22]

2.3 Antibody measurements

Plasma antibodies to the synthetic peptides to GLURP, HRPII , MSP3 and MSP3a were measured by ELISA, as described elsewhere [23].

In brief, microtiter plates (Nunc, Roskilde, Denmark) were coated with recombinant protein 100micrograms per well, incubated overnight at 4^0C, and blocked with 5% skimmed milk for 1 hour at room temperature. Plasma samples diluted 1: 200 were added in duplicate and incubated at room temperature for 1 h, plasma sample of the

mother and the corresponding neonate (mother/baby pairs) were run on the same plate in all cases. Plates were washed 4 times between steps. Plates were developed by Peroxidase conjugated goat anti-human IgG (secondary antibody). Bound secondary antibody was quantified by colouring with ready to use TMB (*3,3', 5,5'-Tetramethylbenzidine*) substrate. Optical density (OD) was read at 450 nm with a reference at 620nm in a plate reader.Value 2 standard deviations above the mean absorbance of the samples from unexposed control donors were used as the negative cut-off. All samples with ODs above 1.4 were diluted further to 1:500 and the OD obtained was then multiplied by the dilution factor.

2.4 Statistical analysis and data presentation

Data were analysed using Stata (Version9). The ELISA OD was considered as antibody level in the mother and baby. When all of the samples were viewed together the distribution of anti-P.*falciparum* IgG levels was not normally distributed in several cases .The statistical significance of the differences between IgG in maternal sera in relation to IPTp uptake and maternal age was therefore evaluated using Mann-Whitney Rank Sum test while parity dependency was evaluated by Kruskal Walles one way analysis of variance. Medians, differences between medians and associated 95%CI were calculated as described in [24, 25].The proportions of IgG transferred from mother to baby was considered as the fraction of the antibody baby to the corresponding mother. The proportions of anti-P.*falciparum* antibodies against GLURLP, HRPII, MSP3 and MSP3a were log transformed prior to regression analysis. In the multivariate analysis, proportion transferred mother to the corresponding baby was the outcome variable, and bed net use, maternal antibody levels ,use of IPTp and the number of doses taken, maternal age HIV status, gravidity (primi-, secundi- or multigravidae) and parasitemia defined as exposure variables. P values of <0.05 were considered as statistically significant.

Proportions of anti-*P. falciparum* antibodies transferred from mother to baby and IPTp use in pregnancy were determined by fraction of the antibody level in the baby to the corresponding mother. In cases where the antibody level was higher in the baby compared to the corresponding mother were not included in the analysis for proportion. Ten mother baby pairs were excluded in the analysis for proportion transferred. In cases where the baby was negative for IgG to particular antigen

proportion was recorded as zero. The use of IPTp and presence of sulfadoxine in blood was left in all models assessing for factors affecting proportions transferred. The influential outliers were determined using Cook's Distance. Values whose Cook's D exceeds 4/n and/or its absolute value for DFITS exceeds 2*sqrt(k/n) were considered influential outliers.

3.0 Results

The study aim was to determine levels of antibodies against *P. falciparum* blood stage antigens in maternal plasma, and proportions transferred from mother to baby and how this is affected by use of IPTp during pregnancy. Two hundred and ninety participants were recruited into a cross sectional study , where the majority of the participants were residing in Kampala and Wakiso districts (79% and 19%, respectively) .Over 98%(286/290) of the study participants attended the antenatal clinic (ANC) at least once during pregnancy with 95% (272/286) of them having ANC card as evidence.

Table 1: Demographic characteristics of the study population

Variable	Frequency (n = 290)	Percentage
Age of mothers (years):		
<20	71	24.48
≥20	219	75.52
Birth weight of babies (kg):		
< 2.5	6	2.1
≥2.5	284	97.9
Gravidity:		
1	97	33.4
2	69	23.8
3	67	23.3
4	30	10.3
5	21	7.2
>=6	6	2.1
WOA*:		
<37	12	4.1
≥37	260	89.7
Don't know	18	6.2
Use of IPTp*:		
No	119	41
Yes	171	59
No of IPTp doses:		
1	135	78.9
2	28	16.4
≥3	8	4.7
Use of mosquito bed-nets:		
Always	239	82.4
Sometimes	20	6.9
Never	31	10.7
Was bed-net treated:		
Yes	109	42.1

Variable	Frequency (n = 290)	Percentage
No	108	41.7
Don't Know	42	16.2
Maternal peripheral parasiteamia		
Negative	270	93.1
Positive	20	6.9
Cord parasiteamia		
Negative	286	98.6
Positive	4	1.4

WOA*; weeks of amenorrhea, IPTp*; self-reported use of during the current pregnancy

The majority of the participants who attended ANC (56%) had the first visit in the second trimester of pregnancy, 37% in the third trimester and only 7% in the first trimester. The mean gestational age for the first ANC visit was 24 weeks with 75% of them coming before 28 weeks of amenorrhea (WOA).

Fifty nine percent (171/290) of the participants reported taking at least one dose of IPTp during pregnancy. Majority of the participants who reported taking IPTp took only one dose (79%) followed by 2 doses (16%) with only 5% taking more than two doses. Primigravidae women were the most predominant with 33.4% ((97/290) of the participants as shown Table 1. Although 82.4% of the participants reported use of bed net, only 108/239 (41%) were using insecticide treated nets Table 1.

Majority of the participants were primigravidae (33.4%) followed by gravidae 2 at 23.8% , Gravidae 3 were 23.3% , and the rest were 19.6% .The prevalence of low birth weight babies was 2.1% and 4.1% of the participants delivered below 37 weeks of gestation .

3.1 Sero-prevalance anti-P.falciparum IgG antibodies in maternal sera

The ELISA absorbances were assumed to be proportional to antibody level in blood at the time of sampling.The antimalaria antibody seoprevalance in the study population was highest against HRPII (92%) Figure 1.

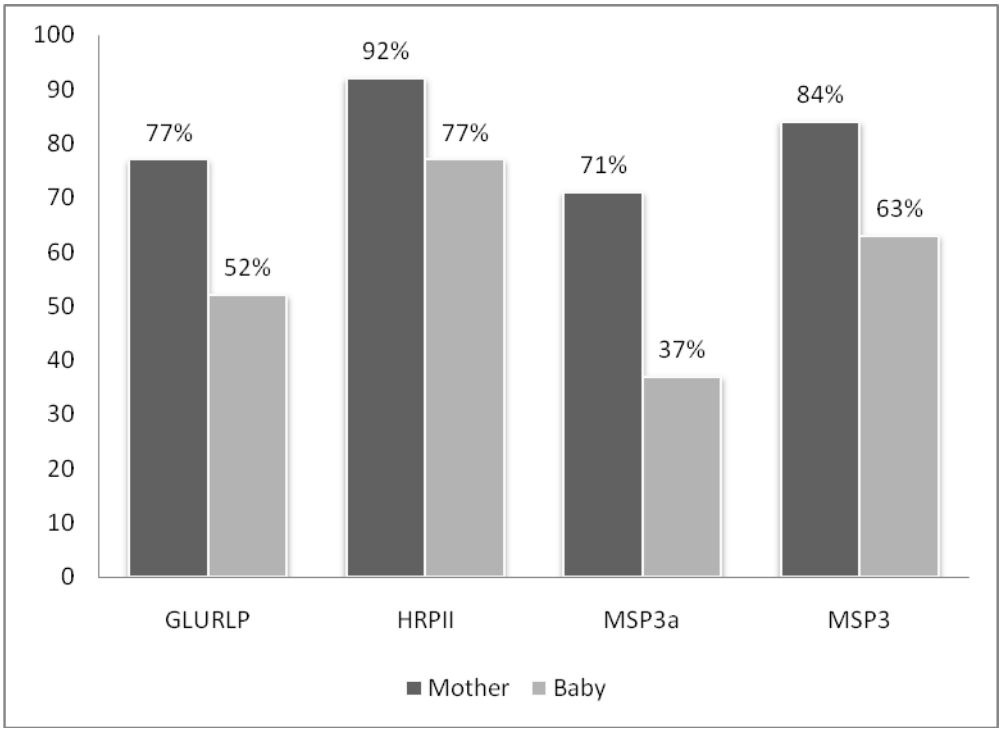

Figure 1: Sero-positivity in mothers and babies to different blood stage synthetic peptides

Table 2,mothers who reported IPTp intake during pregnancy were more likely to be sero-positive for IgG antibodies against MSP3a {P=0.025, OR =1.79} Antibody sero-positivity against other antigens were not affected by IPT use during pregnancy.

Table 2 Sero reactivity of maternal sera and IPTp use during pregnancy

Variable	**Sero-positivity**		Odds Ratio (95% CI)	*P*-value
	Positive	Negative		
Anti –GLURP IgG				
Used IPTp: Yes	121	50	1	
No	90	29	0.78 (0.46, 1.34)	0.340
Anti –HRPII IgG				
Used IPTp: Yes	154	17	1	
No	114	5	0.40 (0.14, 1.11)	0.069
Anti -MSP3a IgG				
Used IPTp: Yes	130	41	1	
No	76	43	1.79 (1.07, 3.0)	**0. 025**
Anti -MSP3 IgG				
Used IPTp: Yes	145	26	1	

	No	98	21	1.20 (0.64, 2.24)	0. 579

3.2 Anti-malarial antibody levels in mothers and babies in relation to IPTp

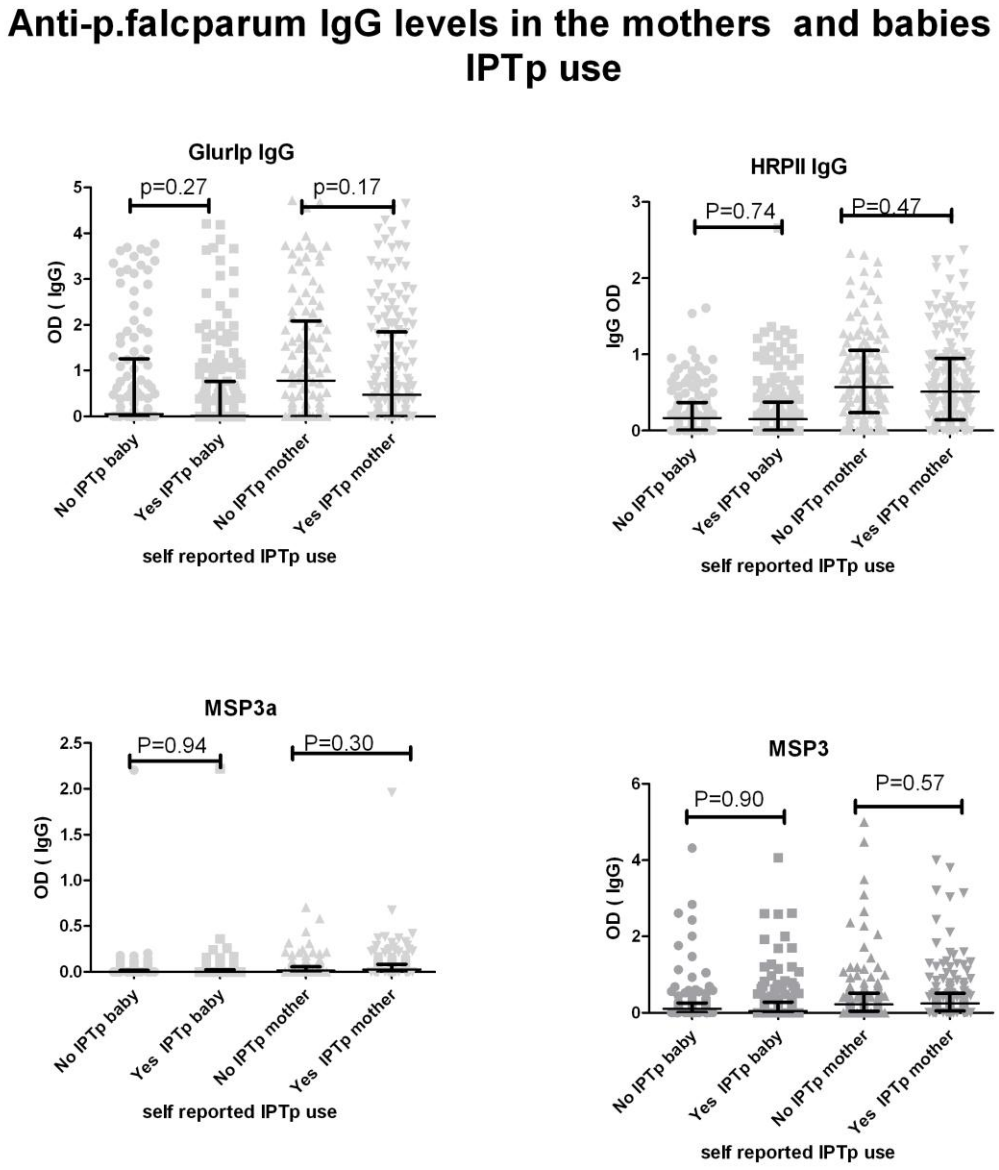

Figure 2: Anti-*P. falciparum* antibody levels in mothers and babies in relation to use of IPTp during pregnancy

The antibody levels in mother and babies in relation to using IPTp by the mother during pregnacy; Using Mann-Whitney rank sum test.

Mothers who reported taking IPTp during pregnancy were more likely to have higher antibody titres than their counterparts shown in Figure 2 . The association between

antibody levels to different antigens and IPTp use did not reach statistically significant levels.

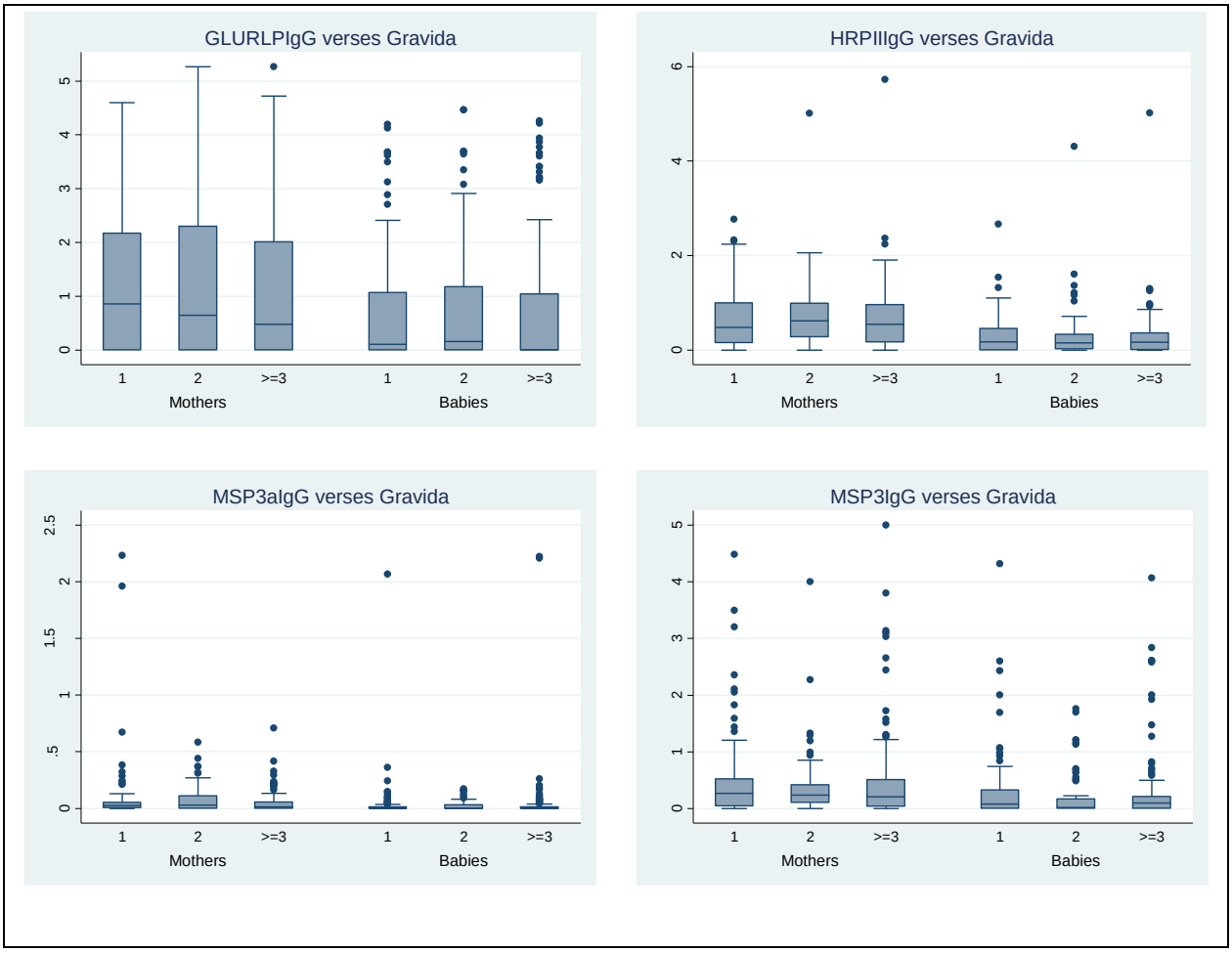

Figure 3: Maternal parity and anti-*P. falciparumP. falciparum*antibodies to different blood stage antigens in mothers and babies.

IgG antibodies against GLURP in maternal sera was higher in the primigravidae compared to their counterparts although this did not reach statistically significant levels. There was generally no effect of parity on antibody levels to all the blood stage antigens tested. The parity of the mother had no effect on the anti-*P. falciparum* anti-bodies in mothers and babies at delivery shown in Figure 3.

3.1.3Proportions of antibodies Transferred from mother to baby

There was a linear relationship between antibody levels in the mother and corresponding newborn. IPTp use and proportion of anti-*P. falciparum*antibodies in mother baby pairs levels are shown in below Figure 4.

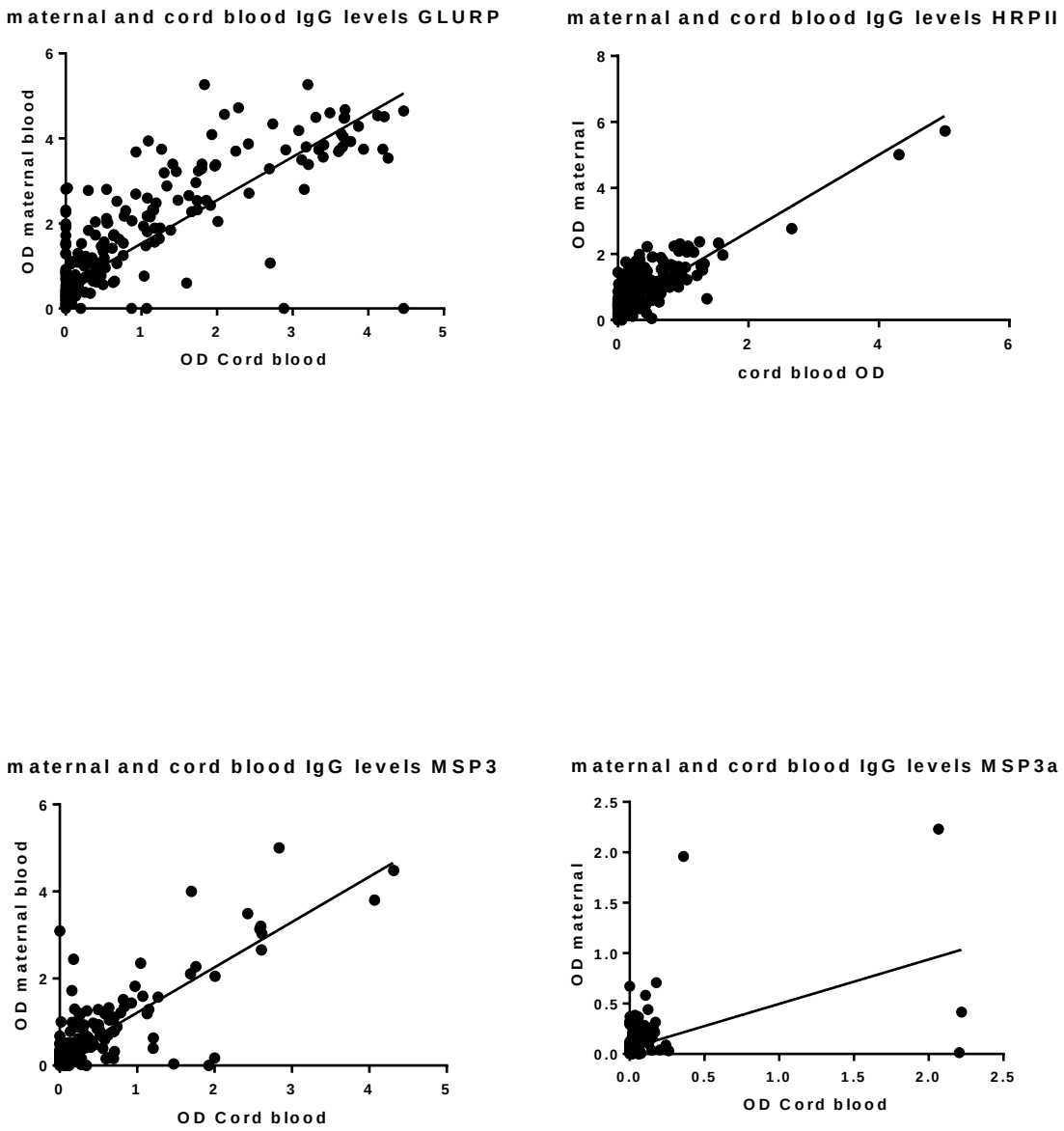

Figure 4 Relationship between maternal and cord blood antibody levels

Linear relationship between maternal and cord blood antibody levels for all antigens GlURP R^2 = 0.68, HRPII R^2 =0.72, MSP3a R^2 =0.24, MSP3 R^2 =0.67

Factors affecting transfer of any amount of antibody from mother to baby. Mothers with *P. falciparum* parasiteamia at delivery were more likely to transfer antibodies against MSP3a compared to their counter after multivariate logistic regression (P=0.043).

Mothers who had sulfadoxine in blood at delivery were more likely to transfer IgG antibodies against MSP3.

Bivariate analysis to assess the relationship between proportions transferred with maternal age, birth weight, gravidity, use of IPTp, number of IPTp doses, presence of sulfadoxine in blood at delivery, parity affected proportions with different blood stage antigens tested. William

Table 3 Factors affecting transfer IgG antibodies from mother to baby

Variable	OR	se	P value	95%CI
GLURP				
Used IPTp	1.070	0.513	0.886	0.41-2.73
HPLC	0.613	0.305	0.327	0.23-1.63
IgG category1*	7.491	3.897	**<0.001**	2.70-20.76
HRPII				
Used IPTp	1.335	0.747	0.605	0.44-3.99
HPLC	1.018	0.657	0.977	0.28-3.61
IgG category1*	1.742	5.177	**0.002**	2.08-28.71
MSP3a				
Used IPTp	0.759	1.015	0.837	0.05-10.43
HPLC	0.219	0.174	0.057	0.04-1.04
BS mother	0.069	0.091	**0.043**	0.01-0.91
IgG category1*	20.952	13.699	**<0.001**	5.81-75.47
MSP3				
HPLC	8.313	6.965	**0.011**	1.60-42.95
Gravidae1	0.432	0.291	0.214	0.11-1.62
Gravidae2	0.303	0.196	0.066	0.08-1.08
IgG Category1*	9.520	5.608	**<0.001**	3.00-30.20

Cat1 category with antibody level above 0 and below median. Median antibody levels for antibodies against different antigens; GLURP IgG=1.31945, HRPII IgG= 0.5877, MSP3a IgG= 0.041, MSP3 IgG= 0.2966; These were used as the levels up to median and then above median* . IgGCat1=antibody level below median, BS Negative =Malaria Blood Slide, HPLC=High performance liquid chromatography (Sulfadoxine in maternal blood). * Factors included in the multivariate analysis model.

3.3 Proportions of antibodies transferred from mother to baby affected by antibody levels in the mother and not use of IPTp.

The outliers for different antibodies were excluded from analysis before fitting the model. Ten participants were excluded from the analysis for proportions because the antibody levels were higher in the newborn compared to the mother thereby having proportions as outliers.

Table 4 Factors affecting proportions of IgG antibodies transferred from mother to baby.

Variable	Coefficient	se	*P*-value	
GLURP				
Used IPTp	-0.292	0.197	0.065	
HPLC	0.170	0.225	0.451	
BS mother	0.241	0.289	0.408	R^2= 0.1271
IgG Category	0.608	0.207	**0.004**	N=77
Constant	-1.161	0.221	**0.000**	
HRPII				
Used IPTp	0.083	0.161	0.606	
HPLC	0.271	0.184	0.144	R^2= 0.023
IgG Category	0.117	0.165	0.482	N=120
Constant	-1.433	0.171	0.000	
MSP3a				
Used IPTp	-0.465	0.311	0.141	
HPLC	0.514	0.524	0.331	R^2= 0.095
IgG Category	-0.841	0.469	**0.079**	N= 55
Constant	-0.296	0.486		
MSP3				
Used IPTp	0.031	0.194	0.870	
HPLC	0.214	0.206	0.303	R^2=0.047
Gravidae1	0.231	0.237	0.334	N= 93
Constant	-1.125	0.187	0.000	

MSP3aIgGcat=1 if MSP3aIgGM >0 and <= median (0.041) else MSP3aIgGcat=2 if MSP3aIgGM>median *GLURLPIgGcat =1 if GLURLPIgGm>0 and <= median (0.041) else GLURLPIgGcat =2 if GLURLPIgGm>0 and >median (IgGm =Immunoglobulin G in maternal serum)*

The participants who had low antibody levels were more likely to transfer higher proportion of antibodies to maternal antibodies than their counterparts after multiple logistic regressions.

4.0 Discussion

Acquisition of protective immunity to malaria is slow and requires repeated parasite exposure to be maintained .The malaria immunity is strain specific and *P. falciparum* parasite stage specific. In endemic areas, children born to immune mothers are protected against disease during their first half year of life by maternal antibodies transferred in-utero [26]. In this study we investigated how antimalarial antibody levels to different P.*falciparum* parasite blood stage antigens in the mother/ baby pairs is affected by use of intermittent Presumptive Treatment (IPTp) during pregnancy. Antibodies to malaria blood stage antigens have been previously been found to be important in protection against clinical malaria [23].

The antibody sero-positivity in the study population was generally higher in the mothers compared to the newborns. Trans-placental transport of IgG begins in the second trimester and increases throughout gestation, with most transport occurring late in pregnancy [27]. In some cases in this study there were no antibodies transferred from the mother to the baby .The factors which may have to failure of transfer of IgG from mother to baby may have been due to integrity of the placenta , placental malaria or maternal heamoglobineamia which were not in the scope of this study [13]. This has been previously shown in a study done in Nigeria in mothers at delivery that mothers had significantly higher levels of anti-*P. falciparum* IgG and IgM compared to their newborn [28].

Studies have indicated gravidity dependant susceptibility to malaria infection in endemic setting with primigravidae being more susceptible to infection than the multiparous counterparts [31]. Studies have indicated higher levels of anti-adhesion antibodies in the multi-gravidae than the Primigravidae [32]. The anti-*P.falciparum* antibody levels in the mother baby pairs were not affected by parity in this study. The effect on parity on anti-P.*falciparum* antibodies may be altered by factors such maternal age and HIV status [33].

We found that the proportions of blood stage anti-P.*falciparum* antibodies transferred from the mother to the newborn were not affected by using IPTp during pregnancy for all tested antigens. The mothers with high levels of antibodies generally transferred less proportions of IgG to the newborn, compared to their counterparts.

The effect of IgG levels on the proportions however was only statistically significant for anti-GLURLP and anti-MSP3a antibodies .This finding from our study is in agreement with another study which found mothers with higher antibody levels tend to transfer less proportions to the corresponding neonates [34, 35]. Gestational age at delivery did not affect the proportion of antibody transferred from mother in this study.We had 12 premature births Table 1, prematurity however did not affect the proportions transferred as previously reported [27]. This could have been due to other confounding factors like placental integrity influencing the effect of prematurity on transfer. The results on the effect of prematurity on proportions transferred may not be conclusive due to low prevalence of premature deliveries in the study population.

Study limitation

The antibodies in the newborn is generally regarded as result of trans-placental transfer of IgG from mother to baby. In some cases however the fetus may be exposed to malaria antigens in utero leading to production of anti-parasite IgG antibodies. Some studies have shown presence of congenital malaria in children born to mothers living in malaria endemic areas [36]. When IgG antibodies are present in cord blood, it is not possible to distinguish whether they are of maternal or foetal origin. The amount of IgG antibody due to foetal priming may have affected the apparent proportions transferred from mother to baby. In cases where the OD levels were higher in baby were higher than the corresponding IgG in the mother were eliminated from analysis for proportions transferred.

In conclusion using IPTp during pregnancy had no effect on the proportions of antibodies to selected P.*falciparum* blood stage antigens transferred from mother to baby. This implies that we can still continue using the available malaria intervention without fear of interfering with immunity of the infant. We recommend further studies to determine the factors affecting transfer of IgG antibodies against malaria across the placenta and the potency of antibodies transferred.

5.0 List of abbreviations

ANC; Antenatal Clinic

FGR; Foetal Growth Restriction

GLURLP; Glutamine Rich protein

HRPII: Histidine Rich Protein

IgG; Immunoglobulin G

IgM; Immunoglobulin M

IPTp; Intermittent presumptive Treatment

ITNs; Insectcide Treated Nets

LBW : low birth weight

MSP3; Merozoite Surface Protein 3

MSP3a; Merozoite surface protein 3a

OD; Optic Density

PTD; Preterm delivery

WOA; Weeks of Ammenorrhea

6.0 Competing interests

Authors declare no conflict of interest.

7.0 Authors' contributions

FN collected the clinical data, carried out data analysis and drafted the manuscript. ME participated in the analysis of samples .FM supervised the clinical data collection and data analysis. MW and FK conceived of the study, participated in its design and coordination, and helped to draft the manuscript.

8.0 Acknowledgement

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PAPER IV

Use of sulfadoxine/pyrimethamine Intermittent Presumptive Treatment in Pregnancy affects prenatal immune priming to malaria-Cross-sectional study.

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Abstract

The effect of using Sulfadoxine/pyrimethamine Intermittent Presumptive Treatment during pregnancy (SP IPTp) on the prenatal immune priming to malaria was assessed. In a cross-sectional study mother/baby pairs were recruited at delivery in Mulago National Refferal Hospital after informed consent. Maternal venous and cord blood was taken off aseptically. Blood was used to make thick and thin blood smears for malaria parasites. Determined IgM antibody levels in maternal and cord sera using indirect Elisa method. This was done against four Plasmodium falciparum blood stage antigens GLURP, MSP3, MSP3a and HRPII. Maternal blood was used to determine the presence of sulfadoxine at delivery using High Performance Liquid Chromatography. Sulfadoxine was used as proxy for SP In blood. Data on use of SP IPTp and number of doses taken was collected using a questionnaire. One hundred and fifty mother/baby pairs were included in the study. The anti- P. falciparum IgM sero-positivity in maternal sera against GLURP, HRPII, MSP3a and MSP3 was 89.9%, 86.3%, 57.5% and 79.9% respectively. The IgM levels in the babies were lower at 5.0%, 10%, 2.9% and 33% against GLURP, HRPII, MSP3a and MSP3 respectively. Presence of IgM antibodies in the mother was the highly correlated to fetal immune priming. Using IPTp during pregnancy as evidenced by self-reports and having sulfadoxine in blood at delivery was protective of prenatal exposure to malaria antigens and malaria in mother towards delivery. In areas with reported resistance to SP in treatment of clinical malaria in children, it is effective in controlling intrauterine exposure of fetus to malaria. Studies to evaluate the effect of prenatal immune priming on development of antimalarial immunity in infants in this setting are recommended.

Key words: intermittent Presumptive Treatment, Malaria, prenatal immune priming

1.0 Introduction

Malaria is one of the leading causes of morbidity and mortality in children under five years. Malaria is responsible for up to six hundred and sixty deaths annually (WHO 2012). In endemic areas malaria affects mainly pregnant women and children under five years. Infants below six months born to mothers living in endemic areas are generally

protected from severe infection because of antibodies transferred from mother in-utero (King et al., 2002) and presence of fetal haemoglobin which does not favour growth of parasites (Amaratunga et al., 2011).

More than 50 million women living in malaria endemic areas are at risk pregnancy malaria every year (Dellicour et al., 2010). In malaria endemic areas pregnancy malaria leads to maternal anaemia, low birth weight and congenital malaria (Steketee et al., 2001). Pregnancy malaria leads to sequestration of parasites in the placenta leading to placental malaria (Fried et al., 1996). This leads to interference of the placental barrier leading to exposure of the fetus to malaria leading to congenital malaria. The World Health organisation recommends effective case treatment of malaria illness in pregnancy, use of insecticide treated Nets and Intermittent presumptive treatment with sulphadoxine /pyrimrthamine for prevention of malaria in pregnancy. Resistance to SP has been widely reported across Africa when used for treatment of symptomatic children. The new WHO recommendation is to give up to four doses of SP IPTp to pregnant mothers up to delivery.

Congenital malaria occurs when malaria parasites cross the placenta either during pregnancy or at the time of delivery. The mechanism of transplacental passage of this infection is not clear. Congenital malaria is confirmed by finding asexual parasiteamia in the first week of life. Congenital malaria in endemic has been found to be rare in endemic areas and has been reported to be rare in some studies (Ouedraogo et al., 2012) but not others (Uneke, 2007).

Human foetus is immunologically active and exposure to antigens leads to sensitization of the humoral and cellular immune response (Metenou et al., 2007). The effect the exposure of the fetus to malaria parasites on subsequent immune response to malaria is not very clear. It has been suggested that babies born to mothers with pregnancy malaria are more susceptible to malaria in the first year of life. This is attributed to the fetus becoming immunologically tolerant and thus increased susceptibility to malaria in infancy. It has also been proposed that intrauterine exposure can lead to activation of the immune system, priming and formation of memory B and T cells.

The aim of this study was to determine the burden of congenital malaria and intrauterine exposure to malaria and how it is affected by use of IPTp during pregnancy. The knowledge on the extent of intrauterine priming in this setting is important in generating considerations for vaccinating the infants below six months. Since antibodies are important in immunity against blood stage infection, data on stage specific immunity induced by in utero-exposure is important in understanding acquisition of immunity. This study focused on response to four blood stage *P.falciparum* antigens. Glutamine Rich Protein (GLURP), Histidine Rich Protein (HRPII), Merozoite surface Protein (MSP) 3 and 3a .These are key malaria vaccine candidates. GLURP and MSP3 are components in the malaria vaccine GMZ2 which is under development for use in malaria endemic areas (Mordmuller et al., 2010).

2.0 Methods

2.1 Study setting

The study was carried out in Mulago National Referral Hospital in Kampala details of the site are described elsewhere (Namusoke et al., 2010)

In a cross sectional study we recruited mothers at delivery after informed oral and written consent and assent. Mothers at 28 weeks of gestation and above were included and excluded, mothers with ante-partum haemorrhage, severe pre-eclampsia and babies with confirmed gross congenital abnormalities and still birth at delivery. All ethical aspects of the study were granted by the Makerere University College of Health Sciences Research and Ethics Committee and the Uganda National Council for Science and Technology. Data on demographic characteristics, obstetric history, order of pregnancy, use of intermittent presumptive treatment during pregnancy and number of doses taken and gestation age at which it was taken was recorded on an interviewer administered questionnaire .Use of bed net during pregnancy and after delivery pregnancy outcome were recorded.

2.2 Sample collection and laboratory studies

Venous blood was drawn asceptically from the study participant at recruitment , for a thick blood smear for malaria examination.A sample was sent to the laboratory where it

was separated using a centrifuge at 3000G for 30 minutes and serum kept at -70degrees till analysis . After delivery 2-4mls of cord blood was taken asceptically for malaria parasite examination and serum kept at -70 degrees . Thick blood films of peripheral and cord blood were stained by Giemsa and malaria diagnosis was assessed by microscopy following standard procedures. Determination of sulfadoxine in blood was done as described in (Namusoke et al 2012).

Synthetic antigens

The synthetic peptides representing p.*falciparum* blood stage *proteins* which included GLURP :(NH2)CGDKNEKGQHEIVEVEEILPEGC(CONH2),

HRP II: (NH2)GCAHHAADAHHAADAHHAADAHHAADGC(CONH2),

MSP3a :(NH2)TLAGLIKGNNQIDSTLKDLV(CONH2),

MSP3: (NH2)AKEASSYDYILGWEFGGGVPEHKKEEN(CONH2) } were used to determine antibody levels .These synthetic antigens were prepared as described (Borre, 1991; Soe, 2004).

2.3 Antibody measurements

Plasma antibodies to the synthetic peptides to GLURP, HRPII, MSP3 and MSP3a were measured by ELISA, as described elsewhere (Theisen et al., 1998).

In brief, microtiter plates (Nunc, Roskilde, Denmark) were coated with recombinant protein 100micrograms per well, incubated overnight at 4^0C, and blocked with 5% skimmed milk for 1 hour at room temperature. Plasma samples diluted 1: 200 were added in duplicate and incubated at room temperature for 1 h, plasma sample of the mother and the corresponding neonate (mother/baby pairs) were run on the same plate in all cases. Plates were washed 4 times between steps. Plates were developed by Peroxidase conjugated goat anti-human IgG (secondary antibody). Bound secondary antibody was quantified by colouring with ready to use TMB (*3,3', 5,5'-Tetramethylbenzidine*) substrate. Optical density (OD) was read at 450 nm with a reference at 620nm in a plate reader. Value two standard deviation above the mean absorbance of the samples from unexposed control donors was used as the negative cut-off. All samples with ODs above 1.4 were diluted further to 1:500 and the OD obtained was then multiplied by the dilution factor.

2.4 Data analysis

Data was cleaned, coded and entered into Microsoft Acess 2007 and exported to STATA (version 9) for analysis. The main outcome variables were Immunoglobulin M (IgM) antibody sero-positivity against respective P. falciparum blood stage antigens in cord and /or maternal blood at delivery

Logistic regression was done to determine factors associated with immune priming (cord IgM sero-positivity). Variables assessed at bivariate included; use of IPTp and the number of doses taken, gestational age, presence of sulfadoxine in maternal sera, parity of the mother, HIV sero-status and maternal age. Variables with P-value of < 0.25 were included in multivariate analysis.

3.0 **Results**

One hundred and fifty mother/baby pairs were recruited were analysed for IgM to blood stage antigens. About 60% of the participants reported taking at least one dose of SP IPTp during that pregnancy. On assessing for presence of SP metabolites; 25% had sulfadoxine in blood at delivery Table 1.

Table 1; Demographic characteristics of the study participants

Variable	Number (n)	Percentage
IPTp Use		
Yes	61	43.88
No	78	56.12
IPTp doses		
1	69	88.46
= >2	9	11.54
HPLC		
Positive	35	25.18
Negative	104	74.82
Age group		
< 20yrs	31	22.30
= > 20 yrs	108	77.70

The anti- *P. falciparum* IgM sero-positivity in maternal sera against GLURP, HRPII, MSP3a and MSP3 was 89.9%, 86.3%, 57.5% and 79.9% respectively. The IgM levels in the babies were lower at 5.0%, 10%, 2.9% and 33% against GLURP, HRPII, MSP3a and MSP3 respectively Figure 1.

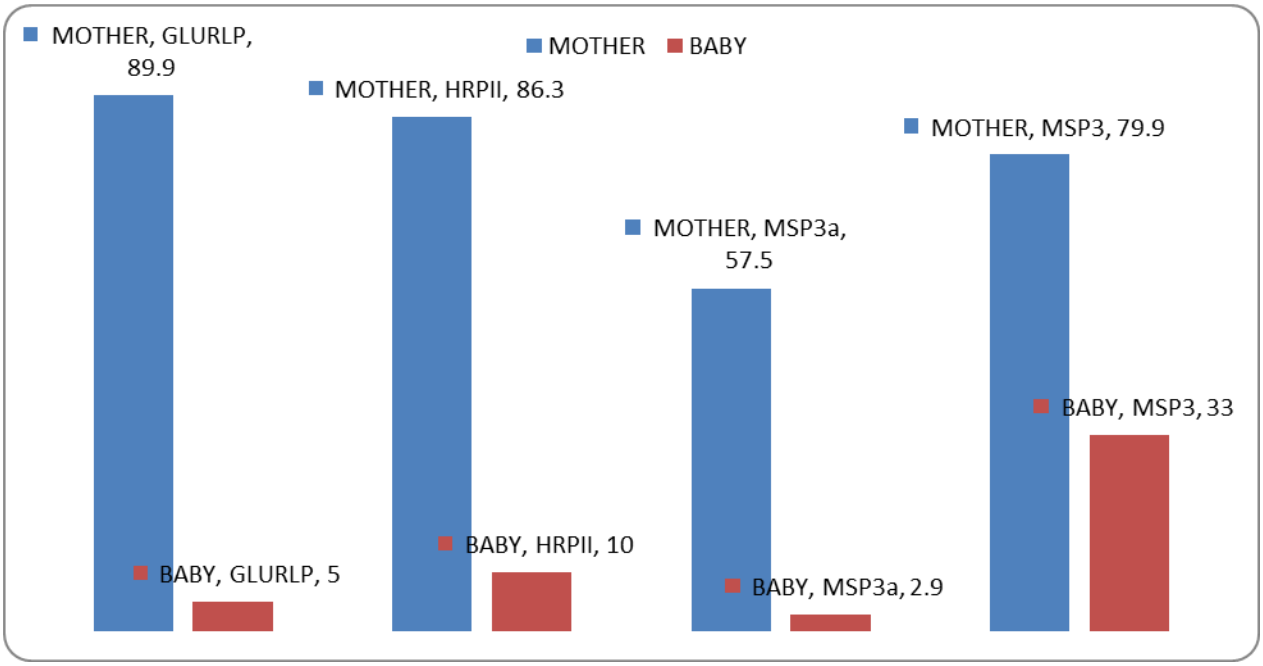

Figure 1; Maternal and cord blood IgM sero-positivity to selected anti-*P. Falciparum antigens*

IgM Sero-positivity of maternal serum and IPTp use

All mothers who had malaria parasites in blood at delivery and those who were HIV positive had IgM antibodies against GLURP. The parity and maternal age did not affect the sero-positivity to IgM in mothers.

Maternal IgM sero-positivity of antibodies against HRPII in was significantly higher in the mothers who had used IPTp and those who had sulfadoxine in blood at delivery than their counterparts. Primigravidae tended have more evidence of recent infection (IgM against HRPII) at bivariate analysis Table 2 .

Antibody sero-positivity against MSP3a antigen however was not affected by IPTp use or presence of sulfadoxine in maternal blood at delivery or malaria parasites at Delivery. All HIV positive participants had antibodies (IgM) against MSP3, and pariticipants who had sulfadoxine in blood at delivery were more likely not to have IgM antibodies at delivery.

Table 2; Maternal IgM sero-positivity and demographics- Bivariate analysis

Outcome	Effect	OR	s.e	P value	95% CI
GLURP					
	Used IPTp	0.477	0.295	**0.231**	**0.14-1.60**
	HPLC positive	0.685	0.460	0.574	0.18-2.55
	Less than 20years	4.105	4.346	**0.182**	**0.51-32.69**
	Gravidae 1	1.037	0.792	0.961	0.23-4.63
	Gravidae 2	0.613	0.393	0.446	0.17-2.15
	<37 WOA	0.693	0.174	**0.147**	**0.42-1.13**
HRPII					
	Used IPTp	8.888	5.838	**0.001**	**2.45-32.20**
	HPLC positive	11.333	6.160	**>0.001**	**3.90-32.89**
	Gravidae 1	7.291	7.817	**0.064**	**0.89-59.62**
	Gravidae 2	0.963	0.503	**0.943**	**0.34-2.68**
	<20years	1.088	0.657	0.888	0.33-3.55
	*BS positive	0.304	0.200	**0.072**	**0.08-1.11**
	HIV positive	1.486	1.611	0.715	0.17-12.44
	<37WOA	1.221	0.255	0.340	0.81-1.84
MSP3a					
	Used IPTp	0.625	0.218	**0.179**	**0.31-1.24**
	HPLC positive	1.010	0.397	0.978	0.46-2.18
	BS positive	1.742	1.093	0.376	0.50-5.95
	<37*WOA	0.835	0.123	**0.225**	**0.50-5.95**
MSP3					
	Used IPTp	1.361	0.577	0.466	0.59-3.12
	*HPLC positive	0.173	0.131	**0.021**	**0.03-0.76**
	*BS positive	1.430	1.143	0.655	0.29-6.85

* BS Positive= mother with malaria parasitemia at delivery; HPLC positive= Presence sulfadoxine in maternal blood at delivery; WOA= Weeks of Ammenorrhea

Maternal IgM sero-positivity and IPTp use in pregnancy

Variables which were fitting perfectly for a particular antigen were not included in the model. After multivariate analysis, participants who reported to used IPTp during pregnancy were more likely to have IgM at delivery. Participants with sulfadoxine in blood at the time of delivery were less likely to have evidence of recent exposure to malaria parasites (Maternal IgM) Table 3.

Table 3; Factors affecting maternal exposure to malaria towards delivery

Outcome	Effect	OR	s.e	*P*-value	95% CI
GLURP					
	Used IPTp	0.481	0.297	0.237	0.14-1.61
	HPLC Positive	0.826	0.567	0.781	0.21- 3.17
HRPII					
	Used IPTp	13.107	9.738	**0.001**	**3.05-56.22**
	HPLC Positive	18.497	12.079	**0.000**	**5.14-66.52**
MSP3a					
	Used IPTp	0.623	0.218	0.178	0.31-1.23
	HPLC Positive	1.053	0.419	0.895	0.48-2.30
MSP3					
	Used IPTp	1.469	0.639	0.376	0.62-3.44
	HPLC Positive	1.176	0.134	**0.023**	**0.03-0.78**

Maternal IgM sero-positivity predicts cord blood recent exposure

All the babies whose cord blood had IgM antibodies against GLURP were born to mothers who reported not to have Used IPTp during pregnancy and presence of IgM in the mothers was generally protective to the babies against all the antigens. All babies whose mothers reported using IPTp and who had sulfadoxine in blood at delivery had no IgM antibodies against MSP3a. Maternal age, gestation age at delivery and parity had no influence on sero-positivity of cord blood against the tested blood stage antigens Table 4.

Factors which fitted perfectly were not included in the multivariate model. Since in Bivariate analysis, all main factors; IPTp and sulfadoxine in blood fitted perfectly and yet none of the other factors was significant no model was fitted for sero-positivity against MSP3a.

Table 4; Factors affecting fetal immune priming to selected *P. falciparum antigens*

Outcome	Effect	OR	s.e	Pvalue	95% CI
GLURP					
	IgM mother positive	0.175	0.138	**0.028**	0.03-0.82
	HPLC positive	0.873	0.751	0.875	0.16-4.71
HRPII					
	IgM mother positive	0.510	0.484	0.479	0.07-3.28
	Used IPTp	2.405	1.601	0.187	0.65-8.86
	HPLC Positive	1.453	1.08	0.617	0.33-6.28
MSP3					
	IgM mother positive	0.128	0.064	**>0.001**	0.048-0.34
	Used IPTp	2.633	1.120	**0.023**	1.14-6.06
	HPLC positive	1.010	0.482	0.982	0.39-2.57

4.0 Discussion

Cord blood *falciparum* parasiteamia in the participants by microscopy was 1.4% although 2-33% had IgM in cord blood as evidence of recent exposure to malaria parasites/antigens. This is lower than what was reported in Hoima district in Uganda of 47% and this was not affected by using Chloroquine chemoprophylaxis (Ndyomugyenyi & Magnussen, 2000). This was done before the IPTp policy was implemented in Uganda. A study done in malaria endemic area in Burkina Faso found prevalance of cord parasiteamia of 1.4% which is comparable to our findings in this study (Ouedraogo *et al.*, 2012) which was significantly associated with parasite density in the maternal and cord blood. Generally trans-placental transmission of *P. falciparum* appears to be low in malaria endemic areas ranging from 1-5% (Uneke, 2007).

The effect of exposure of the fetus to malaria parasites/antigens in utero on the immune response during early infancy is not very clear. Some studies have shown that babies born to mothers with placental malaria are more susceptible to malaria infection in infancy (Malhotra *et al.*, 2009; Schwarz *et al.*, 2008). Trans-placental passage of parasite-derived antigens may leads to, tolerance of the fetal immune system. Another study demonstrated no effect of placental malaria on neonatal immunity (Soulard *et al.*, 2011). It has been postulated that infants born with primed cells may produce secondary response upon exposure to that antigen whereas those who did not produce primary response. This is important in terms of infant immune response and consequently on severity to the acute infections.

Self-reported Use of IPTp (at least one dose) by the mother during pregnancy and presence of sulfadoxine in maternal blood at the time of delivery were protective of congenital exposure to *P. falciparum*. The protective effect was observed for all *P. falciparum* blood stage antigens tested (GLURP, MSP MSP3a) except HRPII antigens. This finding implies that using IPTp during pregnancy is effective in protecting the fetus against congenital exposure to malaria parasites/antigens through control of placental parasiteamia. Placental malaria and maternal anemia have been associated with in utero priming to *P. falciparum* antigens (Gouling *et al.*, 2003). It has been postulated that presence of parasites for an extended period may alter the fetal maternal barrier

leading to congenital malaria. The results of this study are in agreement with the fact that using IPTp protects against maternal infections and consequently fetal exposure towards delivery. The presence of IgM in cord blood indicates that the fetus was exposed in utero since it does not cross the placenta.

The prevalence of cord blood parasitemia was 1.4% and IgM to different *P. falciparum* antigens ranged from 2-33%. A proportion of newborns had IgM in cord blood in absence of parasiteamia. Malaria parasites do not usually cross the placental barrier and such sensitization is most probably caused by trans-placental passage of soluble *P. falciparum* antigens or cross-reactive antigens from other pathogens leading to fetal T and B cell activation (Metenou *et al.*, 2007). Finding IgM in cord blood was significantly associated with having IgM in maternal blood which confirms that the source of fetal infection was maternal.

Pregnant women in malaria endemic areas are often infected with malaria parasites and expose the fetus to malaria antigens. The trans-placental transmission of malaria from the mother to the fetus called congenital malaria has been well documented (Uneke, 2007; King *et al.*, 2002). The mechanisms underlying the trans-placental transfer is not clear but malaria parasites for example has been detected in cord blood (Gouling *et al.*, 2003). *P. falciparum* antigens and possibly cross reactive antigens from the other parasite cross the placenta and activate fetal T and B cells in utero.

Presence of malaria parasites in maternal blood was significantly associated with having IgM against all tested blood stage antigens in maternal blood in this study. Since IgM is the first antibody to be produced after its presence in sera indicates recent exposure to malaria parasite/antigens. All HIV positive participants had IgM antibodies against GLURP and were more likely to have IgM antibodies against MSP. There was however there was no association with HRPII and MSP3a .Since IgM is evidence of recent infection it is clear that HIV infection was highly associated with having malaria infection in the mother towards delivery as shown in previous studies (Nkhoma *et al.* , 2012) .

Participants who had sulfadoxine in blood were less likely to have IgM in maternal sera for MSP3 and HRPII although no effect with GLURP and MSP3a. This implies that the sulfadoxine is still effective in controlling parasiteamia in the mother towards delivery. The new recommendation of WHO in which the pregnant mother should be given SP IPTp up to the time of delivery should be emphasized (WHO 2012).

The cord parasiteamia at delivery, we were not able to delineate the infection got at delivery and that acquired in utero. The cross sectional nature of the study can show association but not cause effect relationship. We recommend longitudinal studies to determine the effect of the intrauterine immune priming on development of immunity in infancy.

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6.0 Disclosure

Authors declare no conflict of interest

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