VAR2CSA Duffy Binding like (DBL) domains and non-specific IgM binding in the acquisition of acquired immunity to Pregnancy-Associated *Plasmodium falciparum* malaria

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DECLARATION

I, Augustina Frimpong, of the department of Pathology, University of Ghana Medical School, do hereby declare that this experimental work entitled "**Var2csa Duffy Binding like (DBL) domains and non-specific IgM binding in the acquisition of natural immunity to pregnancy-associated *Plasmodium falciparum* malaria" is the outcome of my own research work at the Immunology Unit, Noguchi Memorial Institute for Medical Research** under the supervision of Dr. Michael Ofori of Noguchi Memorial Institute for Medical Research All references cited in this work have been fully acknowledged.

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DEDICATION

I dedicate this work to Almighty God whose grace is sufficient for me, my dearest one Samuel E. Danso and especially to my lovely daughter Ama Owusu Danso. Also I dedicate this work to my parents Mr and Mrs Yaw Frimpong, and siblings Theresa Afriyie Frimpong and Samuel Yaw Frimpong. It is also dedicated to the late Dr. Daniel Osei (Clinical Immunologist, Dep't of Pathology) and all Scientists who are committed to the fight against malaria.

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
CD	Cluster of differentiation
CIDR	Cystein-rich InterDomain Region
CSA	Chondroitin Sulfate A
CSP	Circumsporozoite
CXCL	Cytokine Cell Ligand
DBL	Duffy Binding-Like
IE	Infected Erythrocytes
IFN-γ	Interferon Gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MSP	Merozoite Surface Protein
NK cells	Natural Killer Cells
PAM	Pregnancy Associated Malaria
PECAM	Platelet Endothelial Cell Adhesion Molecule
PfEMP1	*P. falciparum* Erythrocyte Membrane Protein1
PfMC	2TM- *P. falciparum* Maurer's Cleft-2
SIgM	Secreted IgM
TGF	Tumor Growth Factor
TNF	Tumor Necrosis Factor
VAR2CSA	Variant 2 Cell Surface Antigen
VSA	Variant Surface Antigens

ABSTRACT

BACKGROUND: *Plasmodium falciparum* malaria throughout history has proved to be a significant menace to human health and pregnancy associated malaria (PAM) has been linked to severe consequences in terms of morbidity and mortality. *P. falciparum* parasites express members of the *Plasmodium falciparum* Erythrocyte Membrane Protein-1 (PfEMP1) on the surface of infected erythrocytes (IEs) which act as ligands binding to a number of different human vascular host receptors such as Chondroitin Sulphate A (CSA) in the placenta. The adhesion to CSA is mediated by VAR2CSA, which also allows for antigenic variation and immune evasion. VAR2CSA has been considered an important target of acquired protective immunity mediated mainly by specific IgG antibodies. In addition to being targets of specific IgGs, IEs have been demonstrated to select natural IgM in addition to CSA.

AIM: This study was designed to determine if binding of VAR2CSA IEs to nonspecific IgM interferes with specific IgG binding to the VAR2CSA epitopes.

METHOD: Plasma samples from 109 pregnant Ghanaian women of varying gestational age and parity were purified by the Dynabeads immunoprecipitation method for IgG and IgM on Mannan Binding Protein and grouped into three (unpurified plasma containing IgG and IgM (IgG+IgM+), purified IgG from plasma (IgG+IgM-) and purified IgM from same plasma(IgG-IgM+). The levels of antibody reactivity to two different domains of VAR2CSA (DBL1 and DBL5) and two domains of IT4var60 not implicated in placental malaria, but mediates rosetting (DBL1α and DBL5ε) were measured using indirect ELISA.

RESULTS: The study showed increased susceptibility to malaria infection in the primigravidae (13%) than in the multigravidae (2.3%). The levels of IgG measured in the presence of IgM was significantly higher than IgG levels measured in the absence of IgM to both DBL1 domains ($p=0.0001$). Also there was a significant increase in the reactivity of IgG to the DBL5 domains (VAR2CSA and IT4VAR60) ($p<0.0001$) in the presence of IgM when compared to the IgG reactivity to the domains in the absence of IgM. However, the decreased IgM reactivity to VAR2CSA DBL5 in the IgG+IgM+ samples may indicate that IgG inhibits IgM binding to VAR2CSA DBL5 domain. Similarly there was an increased reactivity of the antibodies to the domains in the test samples when compared to the control samples. Also among the

multigravidae, there was an increased IgG reactivity to the DBL1 domains and DBL5ε domain during gestation when compared to the primigravidae.

CONCLUSION: The results from the study showed that the VAR2CSA and IT4var60 domains harboured IgM epitopes alongside malaria specific IgGs. Moreover since the levels of IgG binding to the domains were higher in plasma than in the absence of IgM, it may be suggestive that IgM binding may not interfere with IgG binding to the domains. Also a strong immune response against the DBL1 and DBL5ε domains elicited by the multigravidae during gestation may be as a result of their previous repeated exposure or pre-existing memory to *P. falciparum* infections during pregnancy.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria still remains one of the most important global health problems due to it being the leading cause of morbidity and mortality in children and pregnant women especially in developing countries (WHO, 2012). Although protective immunity to *Plasmodium falciparum* malaria develops in the late stages of life, pregnant women become more susceptible especially during their first time in pregnancy due to the sequestering ability of the parasite. The sequestration of *Plasmodium falciparum*-infected erythrocytes in the placenta leads to a severe form of malaria in pregnancy known as Pregnancy-Associated Malaria (PAM). *P. falciparum* has been identified to have the unique ability of cytoadhesion to endothelium adhesion molecules such as CD36 and intercellular adhesion molecule-1 (ICAM-1), which may be involved in severe malaria development in children and non-pregnant women (Ockenhouse *et al.,* 1989; Berendt *et al.,* 1989). The adhesion molecules that have been identified for parasite attachment to placental cells include firstly, the glycosaminoglycan chondroitin sulphate A (CSA) component of the chondroitin sulphate proteoglycan (CSPG) found on the surface of syncytiotrophoblast (Robert *et al.,* 1985; Rogerson *et al.,* 1985) acting as a side chain to tissue anticoagulant thrombomodulin (Saleem *et al.,* 1984), and within the intervillous spaces (Bray *et al.,* 1979), secondly hyaluronic acid and thirdly, non- immune IgG (Flick *et al*., 2001; Beeson *et al*., 2000; Fried and Duffy,1996).

In addition, PfEMP1 (*Plasmodium falciparum* Erythrocyte Membrane Protein-1) identified on the surface of infected erythrocytes (Leech *et al.,* 1984), acts as adhesion ligands binding to a number of different human vascular host receptors which allow them to sequester in various tissues and escape destruction in the spleen. They are also responsible for parasite virulence phenotypes in African children that cause rosetting and sequestration in brain endothelial cells and sequestration to the placenta in pregnant women (Rowe *et al.,* 2002; Chen *et al.,* 2000). The placental binding of infected erythrocytes (IEs) is mediated by VAR2CSA which is a member of the large and diverse *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family (Salanti *et al.,* 2004; Salanti *et al.,* 2003).

The VAR2CSA protein is a large antigenic molecule of 350 kDa, exposed to host immune system on the surface of infected erythrocytes (Gamain *et al.,* 2005; Tuikue Ndam *et al.,* 2005; Salanti *et al.,* 2004). VAR2CSA is structurally composed of six Duffy Binding-Like (DBL) domains (Resende *et al.,* 2008; Avril *et al.,* 2006; Dahlback *et al.,* 2006; Gamain *et al.,* 2005). According to Su *et al.,* (1995) and Francis *et al.,* (2007), each parasite possesses approximately 60 *var* genes and disruption of VAR2CSA results in the loss of CSA adhesion ability of infected erythrocytes (IEs).

In addition, antibodies against VAR2CSA have been identified to be sex-specific and parity-dependent since men from malaria-endemic areas do not develop VSA_{PAM} antibodies and women acquire increasing levels of anti-VSA_{PAM} immunoglobulins during subsequent pregnancies (Salanti *et al.,* 2004; Brabin *et al.,* 1983). High levels of antibodies, including anti-VAR2CSA IgG, have also been found to correlate with protection against the clinical consequences of PAM (Tuikue Ndam *et al.,* 2006; Salanti *et al.,* 2004). These anti-VAR2CSA antibodies block the adhesion of IEs to

CSA after subsequent pregnancies, thus leading to protective immunity (Salanti *et al.,* 2004; Staalsoe *et al.,* 2004).

Nevertheless, along with CSA adhesion, it has been shown that IEs implicated in placental adhesion are also able to bind natural, non-specific/non-immune immunoglobulin M (IgM) according to Creasey *et al.,* (2003). IgM has been identified to be the first antibody to be secreted during an immune response (Boes, 2000). Due to its pentameric structure, it is highly efficient at neutralizing and agglutinating pathogens, and also activates the classical complement cascade with increased avidity than IgG (Burton *et al.,* 1987). Its FcμR helps it in mediating endocytosis, and also aids in antigen processing and presentation (Cho *et al.,* 2006; Sakamoto *et al.,* 2001).

Immunoglobulin M has been found to be associated with rosetting in severe malaria by binding to IEs and helping in antigen interaction between the IEs (Rowe *et al.,* 2002; Scholander *et al.,* 1996). The rosetting and placental parasite isolates may bind nonspecific IgM to allow masking of critical PfEMP1 domains from the destructive action of specific antibodies (Barfod *et al.,* 2011).

A study by Gaboriaud *et al.,* (2004) on structural models of the C1 complex in rosetting phenotypes has shown that both C1r and C1s which are required for activation of the complement pathway bind to IgM where they occupy a large area sitting over the Cμ4 domains of the antibody leading to a conformational change and blocking the C1q site at the Cμ3 domain. This therefore led to a hypothesis that, parasites allow binding of non-immune IgM antibodies in order to prevent binding to specific immunoglobulins, thus avoiding destruction by the immune system (Ashfaq *et al.,* 2008; Rowe *et al.,* 2002).

1.2 Problem statement

The sequestration of *Plasmodium falciparum*-infected erythrocytes in the placenta is responsible for many of the harmful effects of malaria during pregnancy. Such harmful effects affect both mother and child. It accounts for about 200, 000 new-born deaths each year (Brabin *et al.,* 1983). In addition, Pregnancy-associated malaria (PAM) causes immunosuppression in pregnant women and increases the risk of severe anaemia and death. Again, maternal malaria increases the risk of spontaneous abortion, stillbirth, premature delivery, low birth weight (LBW), preterm delivery (PD), and consequently, perinatal and infant mortality (Resende *et al.,* 2008; Gamain *et al.,* 2005; Salanti *et al.,* 2003). Sequestration is made possible because of the presence of parasite adhesion molecules expressed on the surface of IEs binding to host receptors in the placenta such as CSA. These parasite isolates also select non-immune IgM together with CSA (Creasey *et al*, 2003). Therefore there is the need to find out the role played by non-immune IgM upon selection with CSA as it remains unclear whether it promotes adhesion, provides a means of immune evasion for the parasite or affects the acquisition of acquired immunity by interfering with specific IgG binding to the various epitopes. Data from this study will also help in developing an effective vaccine against malaria in pregnancy.

1.3 Justification

This study primarily seeks to determine the role of non-immune Immunoglobulin M in malaria in pregnancy since pregnancy associated malaria has been linked to severe consequences such as increased maternal anaemia, low birth weight and infant mortality. Also, in view of the high number of cases of women who suffer from *P. falciparum* malaria during pregnancy and the effects of this on the health and even the

survival of both mother and child, it is appropriate to know the effects of nonspecific IgM in PAM infections. Again VAR2CSA which has been identified in *P. falciparum* malaria in pregnancy has been considered as a vaccine candidate since its transcription and expression are up regulated in parasites expressing VSA $_{PAM}$ (Hviid *et al.,* 2010; Tuikue Ndam *et al.,* 2005). Moreover antibodies against VAR2CSA are specifically induced during pregnancy and are conserved between parasite genomes. The binding of natural, non-immune IgM to VAR2CSA has also been postulated to either help in the adhesion or enable the parasite evade the immune system (Barfod *et al.,* 2011; Creasey *et al.,* 2003). For this reason this work is going to help provide the necessary baseline information which will be crucial in the design of effective interventions aimed at reducing the prevalence of the infection and promoting the survival of both mother and child in order to impede the global burden of malaria in pregnancy.

1.4 Hypothesis

There is a significant role of Immunoglobulin M (IgM) in acquired immunity to malaria in pregnancy.

1.5 General objective

To determine if nonspecific IgM binding to VAR2CSA infected erythrocytes interferes with specific IgG binding to the VAR2CSA epitopes.

1.6 Specific objectives

I. To purify IgG from plasma collected from pregnant women using Dynabeads® Protein G

II. To purify IgM from same plasma collected from the pregnant women using Mannan Binding Protein.

III. To measure the levels of the purified IgG and IgM to different DBL domains from IT4var60 (not implicated in placental malaria) and Pfl0030c/VAR2CSA (found in placental malaria) using indirect ELISA.

IV. To measure the levels of the IgG and IgM in the plasma to the various DBL domains using indirect ELISA.

V. To compare the antibody responses of the purified and plasma samples

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The disease malaria

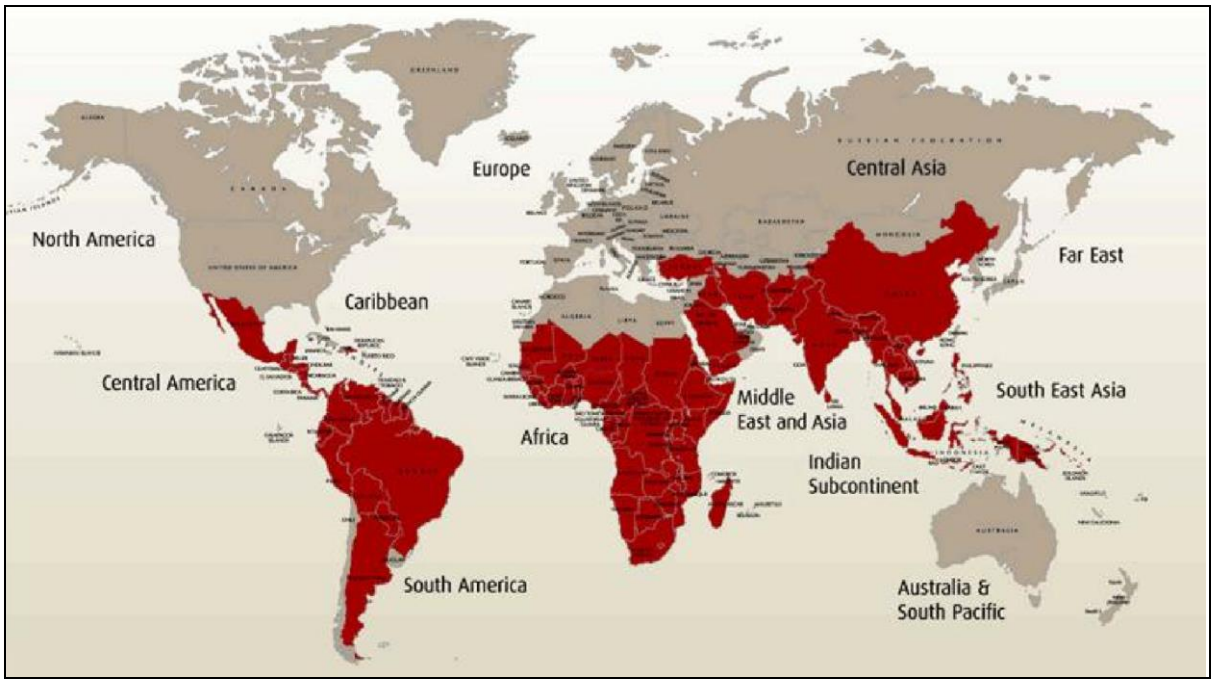
Malaria is an infectious parasitic disease caused by the eukaryotic protists from the genus *Plasmodium* and transmitted by the female *Anopheles* mosquito when taking a blood meal. It is a preventable disease afflicting millions of people leading to socioeconomic suffering such as poverty, as well as physical and mental disabilities including decreased cognition.

In malaria endemic areas, the adult populations have been found to enjoy some level of acquired immunity to the disease and rather have become asymptomatic carriers, with the exception of children, pregnant women and naïve individuals moving to these endemic areas who are normally found to be associated with the severe forms of the disease including mortality (Doolan *et al.,* 2009).

2.2 Global distribution of malaria

Malaria affects about 40% to 45% of the world's population and is endemic in about 100 countries of the world (Fig. 2.1). It is widespread in the tropical and subtropical regions of the world including Africa, Latin America and Asia with about 300-500 million clinical cases annually (Aultman *et al.,* 2002). *P. vivax* is worldwide in tropical regions and some temperate zones causing benign tertian malaria with the ability to relapse after initial infection. It accounts for about 43% of malaria cases in the world (Roberts and Jovanny, 1996). *P. ovale* mainly in tropical West Africa is the rarest of the infection and causes mild tertian malaria in humans (Hall and Canfield, 1972). *P. malariae* is worldwide but with a very patchy distribution causing quartan

malaria accounting for about 7% malaria cases worldwide (Roberts and Jovanny, 1996). *P. falciparum* on the other hand is found throughout tropical Africa, Asia and Latin America causing the most deadly form of the disease. In addition to these, another species which used to infect only monkeys, a fifth type, *Plasmodium knowlesi*, has been found to be causing malaria in people in Malaysia and areas of South-East Asia (Cox-Singh *et al.,* 2008; Luchavez *et al.,* 2008).



Malaria endemic areas in the world

Fig. 2.1: Global distribution of malaria (Adapted from http://www.cedarcrest.edu)

2.3. Malaria as a Public Health crisis

The annual economic burden of malaria in Africa alone is estimated to be around US $12 billion (UNICEF, 2004). The cost of malaria continues to increase due to resistance of the parasite to preventive methods such as insecticide control and prophylactic drug usage. It also decreases a nation's productivity due to complications such as brain damage, decreased cognition and miscarriages. It creates absenteeism in work and schools respectively due to one or more of its morbidities in adults and children alike (Rugemalila *et al.,* 2006). It is also responsible for the slow development in mostly children under 5 years who have survived severe forms of the disease. Moreover, about 90% of mortalities in malaria occur in Africa helping to slow economic growth in the region as well (Sachs *et al.,* 2002).

2.3.1 The malaria situation in Ghana

The malaria situation in Ghana is hyper endemic with the rate of infection ranging between 10-70% with *P. falciparum* being dominant. According to the Ghana Ministry of Health in 1991, three main species of *Plasmodium* are prevalent in Ghana and these are *P. falciparum* with percentage prevalence of 80-90%, *P. malariae* (20%-36%), and *P. ovale* (0.2%) (Ahmed 1989).

The disease is so far the leading cause of morbidity and mortality in the country and accounted for 3.2 million cases in only 2008. It is responsible for 40-60% of outpatient cases and about 20% of mortality in children under five years of age (Rugemalila *et al.,* 2006; Asante and Asenso-Okyere, 2003). A study done by Antwi and his colleague in 1998 recorded that 13.8% of pregnant women visiting the health facilities suffer from malaria whilst 9.4% of them die from the disease whilst another

study done by Ofori *et al.,* (2009) found the prevalence rate to be 19.7% in a coastal savannah region of Ghana.

Malaria accounts for about 44% of reported outpatient visits in the health centres representing a small percentage of the real number of malaria episodes since majority of individuals who are symptomatic treat the diseases at home failing to report to the various health facilities (WHO, 2005). A study done by Agyapong (1992) found that, self-medication practise causes most malaria symptomatic individuals to report cases only when their initial attempts to treat symptoms have failed.

Many working hours are lost when an individual suffers from the disease thereby worsening the economic situation in Ghana and increasing the annual economic burden to 1-2% of the Gross Domestic Product (UNICEF, 2007). A study done in the Northern part of the country identified that the cost to treat malaria in the poor households is about 40% of their income as compared to 1% of the income in rich households (Asante and Asenso-Okyere, 2003).

2.4 The life cycle of the malaria parasite

P. falciparum malaria parasites have a very complex life cycle (Fig. 2.2) involving two distinct hosts, the primary (invertebrates) and the definite (vertebrates). The primary host involves the female *Anopheles* mosquito whilst the secondary host involves humans.

Malaria infection starts with the inoculation of sporozoites intravenously by an infected female *Anopheles* mosquito (Fig. 2.2) or sometimes through exposure to infected blood products (Hoffbrand *et al.,* 2005). The sporozoites initiate the pre-erythrocytic stage by circulating for a short while and localizing in the liver to infect the hepatocytes. It is in the hepatocytes that asexual multiplication (exo-erythrocytic

schizogony) occurs to produce thousands of merozoites. When the infected hepatocytes rupture after 6-16 days, the merozoites move into the blood circulation to begin the asexual erythrocytic stage which likewise commences the pathogenic process. In *P. vivax* and *P. ovale* some parasites (hypnozoites) remain in the liver to cause relapses later.

Malarial parasites (merozoites) are able to invade host cell (erythrocyte) since they belong to the phylum Apicomplexa because they possess apical organelles involved in the invasion. The merozoites in the blood stream affect erythrocytes of various stages for *P. falciparum* but mature RBCs for *P. ovale* whilst *P. vivax* infect immature RBCs. In the RBCs they mature from the ring form, uninucleated and vacuolated parasite known as the trophozoite to the asexual schizonts. The length of this intra-erythrocytic stage is different with each species. It takes place within 48 hours for *P. falciparum, P. ovale* and *P. vivax* whilst in *P. malariae* it takes 72 hours to complete.

P. falciparum is able to cause the deadliest form of the disease because of the possession of adhesive proteins. These adhesive proteins enables infected erythrocytes to sequester in various tissues; including the brain causing cerebral malaria or the placenta (pregnant women) leading to placental malaria. The sequestration enables increase parasitaemia and severe pathology (Rowe *et al.,* 2002; Miller *et al.,* 1994).

Some of the merozoites later develop into micro- and macro-gametocytes, which is the sexual stage of the parasite and is inactive in the vertebrate host. These gametocytes may be picked by a female *Anopheles* mosquito when taking a blood meal. The gametocytes become active in the invertebrate host to sexually produce zygotes which later develop to the ookinete. The ookinete permeates the epithelial lining of the mosquito's mid gut, encysts and forms an oocyts. The oocysts are formed on the outer gut wall where they mature to sporozoites. The oocysts rupture to release

the sporozoites which migrate to the salivary glands ready to infect a human during mosquito feeding. The cycle in the invertebrate host is also referred to as the sporogenic cycle.

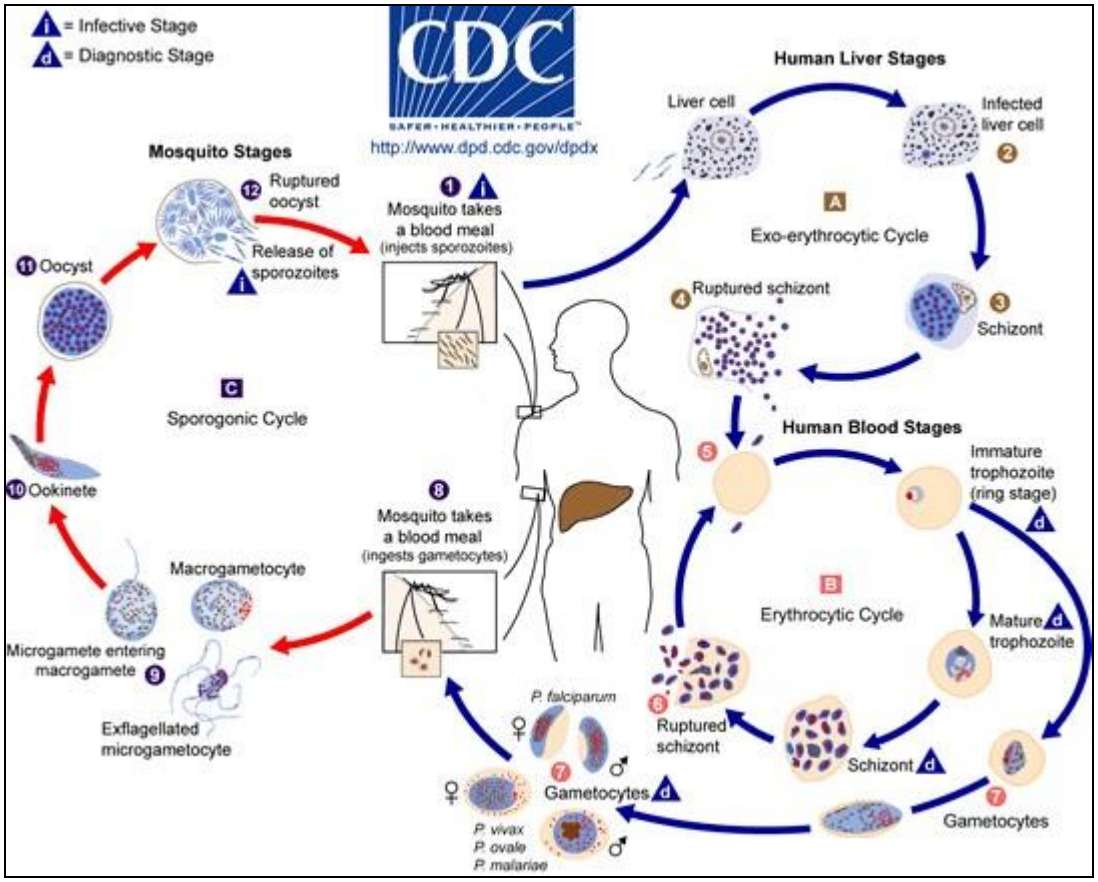


Fig. 2.2: Life cycle of *Plasmodium falciparum* (Adapted from www.dpd.cdc.gov/dpdx)

2.4.1 Signs and symptoms of malaria

Nonspecific clinical symptoms such as headache, fever, vomiting, fatigue, back pains and dry cough are associated with the erythrocytic phase of the disease. The parasite feeds on the haemoglobin in red blood cells to produce haem and globulin. The haem which is toxic to the parasite is converted to haemozoin, a polarized crystal product

which becomes non-toxic to the parasite. Hypoglycaemia, lactic acidosis, pulmonary oedema as well as anaemia are also associated with the erythrocytic phase. The metabolic complications of malaria involving hypoglycaemia and lactic acidosis are as a result of the anaerobic glycolysis of glucose to lactic acid by the parasite to produce energy (Daily *et al.,* 2007). There is also respiratory distress and circulatory failure (Jayakumar *et al.,* 2009). The anaemia and renal failure are as a result of the red cell membranes becoming deformed causing haemolysis and increased splenic clearance (Brattig *et al.,* 2008).

2.5 Pregnancy associated malaria

Pregnant women are mostly vulnerable to malaria as the placenta serves as a preferred site for parasite development and also as a result of immunosuppression during pregnancy (Steketee *et al.*, 1996). This immunosuppression ensures that the foetus is not rejected by the mother's immune system (Wahlgren *et al.,* 1999). Malaria in pregnancy may result in negative outcomes such as maternal anaemia, hypoglycaemia, premature delivery, abortion and even death (Steketee *et al.*, 1996). About 30 million women living in malaria-endemic areas in Africa become pregnant each year (Steketee *et al.,* 2001) putting them at risk of acquiring malaria in pregnancy, therefore making PAM a major health problem. In malaria endemic areas, susceptibility to placental malaria is parity dependent making primigravidae in these areas the most susceptible (Ofori *et al.,* 2009; Shulman and Dorman, 2003; Brabin *et al.,* 1983). These groups of people in endemic areas serve as the main targets of naturally acquired antibodies which is reactive with native VAR2CSA on infected erythrocytes (Fried *et al.,* 1998; Baruch *et al.,* 1996).

Malaria in pregnancy accounts for significantly high morbidity in pregnant women in these endemic areas. The morbidity due to PAM includes anaemia, fever, pulmonary oedema, hypoglycaemia and puerperal sepsis. The high morbidity and mortality is caused by the physiological changes during pregnancy and also pathological changes by the *P. falciparum* as a result of altered immunity and presence of the placenta enabling the adhesiveness of IEs (Fig.2.3). These changes impede oxygen-nutrient transfer and may cause general haemorrhaging contributing to the complications experienced by both mother and child (Claire *et al.,* 2004).

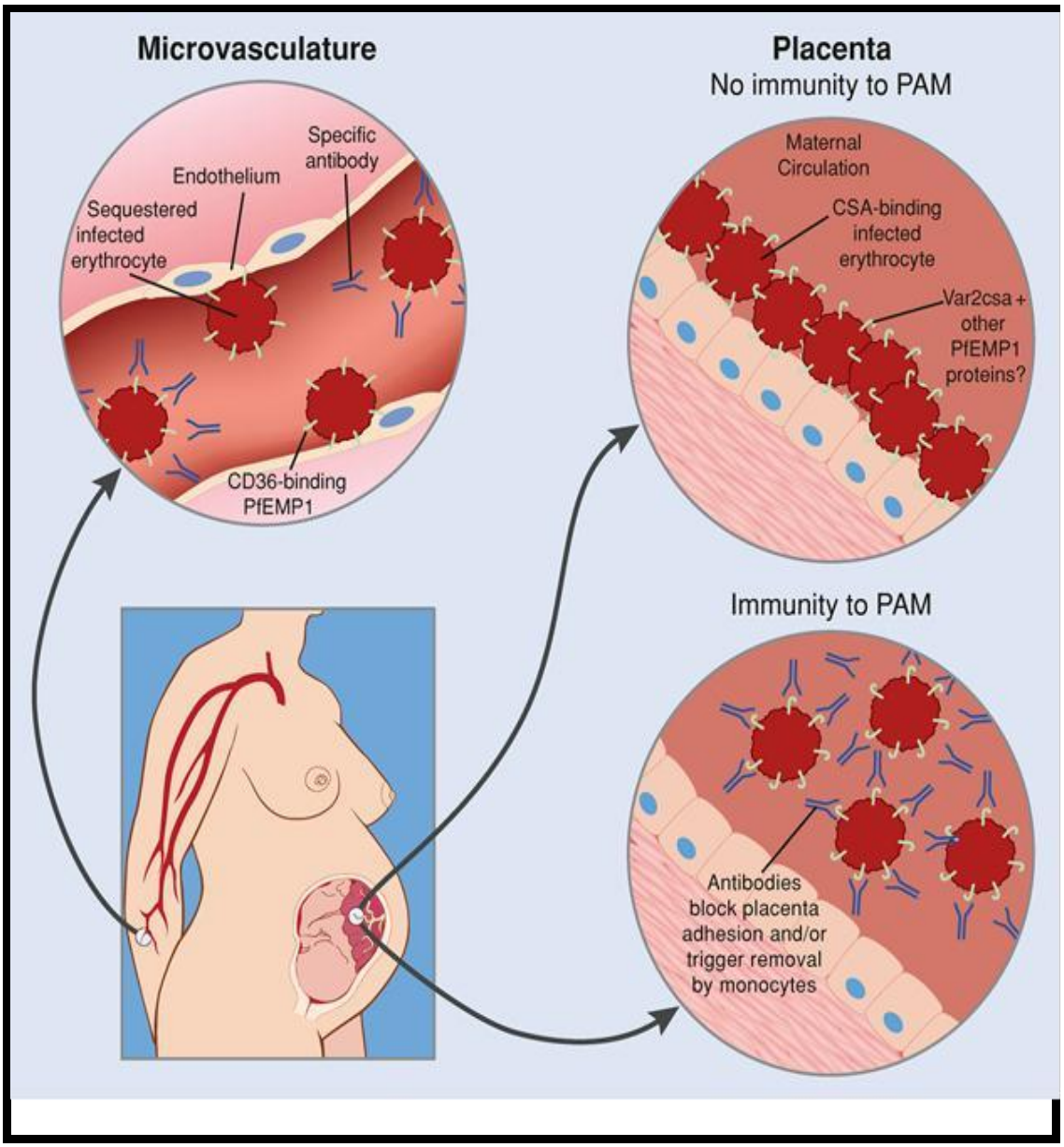


Fig. 2.3: Infected erythrocytes binding to microvasculature endothelium versus placenta (Adapted from Smith and Deitsch, 2004)

The effect of malaria on pregnancy is influenced by the intensity of transmission in a particular area. In stable transmission areas, a high level of acquired immunity to malaria is achieved by frequent transmission by mosquitoes from one person to another. Pregnant women in such areas tend to develop partial immunity resulting in a

low prevalence of peripheral parasitaemia, but a high prevalence of placental infection (Brabin, 1985; Duffy, 2001).

2.5.1 Effects of malaria on the new-born baby

The placenta is an organ responsible for the exchange of metabolic and gaseous products between the mother and foetus. It also serves as a protected site for parasite sequestration and growth (Wahlgren *et al.,* 1999). During the first malaria-exposed pregnancy (that is, in primigravidae), local immunity to malaria develops. This immunity has no effect in the first pregnancy, but is retained in the uterus and increases cumulatively in subsequent pregnancies. This may explain why women in their first and second pregnancies are more affected by malaria than in subsequent pregnancies (Fig. 2.3) (Ofori *et al.,* 2009; Wahlgren *et al.,* 1999).

Placental malaria infection damages placental integrity and interferes with the ability of the placenta to transport nutrients and oxygen to the foetus due to the presence of parasites and macrophages in the intervillous spaces, thereby causing intrauterine growth retardation or foetal growth restriction. Intrauterine growth retardation is defined by a low birth weight of ≤2500g in new-borns following a term gestation (UNICEF, 2001; Sullivan *et al.,* 1999; Walter *et al.,* 1982). Severe maternal anaemia which is a feature in PAM also causes low birth weight in new-borns. In general, low birth weight babies are at a higher risk of dying in infancy (Chigozie, 2007). Also the high level of inflammation may lead to pre-eclampsia helping to account for about 75,000-200,000 of all new-born deaths/still births annually (Steketee *et al.,* 2001).

2.6 Immunity to malaria

Immunity to malaria develops with increasing exposure to infection and age in endemic areas (Doolan *et al.,* 2009). People in high transmission areas develop immunity faster than those in low transmission areas (Doolan *et al.,* 2009). It is developed as a means of protection against severe and life threatening forms of the disease by controlling the level of parasitaemia in the individual (Marsh *et al.,* 2006).

The groups most susceptible to complicated malaria infections include children under the age of five years and pregnant women (Guyatt and Snow, 2004; 2001). Even in the adult population, the level of immunity acquired is partial and short lived due to inconsistency in the malaria specific IgG responses against the parasite antigens. Also, antibodies fail to boost during reinfection due to hypo-responsiveness of the B cells and also require constant contact with some level of parasite. All of these shortcomings could also be as a result of the genetic variability in parasite and human host whereas the partial immunity in adults also (Weiss *et al.,* 2009; Greenwood, 1999) involves both the innate and adaptive immune responses.

Immunity to malaria is mediated by both the innate and acquired immune responses. The acquired response involves both the humoral and cell mediated immune responses through the production of antibodies and cytotoxicity by B and T cells respectively (Abbas *et al.,* 2010).

The immune response against IEs start when dendritic cells interact with IEs through Toll Like Receptors (TLR) and CD36 causing the release of IL-12 (Fig. 4). The IL12 serves as a paracrine cytokine and activates NK cells causing the release of IFN-γ and leading to the activation of macrophages to also interact with the IEs (Stevenson *et al.,* 2004). Activated macrophages secrete IL-12 which supports the differentiation of naïve T cells to release IL-2 to serve as an autocrine and paracrine cytokine to further

activate the NK cells. Macrophages also produce nitric oxide to aid in direct killing of the parasite and TNF to mediate the acute inflammation (Stevenson *et al.,* 2004). T cells also release TGF-β and IL-10 as well as activating B cells to produce Abs.

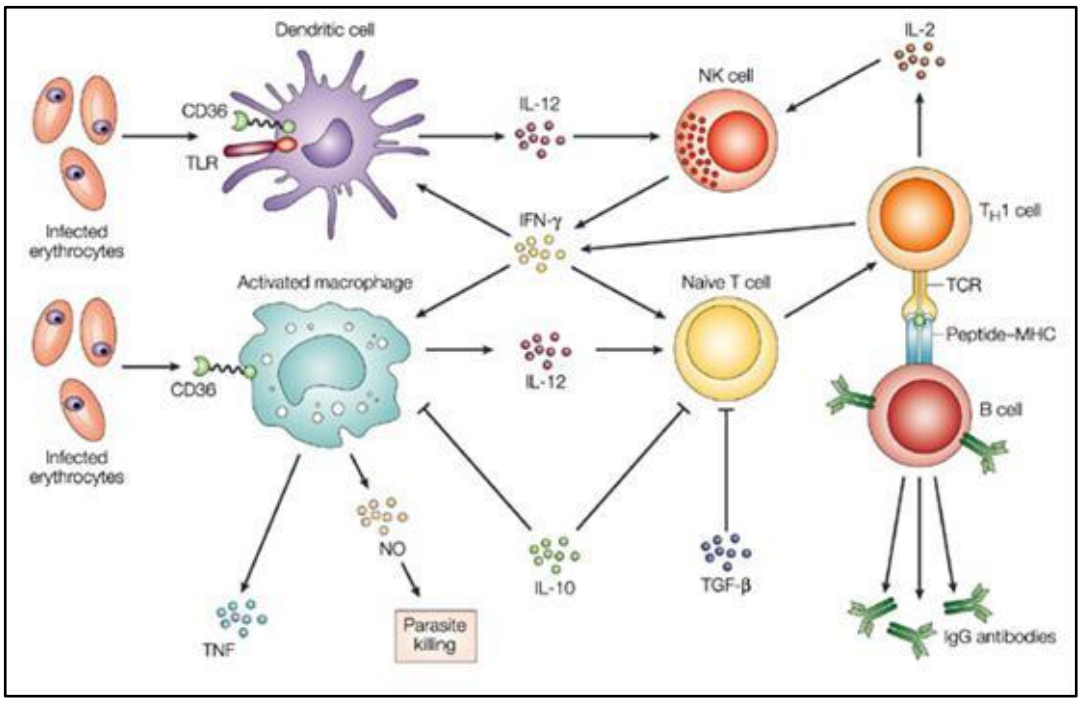


Fig. 2.4: Generation of immune response against IEs (Adapted from Stevenson *et al.,* 2004)

2.6.1 Innate immunity to malarial infection

The innate immune system is made up of a network of cellular and biochemical defence mechanisms that exist in an individual before the acquisition of an infection. Innate immune cells such as macrophages, dendritic cells, natural killer (NK) cells, NK T cells, neutrophils and γδ (gamma delta) T cells play an important role in parasite elimination and the nature of adaptive immune response elicited.

IEs susceptibility to cytotoxicity, parasite inhibition and phagocytosis is enhanced through opsonisation by complement components and antibodies. The phagocytic

activity causes the release of factors such as TNFα (Fig. 4), which may cause tissue lesions but which are also toxic for the parasites, to further mediate the inflammatory process. A study by Couper and colleagues were able to outline the importance of macrophages in blood stage parasite elimination in *P. yoelli* through the production of reactive oxygen intermediaries when activated by IFN-γ (Couper *et al.,* 2007). Also, Eugui *et al.,* (1980) experimentation in different mice strains detected increase phagocytic activity during *P. berghei* infection which corresponded with increase macrophage population, Similarly a study done during placental malaria also found corresponding increase in macrophage population in the intervillous space which is effective in the elimination of parasites (Rogerson *et al.,* 2007).

Another important cell in innate immunity are the NK cells which are mostly located in secondary lymphoid organs as well as places such as in the blood and the lungs. During malaria infection, their role involves the activation and recruitment of other cells and through the production of IL-8 NK cells are an early source of cytokines such as IFN- γ (Fig. 2. 4). Their activation also induces CD25 and CD69 expression as well as the production of CXCL8 to recruit neutrophils to the site of inflammation (Baratin *et al.,* 2005). Moreover a malaria study in infected mice found NK T cells to aid in the clonal expansion of B cells and antibody production (Hansen *et al.,* 2005). Likewise, a work in placental malaria recorded decrease NK cell population and cytotoxicity in primigravidae whilst multigravidae and non-pregnant individuals had increased NK cell cytoxicity (Sartlet *et al.,* 2005; Bouyou-Akotet *et al.,* 2004).

Also other genetic factors including the characteristics and features of the red blood cell such as the glycoproteins on its surface, the red cell metabolic enzyme Glucose6-Phosphate dehydrogenase (G-6-PD) deficiency, the sickle cell trait and type of thalassaemia may confer resistance to malaria in an individual (CDC, 2004). These

inherited traits subject the parasite to oxidative stress, increasing the chances of survival of the individual especially in children inheriting these traits (Allison *et al.,* 1983).

2.6.2 Adaptive immunity

The level of acquired immunity exhibited by an individual infected with malaria is determined by the place of residence of the individual as well as characteristics and the number of infectious bites. Adaptive immunity to malarial infection can be classified into three; anti-disease immunity, anti-parasite immunity and premunition (Doolan *et al.,* 2009; Marsh *et al.,* 1997). Anti-disease immunity protects against clinical disease, anti-parasite immunity protects against high parasitaemia, and premunition protects against new infections by maintaining a low-grade, asymptomatic parasitaemia, with a significant overlap between them (Doolan *et al.,* 2009).

A naïve individual infected with malaria develops acute illness with decreased levels of parasitaemia which may later develop to severe disease or death. Subsequent infections lead to the development of anti-disease immunity which decreases clinical symptoms despite high levels of parasitaemia. A study done by Ladeia-Andrade *et al.,* (2009), was able to establish that individuals with repeated and frequent exposure to malaria infections have a decreased risk of infection and disease which is acquired with increasing age. This suggests that, continuous exposure to malaria may induce anti-parasite and anti-disease immunity over a significant period of time. These levels of immunity acquired have been shown to cause a significant decrease in malaria associated morbidity and mortality (Doolan *et al.,* 2009). Also Riley *et al.,* (1990), were able to demonstrate that the level of parasites in Gambian children did not

decrease till the ages of 10 to 12 whereas at age 6, the incidence of clinical disease reached its highest. This study is therefore suggestive of the fact that anti-disease immunity occurs and reduces the high risks associated with the disease infection.

After numerous and frequent infections, anti-parasite immunity develops leading to very low levels of parasitaemia (Greenwood, 1999). The two main arms of adaptive immunity are the cell mediated and humoral immune response.

2.6.2.1. Cell mediated immunity

Parasite antigen specific T cell responses have been shown to be vital as an effector function in cell mediated immunity. Processed antigenic peptide presented with either MHC class I or MHC class II molecule by an antigen presenting cell is able to elicit the correct T cell response which may inhibit parasite growth or kill parasites (Good *et al.,* 1999). The main cells involved in cellular immunity are the αβ T- cells consisting of the CD4+ and CD8+ T cells. CD8+/ T cytotoxic cells are involved in direct killing of the parasite which helps to prevent or minimize infection and are very effective in the liver stages of the disease (Belnoue *et al.,* 2004; Good *et al.,* 1999). However, the direct activation of CD8+ cells in the erythrocytic stage of the disease may be limited since erythrocytes which harbour the parasites do not express MHC class I molecules. Lack of MHC class I molecules prevent antigen processing and presentation (Perlmann *et al.,* 1998). Unlike CD8+ cells, CD4+ cells have been demonstrated to be very functional in regulating blood stage immunity through the activation, differentiation and proliferation of B-cells to produce Abs through the production of Interleukin 4 (IL-4), IL-5 and IL-10 (Stephens *et al.,* 2005; Anstey *et al.,* 1996). They are also capable of causing the secretion of NO by macrophages to aid in direct killing of the IEs (Anstey *et al.,* 1996).

A study by Fievet and colleagues found levels of interleukin 2, interleukin 4 and interleukin 10 as well as T cell responses to be higher in multigravidae than in primigravidae and in postpartum malaria when they were stimulated with a CSA binding *P. falciparum* strain. Also they found an increase in the activity of interferon gamma (IFN-γ) cytokine activity (Fivet *et al.,* 2002; Fivet *et al.,* 1997). Increase in pro-inflammatory cytokines was found to be detrimental to pregnancies (Taylor Robinson *et al.,* 1999) whilst anti-inflammatory cytokines such as IL-10 was found to be increased in the intervillous spaces during placental malaria (Moore *et al.,* 1999).

Also T cell responses were found to be decreased especially in women in their first pregnancies. A research in pregnant women found increate Th1 response to correspond to increase maternal anaemia, spontaneous abortion and preterm delivery (Kwak-Kim *et al.,* 2005) whilst in normal successful pregnancies, increased Th2 responses were recorded (Wegmann *et al.,* 1993).

Regulatory T cells (Tregs) with the CD4+ and CD25+ molecular markers have been identified to regulate the immune system. An appropriate induction of these cells are needed to regulate the induced anti parasite response to inhibit excessive immune responses which are responsible for severe immunopathology associated with the infections (Hansen *et. al.,* 2010). In humans, transforming growth factor (TGFβ) production and CD4+CD25+FOXP3+ found on Treg cells have been associated with higher rates of *Plasmodium* growth in vivo, indicating that induction of Treg cells could represent a parasite specific virulence factor (Walther *et al.,* 2005). Also an increase in transforming growth factor beta has been recorded in asymptomatic individuals (Beeson *et al.,* 2008). However a decrease in Treg cell population has been found in malaria exposed individuals and individuals with acute uncomplicated malaria. A research work done on mice in 2005 and 2007 found Treg cells to modulate

cell mediated immunity during *P. falciparum infections* (Amante *et al.,* 2007; Vigario *et al.,* 2007).

2.6.2.2. Humoral immunity to malaria

The effector molecules of humoral immune response involve the production of antibodies by activated B lymphocytes. These antibodies are a complex group of glycoprotein, called Immunoglobulins (Igs) and can be found at the body's mucosal surfaces and plasma. There are five types of immunoglobulins which are distinguished by the type of heavy chain they possess. These immunoglobulins are IgG, IgA, IgM, IgD and IgE (Abbas *et al.,* 2010) and they bind with high specificity to the antigen that caused their production. They are involved in effector mechanisms/reactions such as neutralization and elimination of their antigens (Bloom *et al.,* 2003). The elimination of the different malaria antigens depends on the class of antibody or antibody isotype. The protective antibodies target *P. falciparum* antigens such as merozoite surface antigens, erythrocyte invasion ligands and variant surface antigens expressed on IEs (Good *et al.,* 2004).

Individuals living in endemic areas are able to elicit strong humoral response during malaria infection yielding the predominant production of IgM and IgG. Humoral immunity in *P. falciparum* infection was first demonstrated in the study involving passive transfer of antibodies from malaria immune adults to fully treat children with complicated malaria (Bouharoun *et al.,* 1990; Cohen *et al.,* 1961). This also led to other earlier studies reporting that some *P. falciparum* antigens (such as MSP1Merozoite Surface Antigen 1) were found in all *P. falciparum* parasites worldwide (Dodoo *et al.,* 1999; Riley *et al.,* 1992).

Elevated levels of non-plasmodium specific IgE have been demonstrated in human and experimental malaria infections (Troye-Blomberg *et al.,* 1999; Perlmann *et al.,* 1994) but with negative correlation in placental malaria (Desowitz *et al.,* 1993; Maeno *et al.,* 1993) as well as haemoglobin and platelet levels (Maeno *et al.,* 2000). A study by Duarte *et al.,* (2007), showed increased functional activity of IgE in uncomplicated malaria patients. However, the role of malaria specific IgE seems to be in controversy as to either they are involved in protection from malaria or enhances pathogenesis of malaria infection. Immunoglobulin E levels have been found to correlate positively with age (Desowitz *et al.,* 1993; Maeno *et al.,* 1993) and also with increased concentrations in uncomplicated malaria (Farouk *et al.,* 2005; Farnert, 2004).

In another study, high levels of IgE were demonstrated in individuals diagnosed with severe malaria than in those diagnosed with uncomplicated malaria (Perlmann *et al.,* 1994). In cerebral malaria patients, deposits of IgE were found in the brain micro vessels and IEs (Maeno *et al.,* 1993). There is also cellular activation and liberation of tumour necrosis factor (TNF) when IgE forms immuno-complexes with IgG inducing Fcε receptors expression on mononuclear phagocytes such as monocytes (Perlmann *et al.,* 1999; Elghazali *et al.,* 1997). These receptors upon interaction with the immuno-complexes liberate the cytokine which may provide protection but with over production causes tissue damage (Perlmann *et al.,* 1999).

2.6.2.2.1 Immunoglobulin G (IgG) and placental malaria

Recent data has reported increased levels of VSA_{PAM} –specific IgG in response to *P. falciparum* infection in pregnancy. The IgG levels has also been correlated positively to the inhibition of infected erythrocytes to CSA (Beeson *et al.,* 2004; Staalsoe *et al.,*

2001; Ricke *et al.,* 2000), increased haemoglobin levels and increased birth weight in new-borns (Salanti *et al.,* 2004; Staalsoe *et al.,* 2004). Of the IgG produced in pregnancy-associated malaria, IgG1 is the most dominant and acquisition of immunity to PAM is parity dependent and sex specific (Brabin *et al.,* 1983). Serum IgG from primigravidae and men have been reported not to inhibit infected erythrocytes binding to CSA whilst serum from malaria exposed multigravidae are able to inhibit IE binding to CSA (Fried *et al.,* 1998). Furthermore in the acquisition to immunity, VSA_{PAM} –specific IgG in primigravidae were detected around the 20th week in pregnancy in the mid of the second trimester. In contrast, VSA_{PAM} –specific IgG were found to appear earlier, rise faster in multigravidae (Beeson *et al.,* 1999; O'Neil-Dunne *et al.,* 2001).

2.6.2.2.2 Immunoglobulin M (IgM) and placental malaria

The second most abundant protein after albumin is the immunoglobulins which have IgM to be the first to be secreted during a primary immune response (Boes, 2000). IgM is therefore the largest antibody in the circulatory system with a population of about 5-10% and with an average concentration of 1.5 mg/ml in the serum. It however has a higher population in females (about 25%) than in males (McGue *et al.,* 1990; Adinolfi *et al.,* 1978).

IgM may be present as membrane-bound molecules located on B cell surfaces or as a secreted form which is mainly found in the blood (Fellah *et al.,* 1992). Membrane bound IgM is a monomer whilst IgM secreted by plasma cells is a pentamer. Immune IgMs are produced by all other B cells whilst natural IgMs are produced by the long lived, self-renewing B1 cells found in pleural and peritoneal cavities (Hardy and Hayakawa, 1994). Natural IgMs mostly are encoded by the germ line V segments

with no somatic mutation and as result have low affinities and restricted repertoire but have broad specificities (Hardy and Hayakawa, 1994). The pentameric nature of secreted IgM enables its multivalent property (such as increased avidity) of binding to Ags (antigens) and receptors, therefore making it an important mediator of innate immunity (Burton, 1987). It also increases interactions with key ligands such as the complement component C1q. Therefore IgM is effective in complement mediated lysis and one IgM can be compared to a thousand IgGs when it comes to accomplishing a similar task (Czajkowsky *et al.,* 2009; Quartier *et al.,* 2005; Cooper, 1985).

Secreted IgM (sIgM) consists of two classes: an antigen-specific (or immune) IgM which is produced in response to a specific pathogen and a low-affinity, poly-reactive (natural or non-immune) IgM that is synthesized without previous exposure to any particular Ag (Ehrenstein *et al.,* 2010). These unique features of IgM, including its poly-reactivity, increases its capacity to promote the removal of apoptotic cells and enables participation in apparently diverse pathophysiological situations, such as in infection, B cell homeostasis, inflammation, atherosclerosis and autoimmunity (Ehrenstein *et al.,* 2010).

Recent studies have also shown that serum IgM is effective at neutralizing intracellular pathogens by preventing pathogen interaction with cells and also in priming IgG response following an infection. Some of the mechanisms employed by IgM in mediating protective immunity include complements and FcRs interaction with effector molecules (Czajkowsky *et al.,* 2010).

Natural, nonspecific IgM also has a significant role in human malaria since they have been found to bind to the surface of *Plasmodium falciparum*-IEs via the Fc receptors as the levels of IgM correlate with the severity of the disease. *P. falciparum* strains

that bind to nonspecific IgM have adhesion properties conveyed during rosetting (IT4var60) and CSA binding (VAR2CSA) (Barfod *et al.,* 2011; Ghumra *et al.,* 2008; Rowe *et al.,* 2002).

A recent model of human IgM has shown that IgM is a mushroom-shaped molecule, having Cμ3/Cμ4 domains forming the central region which protrudes out of the plane formed by the Cμ2/Fab domains. PfEMP1 has been identified to bind to residues in Cμ4 domain which serves as a binding pocket enhanced by the pentameric nature of IgM (Ghumra *et al.,* 2008). Also, binding of PfEMP1 to the domain causes IgM to retain its non-bound conformational form which does not promote complement activation (Barfod *et al.,* 2011; Czajkowsky *et al.,* 2010).

A recent study by Barfod *et al.,* (2011) found the masking of PfEMP1-specific IgG epitopes by nonspecific IgM. In their experiment they also realised that IgM binding to the VAR2CSA domains DBL3X and DBL5ε did not inhibit IE sequestration to CSA hence enabling the parasite to avoid the protective immunity acquired.

2.7 Antigenic diversity and immune evasion

Antigenic diversity is defined as the expression of antigenically different alleles of a gene in different *P. falciparum* populations to create allelic polymorphism (Ferreira *et al.,* 2004). *P. falciparum* antigenic diversity involves the expression of immunodominant epitopes with repeated sequence of short tandem arrays of its genome to which partially immune individuals have high titres of antibodies against (Reeder and Brown, 1996). Such tandem repeats include Circumsporozoite Protein (CSP) which is highly conserved and (Merozoite Surface Antigen) MSA which is highly variable. These tandem repeats have been noted to be highly involved in

immune evasion since it suppresses antibody response against adjacent antigen (Reeder and Brown, 1991; Schofield, 1991).

Another method of immune evasion is antigenic variation involving the switching of antigenic phenotypes leading mostly to chronic infections with successive parasitaemia waves and also serving as the principal cause for functional diversity (Ferreira *et al.,* 2004; Reeder and Brown, 1996). Most antigenic variations are linked to the surface proteins on the IE such as PfEMP-1 (*Plasmodium falciparum* Erythrocyte Membrane Protein-1) and Merozoite Surface Protein (MSP) (Reeder and Brown, 1996).

One major surface protein ascribed with immune evasion is the PfEMP-1 family of antigens. PfEMP-1 enables IEs to escape the bodies defensive mechanism by sequestrating to host endothelial molecules such as ICAM-1, Platelet Endothelial Cell Adhesion Molecule (PECAM), CD36, CSA and hyaluronic acid (Udomsangpetch *et al.,* 1997; Berendt *et al.,* 1989; Ockenhouse *et al.,* 1989). Also PfEMP-1 can bind to dendritic cells to down-regulate the immune system as well as reducing the efficiency of the immune response (Bir *et al.,* 2006). The well characterised genes encoding the PfEMP1 genes are by the variant surface antigens (var genes).

2.7.1 Variant surface antigens expressed on infected erythrocytes

Var genes are a large variant of highly conserved genes containing DBL domains and with different expressions results in antigenic variation. They also relate to the erythrocyte binding antigen (EBA-175) and to the Duffy Antigens of *P. vivax* and *P. knowlesi* (Su *et al.,* 1995; Sim *et al.,* 1994).

Each individual parasite possesses about 60 of the var genes but expresses a single var gene at a time. The expression of a single variant enables the parasite to maintain the

remaining *var* genes found in its genome in a transcriptionally silent state (Guitard *et al.,* 2008; Smith *et al.,* 1995). These var genes also encode PfEMP1. The genes consist of two exons with exon 1 coding for the variable extracellular domain and the trans membrane region whilst exon 2 codes for the highly conserved acidic terminal domain which anchors PfEMP1 at knobs (Horrocks *et al.,* 2005; Su *et al.,* 1995).

Mostly, var genes are located on the sub telomeric loci of *P falciparum*. Apart from the var genes, there are four other distinct antigens on the loci of *P. falciparum* and these are the rifin, stevor, *P. falciparum* Maurer's Cleft-2 trans membrane (PfMC-2TM) and Surfin (Fig. 2.5) (Kyes *et al.,* 2000).

Repetitive Interspersed Family Proteins (RIFINs) consists of over 200 rif genes in a *P. falciparum* haploid genome and are transcribed by immature trophozoites for a shorter period. They are the second family of clonally variant proteins encoded by the parasite on IEs surfaces with a molecular weight of about 45kDa and about 17kDa variable domain (Kyes *et al.,* 2000). They have no known function in cytoadherence even though they are found in close proximity to the var genes (Kyes *et al.,* 2000). The Rifins, Stevor and PfMC-2TM make up the two trans membrane type supergene families. These surfaced exposed antigens/variants are also determinants in the level of clinical immunity acquired to malaria in Africa (AbdelLatif *et al.,* 2003).

The other group of antigens apart from the *rif* genes are the Sub telomeric Variant Open Reading Frame (Stevor). Stevor genes unlike the rifins are transcribed by mature trophozoites, sporozoites and gametocytes for a short period of time and more conserved among parasite strains (Lavazec *et al.,* 2007). They consist of over 30 genes coding for about 30 kDa proteins with about 12kDa variable domains (Blythe *et al.,* 2004). The *rif* and stevor antigens are located within the *P. falciparum's* Maurer's cleft in the cytoplasm of the IE (Kaviratne *et al.,* 2002).

The PfMC-2TM has a molecular weight of about 27kDa and a variable domain of about 1.7kDa. It has no known function yet undergoes clonal variation (Abdel-Latif *et al.,* 2003).

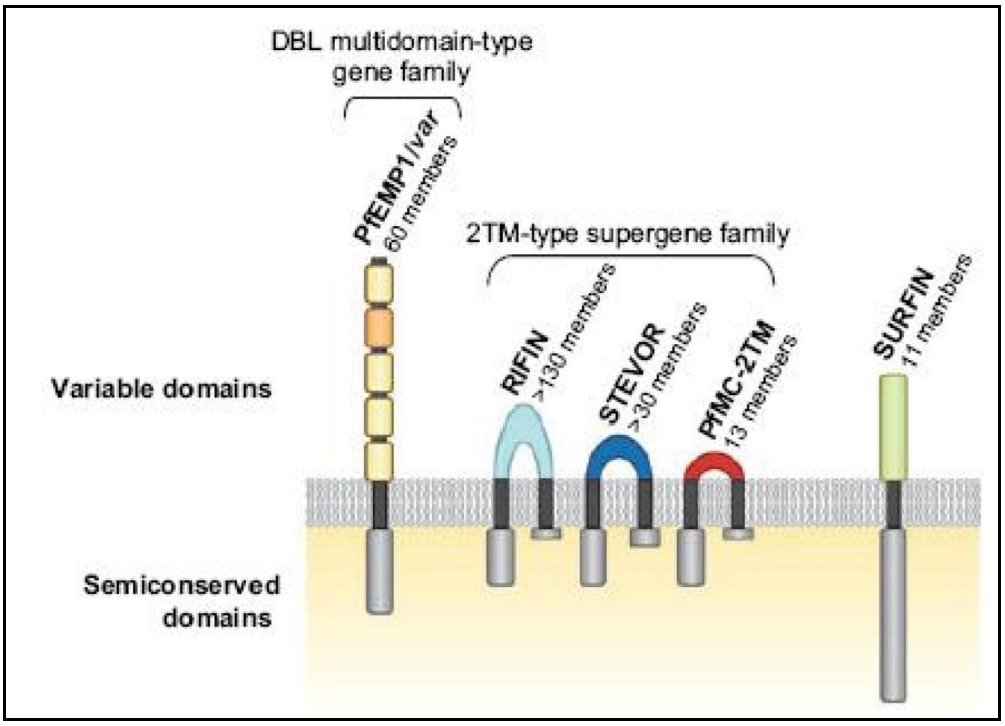


Fig. 2.5: Variant Antigens on the surface of infected erythrocytes (Adapted from Scherf *et al.,* 2008)

2.7.2 *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1)

The protozoan parasite, *Plasmodium falciparum*, causes the deadliest form of human malaria that affects millions worldwide every year. The virulent nature of *P. falciparum* is attributed to its ability to evade the human immune system by modifying infected host erythrocytes and directly mediating adhesion to a variety of host cells and also undergoing antigenic variation (Horrocks *et al.,* 2005). A key virulence factor for *Plasmodium falciparum* is *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is expressed on the surface of infected

erythrocytes (IEs). Structurally, PfEMP1 protein family consist of an N-terminal segment (NTS), Duffy binding-like (DBL), Cysteine-rich inter domain region (CIDR), C2, trans membrane (TM), and acidic terminal segment (ATS or exon2) grouped into different types (Nelson *et al.,* 2007) but the common domains in PfEMP1 are the NTS, CIDR, C2 and the DBL (Smith *et al*., 2000) Fig. 2.6. The NTS is located at the amino terminus of PfEMP1 and consists of 75-107 amino acids. Its shape is predicted to be globular alpha helical fold. In contrast the C2 domain consists of 140-217 amino acids and binds to Intracellular adhesion molecule-1 (ICAM-1) (Smith *et al*., 2000). It has also been predicted to have an alpha helical structure (Smith *et al*., 2000). The DBL domains are however are found in erythrocyte binding antigen (EBA) as well as in PfEMP1. In the EBA, it is used for parasite invasion of the red blood cells which is an essential property of all the *Plasmodium spp* whilst sequestration by the DBL is a property specific to only *P. falciparum* (Smith *et al*., 2000).

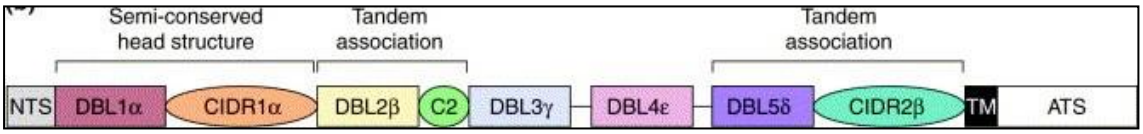


Fig. 2.6 A structure of the PfEMPI protein coded by var genes (Adapted from Smith *et al.,* 2001)

A number of other parasite-encoded proteins are similarly exported to the IE membrane and play an indirect role in this adhesion process through the modification of the erythrocyte cytoskeleton and the formation of electron dense knobs into which PfEMP1 is anchored **(**Horrocks *et al.,* 2005; Su *et al*., 1995). These knobs consisting

mainly of knob-associated histidine rich protein (KAHRP) binds to PfEMP1 ATS (Rug *et al.,* 2006).

PfEMP1 enables the adhesion of infected erythrocyte to various host receptors such as CD36, Intercellular adhesion molecule-1 (ICAM-1), Vascular adhesion molecule1 (VCAM-1) and chondroitin sulphate A (CSA) (Baruch *et al.,* 1996) during cytoadhesion to endothelium. PfEMP1 variants such as IT4var60 containing domains such as DBL1α located in the N-terminal end has been implicated in severe malaria by enhancing rosetting between IEs and non-infected erythrocytes. They are able to bind to heparan sulphate proteoglycan (Barragan *et al.,* 2000; Chen *et al.,* 2000; Chen *et al.,* 1998) and CR1/CD35 (a regulatory protein for complements) on non-infected RBCs (Khera *et al.,* 2009; Rowe *et al.,* 2000; Rowe *et al.,* 1997) and the rosetting IEs are able to interact with the C3b-binding domain on the CR1 (Rowe *et al.,* 2000).

As the antibody response against the single expressed PfEMP1 develops, small subpopulations of parasites switch expression to alternative forms of PfEMP1 and re-establish the infection (Smith *et al.,* 1995).The different binding phenotypes cause sequestration in different organs and contribute to life threatening manifestations of the disease. This process is referred to as antigenic variation and is responsible for the persistent nature of the disease, as well as the waves of parasitaemia, typical for *P. falciparum* infections (Miller *et al.,* 1994). This has therefore established PfEMP1 as a key player in the pathogenicity of *P. falciparum* (Pasternak *et al.,* 2008).

Nonspecific immunoglobulin M (IgM) antibodies binding to infected erythrocytes (Creasey *et al.,* 2003) have been observed on rosetting parasites (Rowe *et al.,* 2002; Scholander *et al.,* 1996) to be mediated by PfEMP1 (Chen *et al.,* 2000) and may also be involved in binding on CSA binding parasites (Barfod *et al.,* 2011; Creasey *et al.,* 2003). In the case of rosetting, it has been suggested that IgM could act as a bridge

between infected and uninfected erythrocytes to strengthen the rosettes (Scholander *et al.,* 1996).

2.7.3 VAR2CSA as a vaccine candidate

VAR2CSA is a polymorphic protein of approximately 3,000 amino acids made up of three Duffy-binding-like type X (DBL-X) domains and three DBL epsilon (ε) domains (Smith *et al.,* 2000). This var gene unlike other var genes lacks a DBL-α, CIDR and DBL-γ domains which were first thought to be involved in CSA binding (Salanti *et al.,* 2003). PfEMP1 encoded VAR2CSA has low similarity with other PfEMP1s and has its transcription upregulated in CSA-binding parasites (Salanti *et al.,* 2003). It has a two-exon structure (Su *et al.,* 1995) in which the first exon is the larger (~3.5 to 9.0 kb) and the second exon is smaller (~1.0 to 1.5 kb), and codes for a more conserved cytoplasmic tail (Su *et al.,* 1995).

Mostly variant surface antigens are located on the sub telomeric region of most chromosomes and they occur in duplicates (Fischer *et al.,* 1997). For instance the VAR2CSA PFL0030c has its duplicate being a pseudo-gene (MAL13P1.354) situated at opposite ends of chromosome 13 having no effect on antigenic variation nor adhesive properties of *P. falciparum* (Flick *et al.,* 2004, Salanti *et al.,* 2003).

PfEMP1 encoded by PFL0030c has low similarity with other PfEMP1s and has its transcription up regulated in CSA-binding parasites (Salanti *et al.,* 2003). Also IT4var60 has the ability to cytoadhere after *in vitro* adaptation (Bourke *et al.,* 1996; Day *et al.,* 1993; Biggs *et al.,* 1992; Roberts *et al.,* 1992). It is found on chromosome 12 and unlike PFL0030c, functions in rosetting and belongs to the group A1 types of var based on the transcriptional orientation (Nelson *et al.,* 2007).

VAR2CSA has been considered a prominent vaccine candidate owing to a number of reasons. Firstly, its transcription and expression is markedly up regulated in *P. falciparum* in PAM (Tuikue Ndam *et al.,* 2005). Again, antibodies against it have been found to be specifically induced during pregnancy (Salanti *et al.,* 2004; Staalsoe *et al.,* 2004). In addition, women with high levels of these antibodies are protected against some of the serious complications of PAM (Fried *et al.,* 1998) whilst Salanti *et al.,* (2003) has also shown VAR2CSA to be highly conserved between parasite genomes. VSA_{PAM}-expressing IEs have been shown to have a generally increased reactivity with IgG and IgM (Creasey *et al.,* 2003). Also 3D7 *VAR2CSA*-specific IgG have been detected in the plasma of pregnant women from both West and East Africa. The fact that levels of VAR2CSA-specific IgG correlated with sex and parity in plasma from *P. falciparum*–exposed individuals is consistent with the theory that PAM develops in otherwise malaria-immune women who do not have specific protective immunity against parasites binding to CSA (Salanti *et al.,* 2004). In addition, CSA binding infected erythrocytes have been shown to bind non-specifically to IgM, a process that may facilitate placental adhesion or promote immune evasion (Barfod *et al.,* 2011; Creasey *et al.,* 2003).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study design

This was a cross-sectional study involving pregnant women attending ante-natal clinics at the Asutsuare Health Centre. It was part of a bigger study titled "Mother-child Health in Southern Ghana: Malaria in pregnancy and in childhood" involving 820 pregnant women. In this study, a subset of 109 pregnant women were randomly selected and constituted the study population

3.1.1 Inclusion and exclusion criteria

All pregnant women attending antenatal clinic at the Asutsuare Health centre and consented to participate were included. All the pregnant women who refused to sign the consent form were excluded likewise all those who were not sure they will continue their monthly antenatal visits at the centre.

3.1.2 Study area and population

The study was conducted in the Dangme West district of the Greater Accra Region with Dodowa as the district capital. It has a population of 122836 according to the Ghana Statistical Service in 2010. It has a landmark area of 1.522km^2 bounded by the Dangme East district, parts of Volta and Aburi in the Eastern Region of Ghana.

The inhabitants of the area are mostly peasant fishermen, rice and food crop farmers. There are four sub districts including Dodowa, Osudoku, Pampram, and Ningo with each having a Health Centre. The Osudoku sub-district where the study was

undertaken has Asutware to be its capital. Asutware owns one of the four Health Centres found in each sub-district. Participants for the study were recruited from the Osudoku Health Centre located in the forest area. The region has stable malaria transmission with *P. falciparum* infection constituting 98% of all infections (Afari *et al.,* 1995).

3.1.3 Blood sampling strategy

After obtaining informed consent from the participants, about 4.5ml venous blood was taken from each participant into a heparinised vacutainer tubes. The blood was transported from the field to the laboratory where they were centrifuged at 2000rpm for 10 minutes. After centrifugation the plasma was collected and placed in cryotubes and stored at -80^{o}C until ready to be used.

3.1.4 Blood film preparation

Both thick and blood films were also prepared for each sample collected for parasite identification. The glass slides were soaked in 70% ethanol, cleaned well with cotton before the films were prepared then allowed to dry, and the thin film fixed in methanol. After drying the film was stained with 2.0% Giemsa (BDH Laboratory Supplies, Poole BH15 LTD, England), allowed to stain for10 minutes, washed with water, dried and viewed under the microscope (Olympus BH2 Microscope) at 100X magnification with oil immersion. The parasitaemia, species type and growth stages of the parasites were recorded

3.1.5 Blood grouping determination of the samples

Direct blood grouping technique was done using the principle of agglutination. A clean microscope slide was marked into three equal parts using a wax pencil. One drop each of the commercial anti A serum was added to one part, commercial anti B serum to the other part and a drop of commercial anti Rhesus factor was added to the last marked area. A small drop of well mixed blood was placed on each side of the slide using an applicator stick. The blood sample was each mixed with corresponding anti-serum or Rhesus factor using a clean applicator. The slide was rocked gently for about three minutes and observed under light to check for agglutination. It was recorded positive if there was agglutination and negative for no agglutination. This helped to group the samples into blood groups: A positive (+)/A-, AB+/AB-, B+/B- and O+/O-.

3.2 Purification of Plasma

Plasma samples were purified and grouped into three, according to the procedure described in **3.2.1** and **3.2.2** containing; only IgG, only IgM and non-purified plasma.

3.2.1 Purification of IgG from Plasma using Dynabeads® Protein G

The Dynabeads Protein G (Invitrogen, USA) was washed by re-suspending thoroughly to obtain a homogenous suspension. The desired volume of Dynabeads® Protein G was transferred to an Eppendorf tube at room temperature. The beads were washed by placing the tube on the magnet for 1 minute and the supernatant discarded by aspiration with a pipette while the tube still remained on the magnet. It was then removed from the magnet and 0.5 ml of Citrate-Phosphate Buffer, pH 5.0 was added

to re-suspend the Dynabeads® Protein G. the washing procedure was repeated to condition the beads before the addition of sample.

For capturing the IgG immunoglobulins, 100µl sample containing the immunoglobulins was added to the washed Dynabeads Protein G. It was then incubated with gentle mixing for 40 minutes at room temperature. The test tube was placed on the magnet for 2 minutes and the supernatant was discarded. The test tube was removed from the magnet and 0.5 ml Citrate-Phosphate Buffer, pH 5.0 was again added to the beads. The Dynabeads® Protein G-immunoglobulin complex was again washed to remove the non-bound proteins. The washing procedure was repeated by the addition of 0.5 ml Citrate-Phosphate Buffer, pH 5.0 to the complex.

The test tube was placed on the magnet for 2 minutes and supernatant was discarded.

In eluting the captured immunoglobulin, 30µl of 0.1 M citrate (pH 2-3) was added to the Dynabeads Protein G-IgG complex and mixed well by tilting and rotation for 2 minutes. The test tube was placed on the magnet for 1 minute and supernatant containing purified immunoglobulins transferred to a new tube. The above steps were repeated in order to elute any remaining immunoglobulins. The supernatants containing the pure immunoglobulins were pooled giving a total volume of 60 µl. the purified IgG was stored in Eppendorf tubes at -20°C until ready to be used.

3.2.2 IgM Separation from Plasma using Mannan Binding Protein

For the column preparation, the immobilized Mannan Binding Protein Column was set up and the storage solutions drained. The column was washed with 5ml of the column preparation Buffer. The immobilized Mannan Binding Protein Column, binding buffer and sample were kept at 4°C as instructed by manufacturer. For the binding of the immunoglobulin to the Mannan Binding Protein, a 20ml of IgM

binding buffer was added to the column and allowed to drain through. Then 500 µl of plasma was diluted in 500µl of the IgM Binding buffer in an Eppendorf tube. The diluted sample was then applied to the immobilized Mannan Binding Protein and allowed to completely enter the gel. Another 500µl of the binding buffer was added to the column and incubated at for30 minutes at 4°C. after incubation, the Mannan Binding Protein was washed with 42ml of Binding buffer to remove unbound proteins. A 3ml volume fraction was collected to check for their absorbance. Then fractions with absorbance >0.1 were pooled and reapplied to the column to increase yield. In eluting the bound IgM, the column was brought to room temperature and 3ml of elution buffer was added to the column, capped and incubated for 2 hours. Eluates were collected and 0.5ml fractions were collected and their absorbance checked.

Absorbance measurements ≥ 0.02 were pooled and dialysed at 4°C with plain PBS. PBS was changed every after every 2 hours and left overnight. After dialysis, the purified IgMs were stored in labelled Eppendorf tubes with glycerol at -20°C until ready to be used.

3.3. Measurement of IgG levels using Indirect ELISA method

Nunc maxisorp 96 well microtitre plates (Thermoscientific, USA) were coated with 100µl/well of the respective VAR2CSA/IT4var60 protein constructs (3D7 DBL1; 3D7 DBL5; FCR3 DBL5e; FCR3 DBL1α) at a concentration of 1µg/ml. The plates were sealed and incubated overnight at 4^{O}C. The next morning, the plates were brought to room temperature and washed 4 times with plain Phosphate Buffered Saline (PBS) /0.05% Tween 20 and then blocked with 5% non-fat dry milk in plain PBS at 200µl/well for 1 hour at room temperature. After incubation, the plates were washed again 4 times and padded dry on a paper towel.

The samples were then diluted in blocking buffer. The purified IgG samples were diluted in a dilution rate of 1:50 whereas the plasma samples were diluted 1:5000. They were added at 100µl/well and incubated at room temperature for 2 hours at room temperature. Plates were washed 4 times after incubation and padded dry on a paper towel. A goat anti-human IgG secondary antibody conjugated with HRP (Horse radish peroxidase) (Invitrogen, USA) was diluted in sample diluent (1:1000) and added at 100µl/well and incubated for 1 hour. The substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Thermoscientific, USA) was added and incubated for 20 minutes. The reaction was stopped with 0.2 M sulphuric acid at 100µl/well and the plates read with an automated ELISA plate reader (Biotek, USA) at 450nm wavelength. The optical densities were converted to concentration using the ADAMSEL software programme.

3.4 Measurement of IgM levels using Indirect ELISA method

Nunc maxisorp 96 well microtitre plates (Thermoscientific, USA) were also coated with 100µl/well of the respective VAR2CSA/IT4var60 protein constructs (3D7 DBL1; 3D7 DBL5FCR3 DBL5e; FCR3 DBL1α) at 1µg/ml concentration. The plates were sealed and incubated at 4^{O}C overnight. Plates were brought to room temperature and washed 4 times with plain PBS (phosphate buffered saline)/0.05% Tween 20. After washing, they were padded dry on clean paper towel. They were then blocked with 5% non- fat dry milk in plain PBS at 200µl/well and incubated for 1 hour at room temperature in an incubation chamber. After incubation the washing procedure was repeated.

Purified samples were diluted containing IgM were diluted in 1:50 with blocking buffer whereas the plasma samples were diluted in 1:5000. The samples were added at

100µl/well and incubated at room temperature for 2 hours in an incubating chamber. Plates were washed 4 times after incubation and padded dry on a paper towel. A goat anti-human IgM secondary antibody conjugated with HRP (Horse radish peroxidase) (Invitrogen, USA) was diluted in sample diluent (1:1000) and added at 100 µl / well. It was incubated for 1 hour at room temperature and washed after incubation. The ELISA substrate, 3,3′,5,5′-tetramethylbenzidine (TMB) (Thermoscientific, USA) was added to develop the end results and incubated for 20 minutes at room temperature. The reaction was later stopped with 0.2 M sulphuric acid at 100 µl / well. The plates were read with an automate ELISA reader (Biotek, USA) at 450nm wavelength to determine the optical densities. The optical densities were converted to concentration using ADAMSEL program software before statistical analysis.

3.4 Statistical Analysis

Screen data were analyzed using SPSS (Statistical Product and Service Solutions) software. Mann-Whitney test for nonparametric data was used to test for significance for the antibody reactivity to the domains. To determine if there was any significance on the effect of gestation on the antibody reactivity to the various domains the Kruskal-wallis one way analysis of variance for nonparametric data was used. Likewise, linear regression was used to determine the association between the ABO blood group phenotypes to the DBL domains. To determine the relationship between antibody responses to the various domain pairs the Coefficient of correlation was used. A significant level of p value less than 0.05 ($p<0.05$) was considered significant for all data analyzed.

CHAPTER FOUR

4.0 RESULTS AND STATISTICAL ANALYSIS

4.1 Characteristics of the study sample

Out of 109 samples randomly selected for this study, 23 were primigravidae with 86 of them being multigravidae, constituting 21% and 79% respectively (Table 4.1). Among the primigravidae, 13% had malaria parasites whilst 2.3% of the multigravidae had malaria parasites (Fig. 4.1a). Furthermore, the highest ABO blood group phenotype observed in the study samples was blood group O with 44% of the study samples belonging to the group. The blood group B phenotype was the second highest representing 34% whilst blood group A was found in 20% of the study sample. Blood group AB was the least with only 4% of the study sample belonging to that group (Fig. 4.1b).

Table 4.1- Characteristics of the study sample

Parity	Number of study samples n (%)	Mean age (range) in years
Primigravidae	23 (21)	21 (16-28)
Multigravidae	86 (79)	28 (17-40)

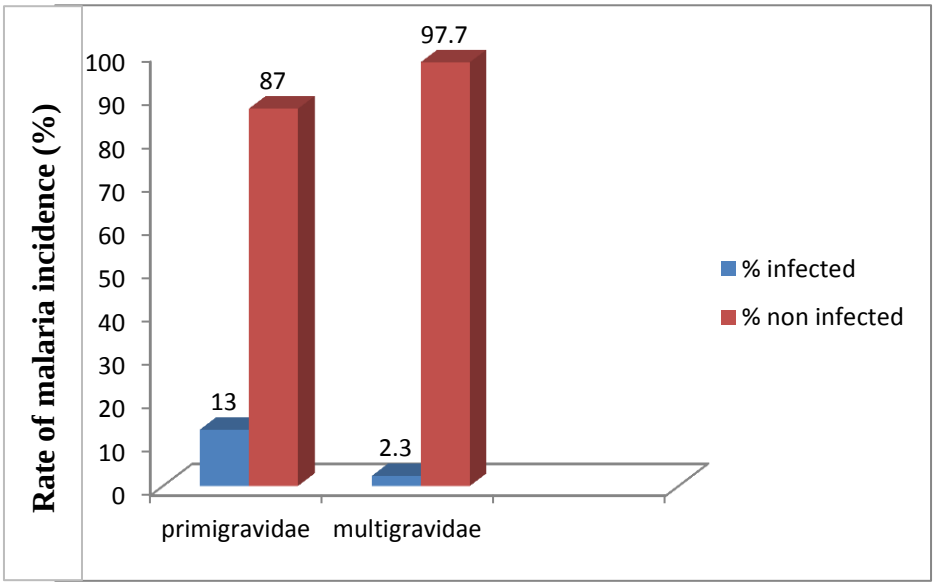


Fig. 4.1a: A bar chart showing the percentage of malaria infection in primigravidae and multigravidae.

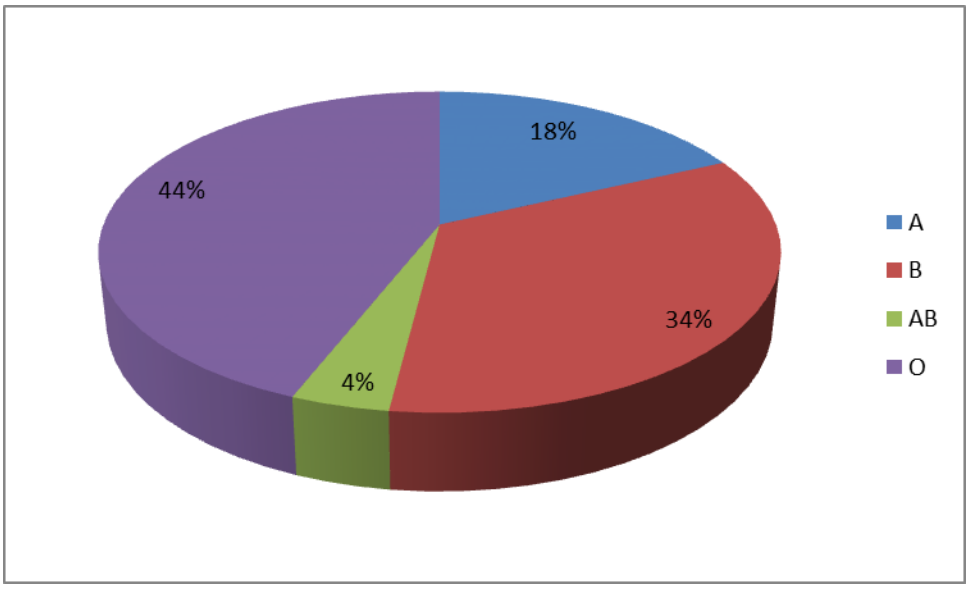


Fig. 4.1b: A pie chart showing the distribution of the ABO phenotypes of the study sample

4.2 Antibody reactivity to the IT4VAR60 DBL1α domain

To determine whether there was a significant difference between the reactivity of the antibodies to the domain, the responses of the various purified antibodies were compared to their corresponding non-purified antibodies. The levels of purified antibodies raised against the IT4VAR60 DBL1α (control antigen) domain were found to be lower when compared to the levels of plasma antibodies raised against the same domain (Fig. 4.2). A significant difference was observed between the reactivity of IgG purified from the plasma samples (392.6ng/ml) and the reactivity of IgG measured directly from same plasma (11429.6ng/ml) ($p<0.0001$). Similarly, the plasma IgM was also found to have a higher reactivity (40216.4ng/ml) to the DBL1α than its corresponding purified IgM (1632.4ng/ml), yielding a higher statistical significance value of $p<0.0001$ (Fig. 4.2).

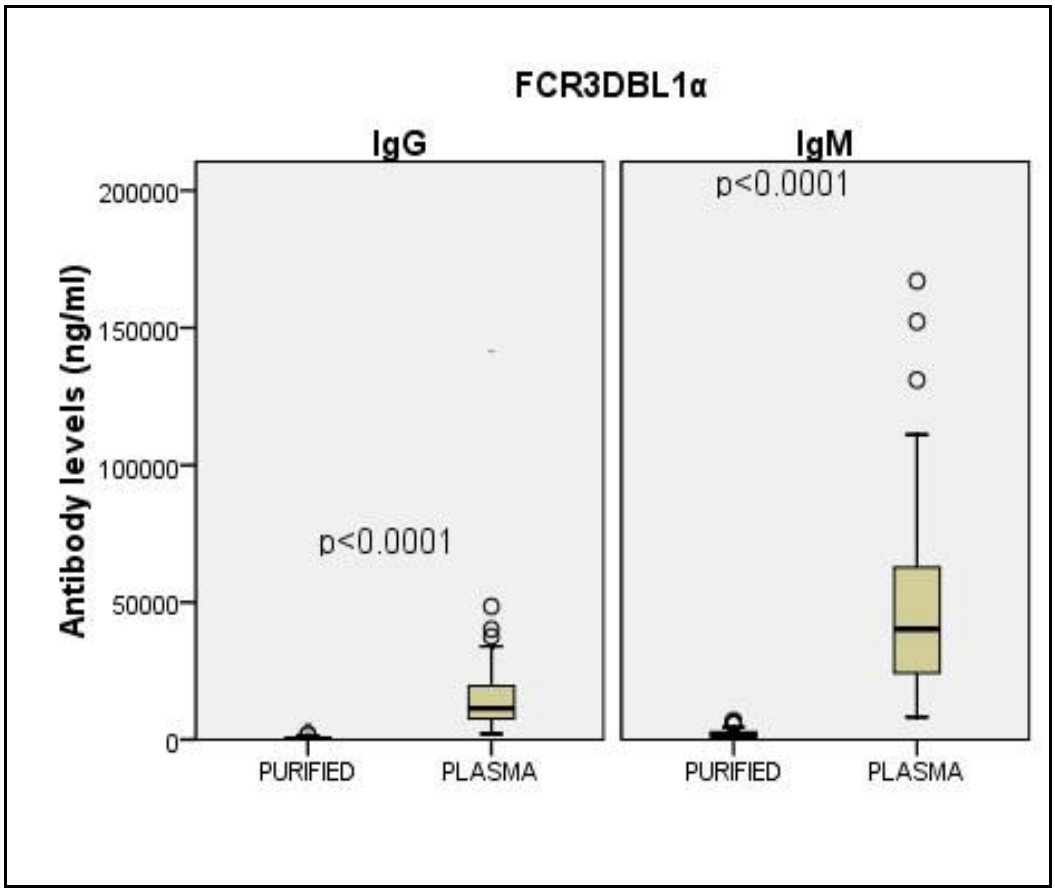


Fig. 4.2: Purified and plasma samples antibody reactivity to the IT4var60 DBL1α domain

4.3 Antibody reactivity to the VAR2CSA DBL1-X domain

The reactivity of IgM purified from the plasma samples against the VAR2CSA DBL1-X (test antigen) domain were found to be lower (2118.5ng/ml) when compared to the IgM responses measured directly from plasma (20837.7ng/ml) giving a statistically significant difference between the two immunoglobulins ($p<0.0001$).

Correspondingly, the IgG levels in the plasma samples were also found to be higher to the IgG levels purified from the same plasma to the DBL 1-X. The median IgG level in plasma was (28,108ng/ml) and that of the IgG measured from the purified sample level was (299.8ng/ml) (Fig. 4.3) and the difference was statistically significant ($p<0.0001$) using the Mann-Whitney test. The difference between the IgGs and IgMs measured in plasma were also statistically significant ($p=0.018$).

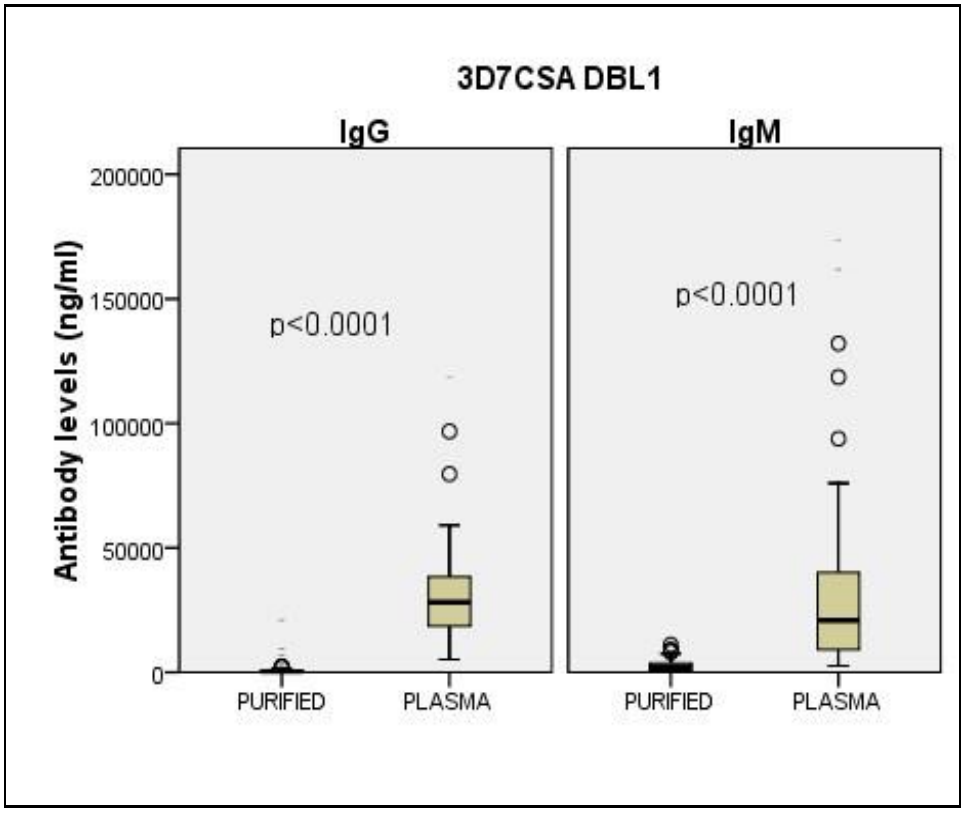


Fig. 4.3: Purified and plasma samples antibody reactivity to the VAR2CSA DBL1 domain

4.4 Antibody reactivity to the IT4VAR60 DBL5ε domain

Increased plasma antibody levels were observed against the DBL5ε domain (Fig. 4.4). The median reactivity of the IgG purified from the plasma samples was found to be 344.7ng/ml, significantly lower to the corresponding reactivity of the IgG measured from the plasma samples (33635.3ng/ml) ($p<0.0001$). Similarly, there was significant difference between the reactivity of the IgM measured from plasma (43144.6ng/ml) and that purified from the same plasma (620.6ng/ml) with $p<0.0001$. In comparison to the test samples, the naïve samples recorded lower antibody reactivity to the DBL5ε for both IgG (0.046ng/ml) and IgM levels in the naïve samples (0.0029ng/ml).

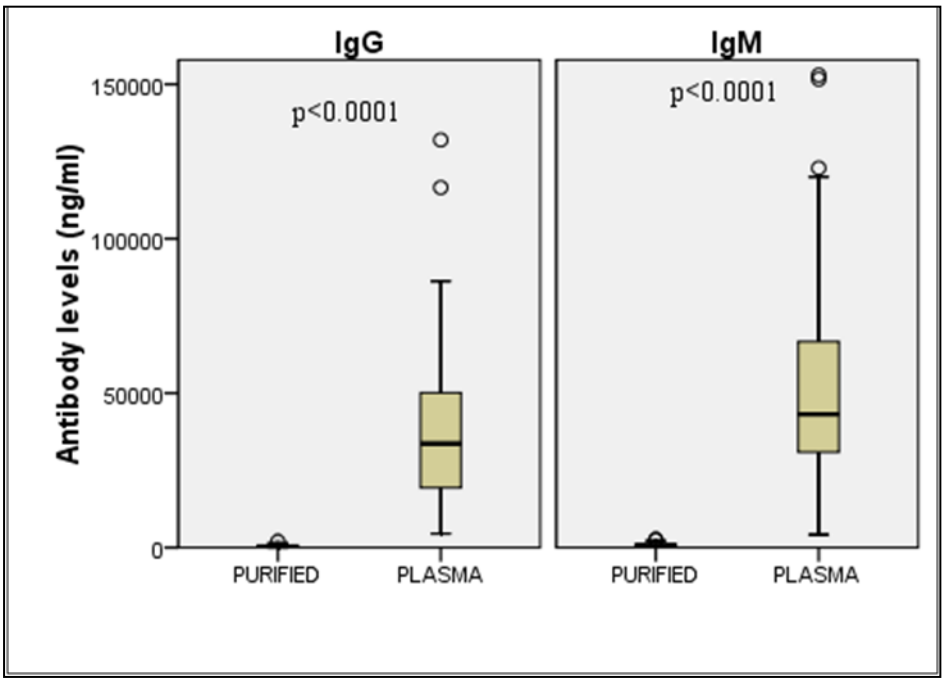


Fig. 4.4: Purified and plasma samples antibody reactivity to the IT4var60 DBL5ε domain

4.5 Reactivity of antibody to the VAR2CSA DBL5 domain

To determine whether there was a significant difference between the reactivity of the various antibodies to the DBL5, responses of the various purified antibodies were compared to their corresponding non-purified antibodies. The levels of purified IgG raised against the domain were found to be lower to the levels of IgG measured directly from plasma (Fig. 4.5). There was significant difference between the reactivity of IgG purified from the plasma samples (697ng/ml) and the reactivity of IgG measured directly from plasma (13013.2ng/ml) ($p<0.0001$). However, the levels of IgM measured from the non-purified samples were found to be lower (3968.4ng/ml) than the levels of IgM purified from the same plasma (4345.5ng/ml), yet there was no statistical significance between the antibody responses ($p>0.05$). (Fig.4.5). Likewise the differences between the reactivity of the IgG and IgM measured in plasma were also statistically significant ($p=0.0001$). Notwithstanding, the levels of IgG (0.021ng/ml) and IgM (0.002ng/ml) in the negative control samples were lower than those measured from the pregnant women donor samples.

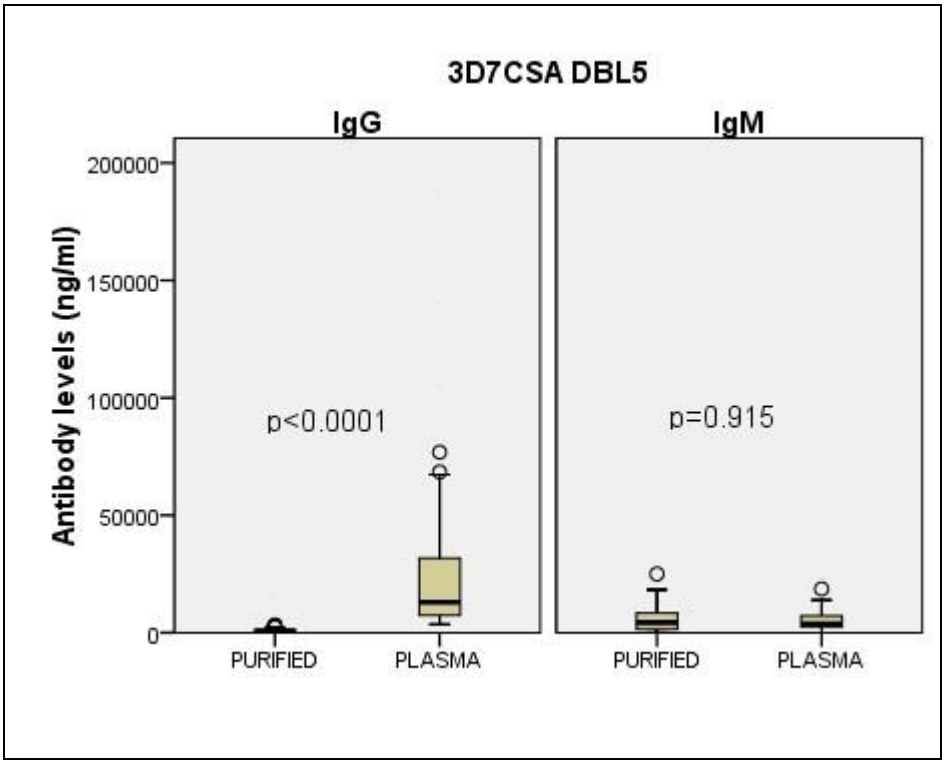


Fig. 4.5: Purified and plasma samples antibody reactivity to the VAR2CSA DBL5 domain

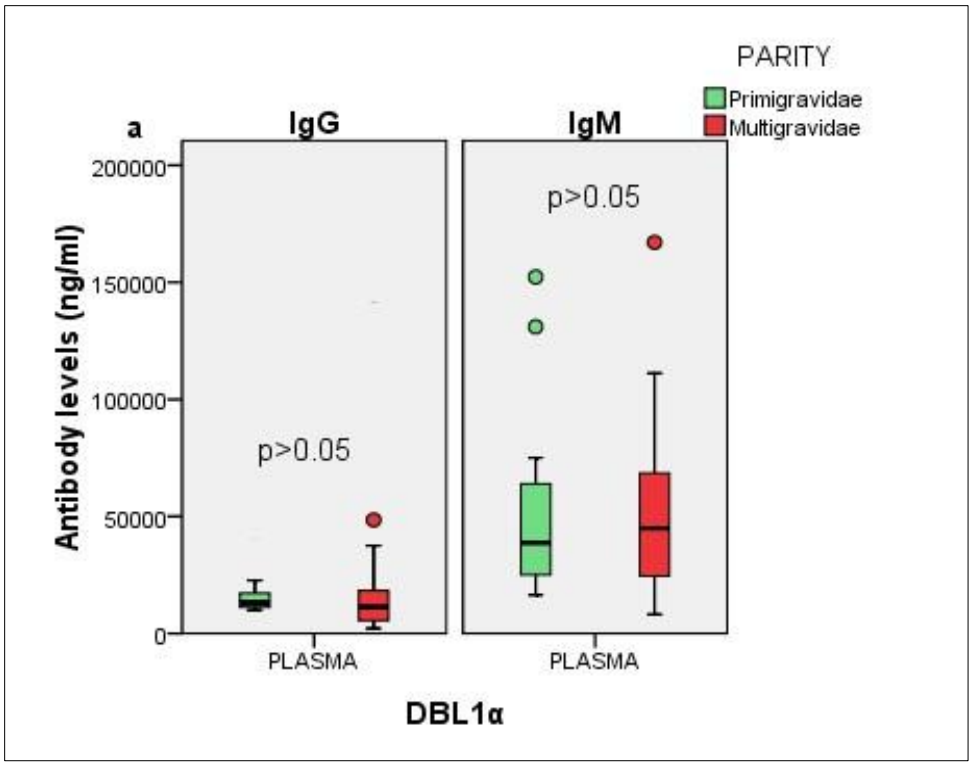
4.6 ABO phenotypes and antibody reactivity to the various domains

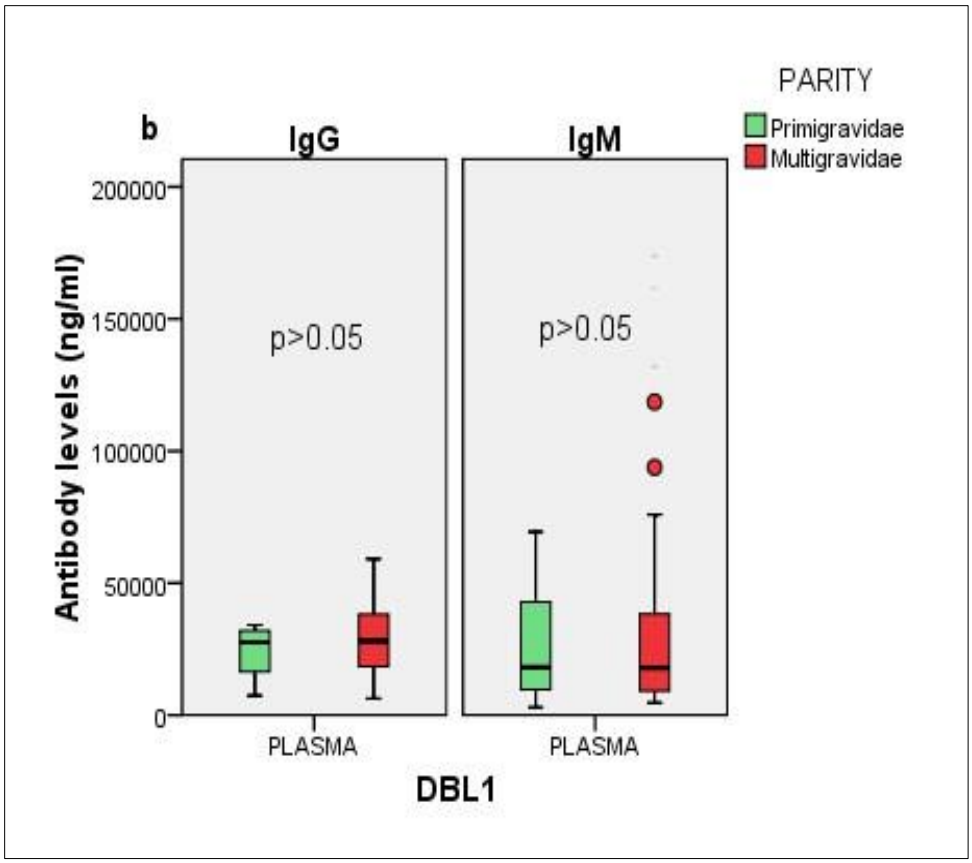
Taking parity into consideration, the effect of ABO phenotypes on the levels of antibody to the DBL domains was found to be statistically significant only in group O phenotypes ($p=0.031$) between the primigravidae and the multigravidae. The primigravidae had increased IgG levels (116,303.6ng/ml) when compared to the multigravidae (10,480.96ng/ml). There was however no statistical significant effect of the blood group phenotypes on the IgM levels to any of the domains between the primigravidae and multigravidae ($p>0.05$).

Also, no significant difference was observed for the ABO phenotype effects on the levels of antibodies to any of the domains when parity was not taken into consideration ($p>0.05$).

4.7 Effect of parity on levels of antibodies to the various domains

The Fig. 4.6 (a-d) shows the antibody levels for IgG and IgM between the primigravidae and multigravidae. There was significant difference between the primigravidae and multigravidae for IgG and IgM antibody levels to VAR2CSA DBL5 with the primigravidae having higher median total combined IgG and IgM levels (47,407.47ng/ml) than the multigravidae (10,565ng/ml) ($p=0.004$). Nonetheless there was no statistical significance between IgM levels for the primigravidae and multigravidae to the same domain ($p=0.06$) (Fig. 4.6d). Also, there was no statistical significance between the levels of IgG between the primigravidae and multigravidae for the DBL1 domain ($p>0.05$) likewise the IgM levels (Fig. 4.6b). However, the IT4VAR60 DBL1α and DBL5ε domains had no statistical significance even though the IgG levels to the multigravidae were higher than the primigravidae in the IT4VAR60 DBL domains ($p=0.16$ for 1α and $p= 0.13$ for 5ε). Also no statistical significant was observed in the IgM levels to any of the domains.





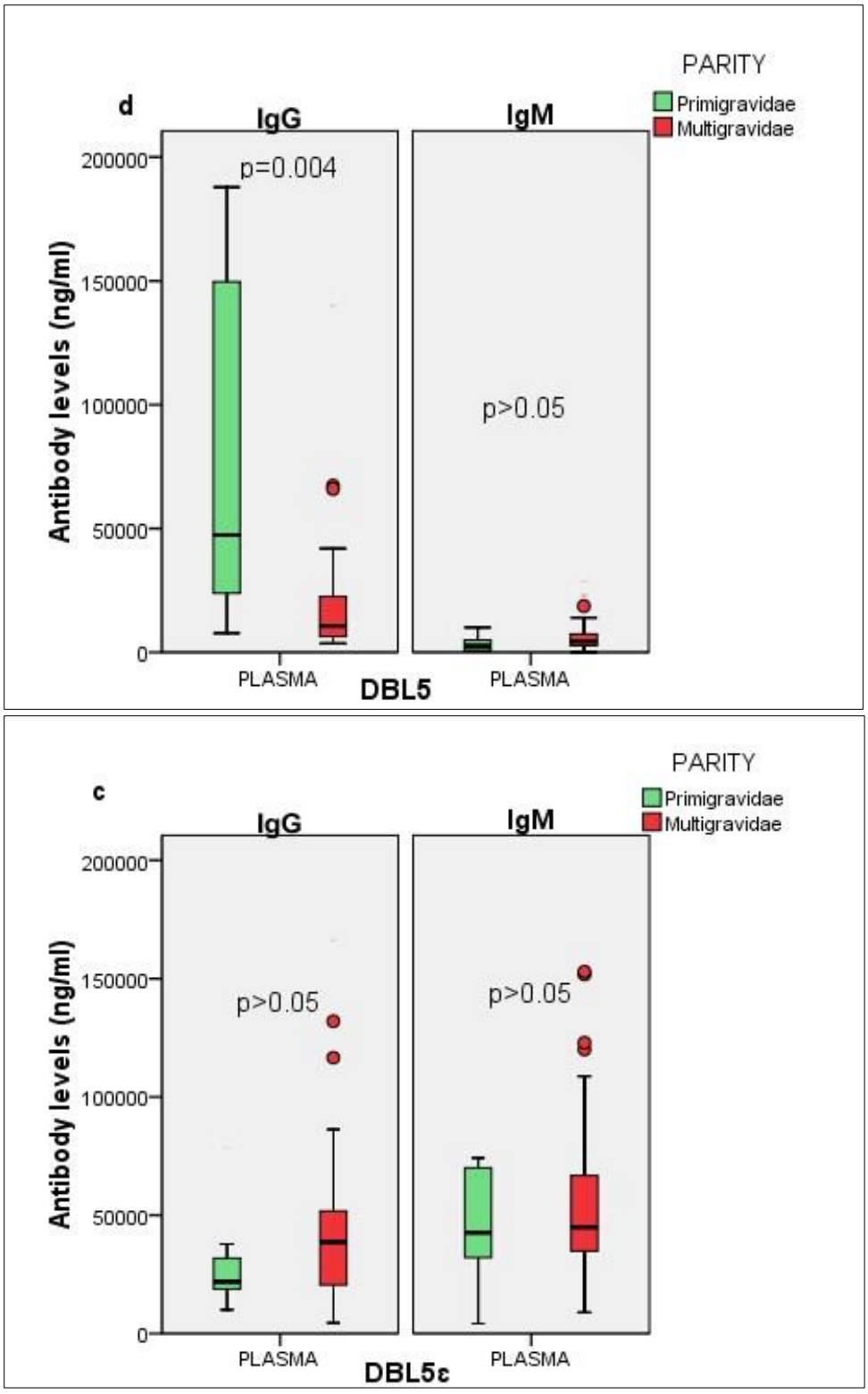


Fig. 4.6: Plasma levels of IgG and IgM to the IT4VAR60 DBL1α and VAR2CSA DBL1 domains (a&b) and IT4VAR60 DBL5ε and VAR2CSA DBL5 (c&d) domains

4.8 Effect of gestation on the antibody reactivity to the various domains

For the VAR2CSA DBL1 domain, there was found no statistical significance for IgG levels across the gestation periods in the primigravidae (p=0.3) as well as for the IgM (p=0.07) using the Kruskal-Wallis test but it was observed that levels of IgG decreased across the gestation periods. Similarly no statistical significance was found in the IgG levels in the multigravidae likewise the IgM levels (p=0.07) (Fig. 4.7ii). Also the levels of IgM in the VAR2CSA DBL1 domain decreased in the second trimester but increased in the third trimester as compared to its corresponding IgG levels but showed no statistical significance (p=0.08) when parity was not taking into consideration. Furthermore the multigravidae had higher IgG levels in the third trimester than the primigravidae (Fig. 4.7i).

For the IT4var60 DBL1α domain, IgG levels were found to be higher in the third trimester for the multigravidae than the primigravidae whilst the levels of IgM decreased across the gestation periods for both primigravidae and multigravidae. However, there was no statistical significance in the IgG and IgM levels with or without parity to the DBL1α (Fig. 4.7i).

In addition, the plasma IgG levels across the gestation period for the 3D7 (VAR2CSA) DBL5 domain for the primigravidae was highest at the second trimester with the third trimester being the least yet there was no statistical significance between the levels when the Kruskal Wallis test was used (p>0.05). Also there was no statistical significance between the IgM levels across the gestation periods (p=0.2) (Fig. 4.7(ii)). Nevertheless the levels of IgG increased greatly from the first to third trimester than IgM for the DBL 5 domain when parity was not taking into consideration but there was no statistical

significance (p=0.3) whilst significance was found between the IgM levels across the gestation periods (p=0.01) without parity. No statistical significance was found between the IgG and IgM levels to the VAR2CSA DBL5 domain. On the other hand, the IgG levels were higher for the primigravidae than the multigravidae for the first and second trimesters with both having their highest levels at the second trimester whilst the levels for the third trimesters were not different for each other. Also the primigravidae IgG levels decreased markedly from the second to third trimester.

Likewise, levels of IgG and IgM to the IT4VAR60 DBL5ε domain across the gestation periods was not statistically significant (p>0.05) (Fig. 4.7ii) in the primigravidae as well as in the multigravidae. However in the multigravidae, IgG levels to the DBL5ε domain were higher throughout the gestation periods than the levels for the primigravidae. In the second trimester IgG levels were higher when compared to the first and third trimesters for both primigravidae and multigravidae. On the other hand, IgM levels against the DBL5ε domain were observed to be decreasing across the gestation periods for both groups (Fig 4.7(i&ii)).

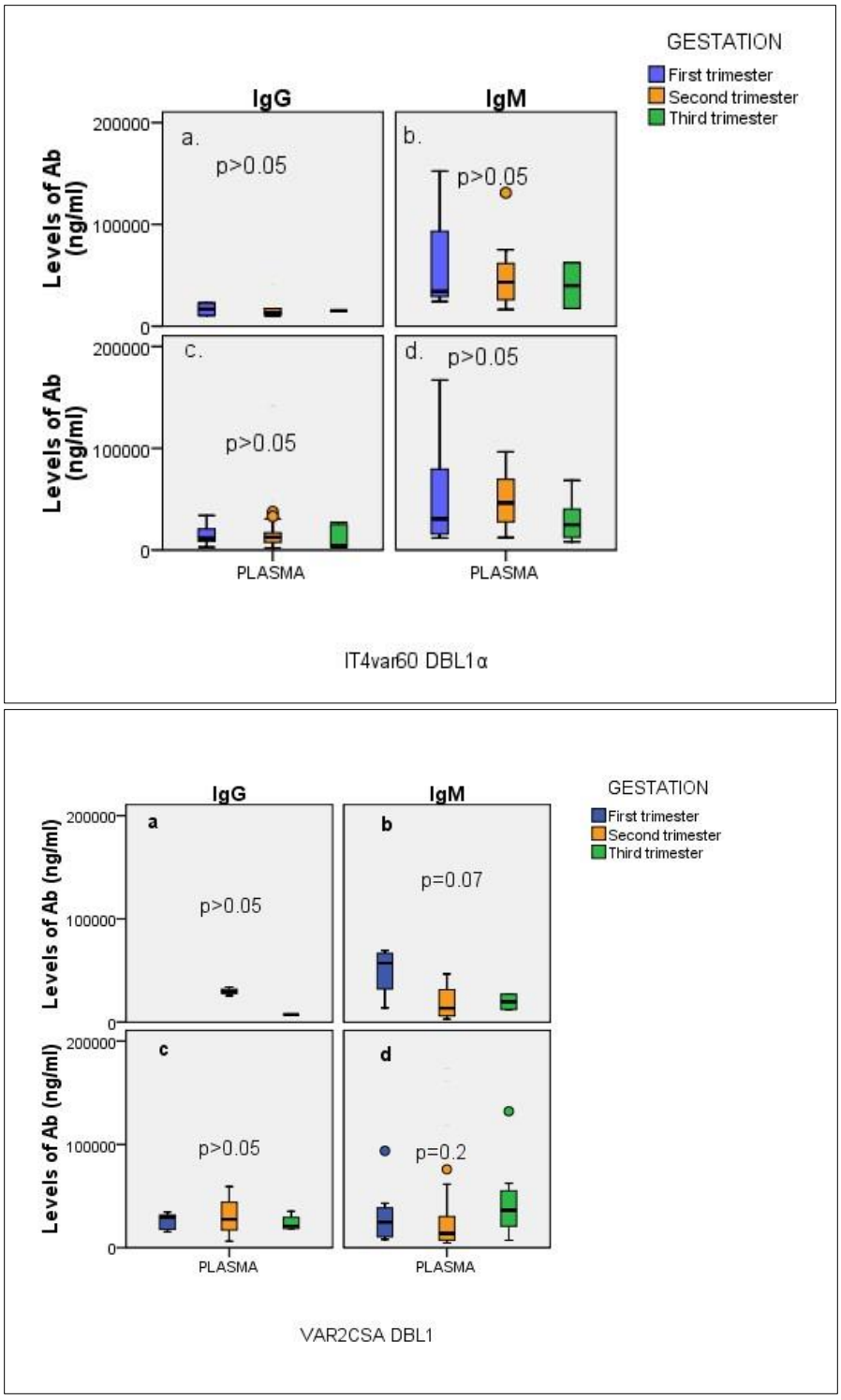
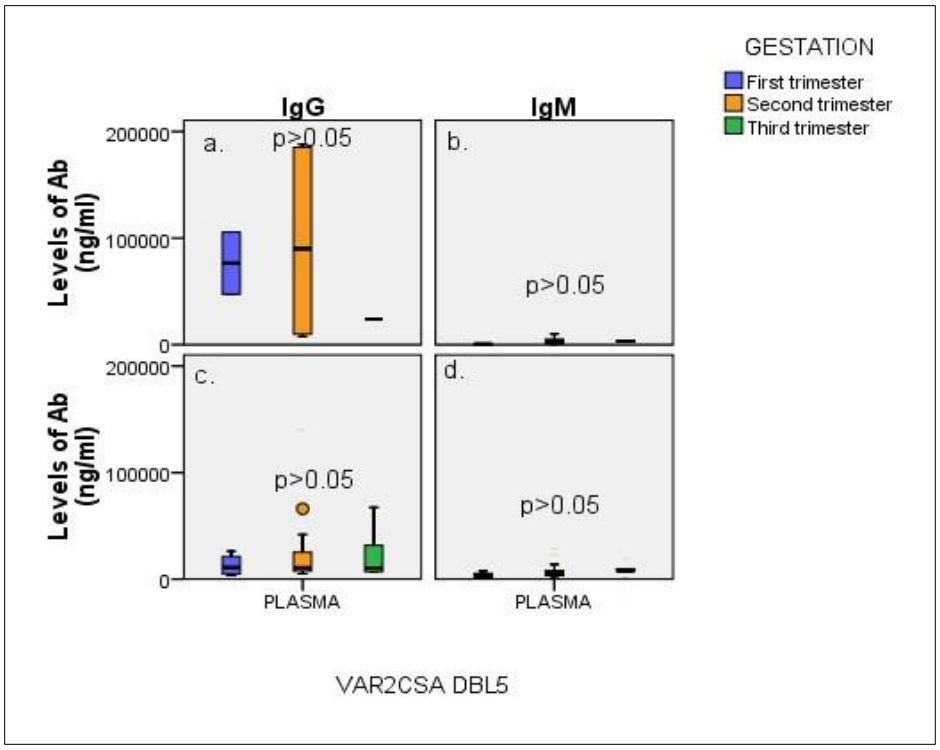


Fig. 4.7(i): A boxplot showing the levels of antibody distribution between the primigravidae (a&b): and multigravidae (c&d) for the IT4VAR60 DBL1α and VAR2CSA DBL1 domain across the gestation periods



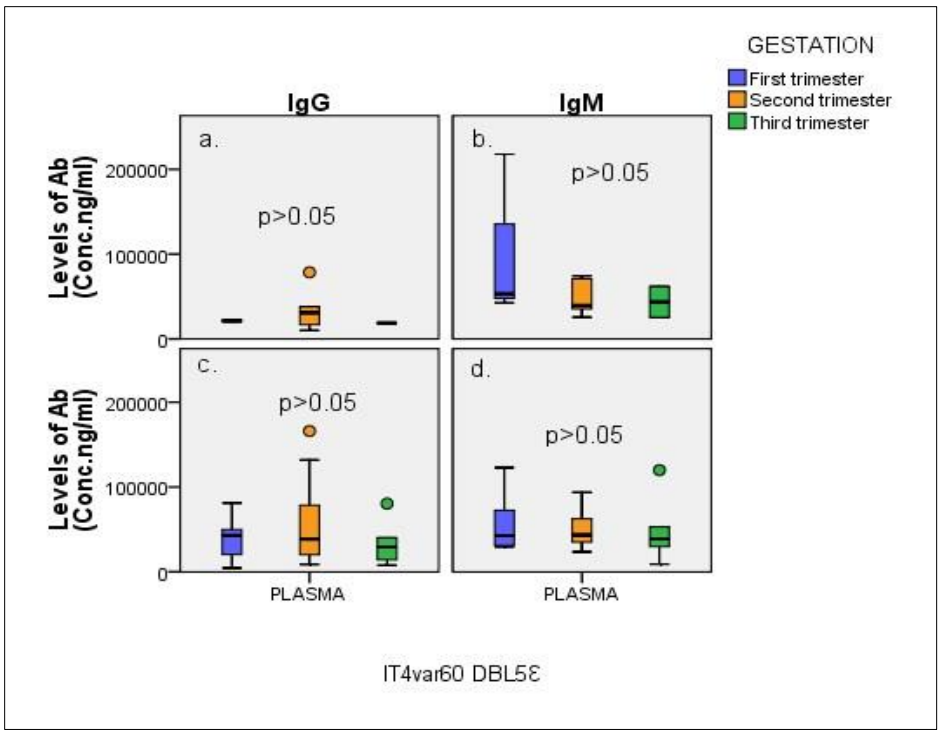


Fig. 4.7(ii): A boxplot showing the levels of antibody distribution between the primigravidae (a&b) and multigravidae (c&d) for the IT4VAR60 DBL5ε and VAR2CSA DBL5 domain across the gestation periods

4.9 Relationship between antibody responses to the various domain pairs

The IgG responses to the various domain pairs were positively correlated (Table 4.2). Among the domain pairs, the strongest correlation was observed between IgG responses to DBL5ε and DBL1 domains (r^2=0.42). However the weakest correlation was found between the IgG responses to DBL5ε and IgG responses to DBL5 (r^2=0.026).With regards to the IgM levels, responses to DBL5ε was highly correlated to IgM responses to DBL1α (r^2=0.777). This was followed by the responses of IgM against DBL5ε/ DBL1 (r^2=0.51). Nonetheless IgM responses to DBL5 and DBL1α showed the weakest correlation (r^2=0.00031) (Table 4.2).

Table 4.2: Antibody responses against the domain pairs

DOMAIN PAIR	r (IgG)	r (IgM)
DBL5ε/DBL1α	0.112	0.777
DBL5ε/DBL1	0.42	0.51
DBL5ε/DBL5	0.026	0.00031
DBL1α/DBL1	0.098	0.211
DBL5/DBL1α	0.056	0.00031
DBL5/DBL1	0.338	0.017

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

There have been many extensive studies on different malaria antigens and their corresponding circulating antibodies in regard to their roles in acquired immunity (Doolan *et al.,* 2009; Good *et al.,* 2004; Riley *et al.,* 1992). The immunity acquired by an individual in respect to the various phenotypes they are exposed to becomes non-functional during pregnancy (Steketee *et al.,* 1996). This is due to the fact that in the course of pregnancy, a subpopulation of *P. falciparum* parasites acquires the capability of sequestration and growth. This new endowed ability is due to the presence of a new organ introduced into the body, called the placenta. This cytoadherence ability of *P. falciparum* has been identified to promote immune evasion and also cause an increase in parasitaemia which is associated with most complications in *P. falciparum* pregnancy-associated malaria infections (Barfod *et al.,* 2011). These phenotypes of *P. falciparum* express the VAR2CSA antigen making the antigen the most extensively studied in placental malaria. The antigen consists of Duffy Binding Like domains which have been studied extensively and have been found to stimulate the immune system to produce specific IgG responses once the body's immune system is exposed to it (Salanti *et al.,* 2004; Staalsoe *et al.,* 2004). These antibodies produced are therefore crucial in the development of protective immunity during placental malaria. The antibodies have been found to be involved in effector mechanisms such as direct neutralization of parasites or

their toxins, complement mediation, cell cytotoxicity and inhibition of host cell invasion by malaria parasites (Czakowsky *et al.,* 2009; Blackman *et al.,* 1994; Cooper *et al.,* 1985). Apart from binding of specific IgG to the exposed epitopes/domains of VAR2CSA, non-specific IgM has similarly been identified to bind to some of the exposed epitopes of the antigen and has been implicated in the severity of the disease (Ghumra *et al.,* 2008; Rowe *et al.,* 2002).

Also according to Creasey *et al.,* (2003), this VAR2CSA antigen does not only have IgG binding sites but also harbours sites for non-specific IgM. This has also been further demonstrated in many studies (Barfod *et al.,* 2011; Rasti *et al.,* 2006; Semblat *et al.,* 2006). For this study, the levels of IgG and IgM reactivity to two different domains of VAR2CSA implicated in placental malaria and two domains of IT4var60 not implicated in placental malaria, but that mediate rosetting were measured using indirect ELISA.

In this study those diagnosed with malaria during the enrolment were mainly the primigravidae which was in conformity with previous studies done in the same community (Ofori *et al.,* 2009) and studies done in other areas (Shuman and Dorman, 2003).

It was noted that among the domains studied, IgM reactivity to the VAR2CSA DBL5 domain was the lowest when compared to the other domains. The levels of IgM in the absence of IgG against DBL5 was found to be higher when compared to the IgM levels in the presence of IgG even though they were not statistically significant ($p>0.05$). Despite the fact that IgG reactivity to DBL5 in the presence of IgM was higher than IgG levels in the absence of IgM, the levels of IgG against DBL5 were found to be lower when compared to IgG levels against VAR2CSA DBL1 and IT4var60 DBL5ε domains. The

lower IgM reactivity to VAR2CSA DBL5 could probably be that DBL5 expresses less IgM binding epitopes. Nonetheless, it may also imply that IgG binding inhibits IgM binding to DBL5. Interestingly, the significant increase in IgG reactivity to DBL5 in the primigravidae may also imply that DBL5 domain is highly expressed during primary placental malaria infection in the primigravidae resulting in the high levels of IgG antibodies to the domain.

Moreover, the decreased IgM reactivity to DBL5 may indicate that DBL5 may not be involved in sequestration by the parasite hence the lower IgM epitopes on the domain when compared to VAR2CSA DBL1. Furthermore, a study in rabbits by Avril *et al.,* (2010) observed that VAR2CSA DBL5 did not induce adhesion inhibitory antibodies. From this current study, it may be suggested that the VAR2CSA DBL5 domain is not involved in sequestration but is exposed to the host immune system. This however seems to support a current study by Dahlback *et al.,* (2012) in which only DBL1X, DBL2X and DBL3X were identified to be involved in sequestration.

Interestingly, it was observed that IgG levels against the VAR2CSA DBL1 and DBL5 domains were present in the primigravidae during gestation which is contrary to a previous study by Fried *et al.,* 1998 which reported that primigravidae do not produce anti-VAR2CSA antibodies. The presence of IgG to the domains in the primigravidae may suggest that, once the placenta has been introduced, it is able to isolate VAR2CSA expressing parasite isolates from the periphery to the placenta which are able to stimulate an immune response (Adam *et al.,* 2005).

Nonetheless, the IgG reactivity to VAR2CSA DBL1 was parity dependent since they were found to be higher in the multigravidae than in the primigravidae. Also a previous work

by Salanti *et al.,* 2004, observed IgG reactivity to VAR2CSA domains to be parity dependent and that it conferred protection to the pregnant women against severer consequences of PAM such as low birth weight and maternal anaemia. Furthermore the higher IgG reactivity to DBL1 in multigravidae observed in this study may be as a result of their previous repeated exposure to the infection which enabled them to elicit strong immune responses against the domain.

In addition, the increased in IgG levels against DBL1 during gestation, in the multigravidae than in the primigravidae is in conformity to a previous study by O'Neil *et al.,* (2001), which showed that IgG levels against VAR2CSA in multigravidae appear and increase faster than in the primigravidae during gestation. The rapid response of the multigravidae to the domains could also be as a result of the accumulation of PAM specific IgG or pre-existing memory upon previous repeated exposure to the infection. Also the absence of IgG levels in the primigravidae to the VAR2CSA DBL1 domain may indicate that, during primary initial infection, primigravidae lack antibodies to the domain since there has not been a previous exposure to it and when compared to VAR2CSA DBL5, DBL1 may be involved in sequestration.

Also, IgG levels measured against the IT4var60 DBL5ε (non-placental binding isolate) domain in the presence of IgM antibodies were higher than the IgG levels measured in the absence of IgM. This is contrary to a previous study by Barfod *et al.,* (2011), where they found decreased IgG reactivity in the presence of IgM as compared to an increase IgG reactivity in the absence of IgM against VAR2CSA DBL5ε. The differences in the antibody reactivity levels to DBL5ε may be due to the fact that in the previous work placental binding isolate (VAR2CSA) DBL5ε and monoclonal antibodies were used

whilst in this study (polyclonal) antibodies from pregnant women in a malaria endemic area were used. Also the lower IgG reactivity observed in the absence of IgM may be as a result of the method of purification used or alternatively the total IgG purified had lower IT4var60 specific IgGs.

Likewise the IgG reactivity to the non-placental binding isolate (IT4var60) DBL5ε was parity dependent since they were found to be higher in the multigravidae than in the primigravidae even though the difference was not statistically significant ($p>0.05$). This seems to support a previous study by Mayor *et al.,* (2011) in which they found increase IgG responses to non-CSA binding parasites in multigravidae than in primigravidae. This may imply that malaria infections during pregnancy may enhance other antibody responses against different *P. falciparum* isolates.

Also, IT4var60 DBL5ε was observed to have a higher IgM reactivity to its domain when compared to the reactivity of VAR2CSA DBL5. It was observed that the level of binding of IgM to the DBL5ε in the absence of IgG was lower when compared to IgM reactivity to the domain in the presence of IgG. The increased IgM reactivity to DBL5ε may indicate that 5ε harbours more IgM epitopes and may be involved in an immune evasion mechanism for the parasite since it has been implicated to mediate rosetting, increase the severity of the disease and help the parasite to evade the host immunity (Barfod *et al.,* 2011; Czakowsky *et al.,* 2010).

Similar to the DBL5ε, there was an increased IgG reactivity to DBL1α in the presence of IgM even though IgG reactivity to this domain was the lowest when compared to the other domains. In addition the reactivity of IgM to DBL1α was relatively higher in the presence of IgG. Also other studies have identified IgM and DBL1α to be involved in

rosetting in children with cerebral malaria and found to serve as a source of preventing immune IgGs from binding to the epitope to aid in sequestration to host receptors (Chen *et al.,* 2004; Rowe *et al.,* 2002; Smith *et al.,* 2001). The result obtained from this study could then suggest that IgM binding to IT4var60 DBL1α during pregnancy may aid in the immune evasion mechanism of *P. falciparum* expressing this isolate. Moreover, the lower IgG reactivity to the domain could suggest that during mixed infections with different *P. falciparum* isolates during pregnancy, the total IgG reactivity to some non-CSA binding parasite antigens such as IT4var60 DBL1α is reduced which may be as a result of pregnancy associated immunomodulation to protect the foetus.

Also, no significant difference was found between the reactivity of IgM in the primigravidae and multigravidae to the domains ($p>0.05$). However, previous studies have found IgM to be involved in immune evasion mechanism or severity of infection in cerebral malaria (Czakowsky *et al.,* 2010; Rowe *et al.,* 2002) by binding to infected erythrocytes. Even though in this study, the domains used were found to bind to IgM, the decreased consequences of PAM in the multigravidae may be accounted for that the IgGs they produce have a higher avidity than the IgM antibodies whereas the IgGs produced by the primigravidae have a lower affinity hence the primigravidae suffering more from the consequences of PAM.

Although there have been studies that have analysed ABO blood group association with malaria risk in non-placental (Min-Oo *et al.,* 2005; Udomsangpetch *et al.,* 1993) and placental malaria cases (Maubert *et al.,* 1998), none has been done on specific domains of the VAR2CSA such as the DBL1 and DBL5. In this study, the association of ABO blood group phenotypes to DBL domains were analysed using linear regression. A positive

association was found for levels of IgG in the multigravidae for individuals of blood group O (p=0.03) as compared to the other ABO phenotypes in the DBL1 domain indicating that the ABO phenotype system may be a determinant factor in the nature of antibodies produced since the red blood group antigens found in the sialoglycoprotein are found on the red cell surface.

Also the positive correlation observed among the VAR2CSA domain pairs may suggest that, immunity to VAR2CSA is acquired synergistically, confirming an earlier study by Oleinikov *et al.,* (2007). This may also indicate that an individual vaccinated with DBL5 may elicit a positive immune response against the DBL1. Likewise an immune response elicited by an individual vaccinated with DBL5 will be poor to immune responses against DBL1α.

5.2 Conclusion

The results from the study showed that all the four domains harboured IgM epitopes alongside placental malaria specific IgGs. Also since the levels of IgG binding to the domains were higher in plasma than in the absence of IgM, it may suggest that IgM binding may not interfere with IgG binding to the domains. Also a strong immune response observed in the multigravidae against DBL1α, DBL1 and DBL5ε domains during gestation may have resulted from a pre-existing memory or their previous repeated exposure to *P. falciparum* infections during pregnancy. Also the positive correlation observed among the IgGs generated against the domains may indicate that immunity to VAR2CSA and IT4VAR60 is acquired in unison. Furthermore the ABO blood group system may be a determinant factor in the levels and strength of antibodies produced to

the DBL domains since VAR2CSA which mediates the adhesion of infected erythrocytes to the CSA involves modulation of the proteins, such as sialoglycoprotein which contains the blood group antigens on the membrane surface of the IEs and also IT4var60 domains like DBL1α interacts with CR1 which is located on the red cell membrane.

5.3 Recommendations

VAR2CSA has been identified as the leading vaccine candidate against PAM due to its low level of polymorphism and the speedy acquisition of specific IgG after primary infections. Hence the binding of VAR2CSA to nonspecific IgM needs to be further studied in an increased sample size to determine their effect on IgG.

Furthermore, investigation into the role of anti-DBL5 antibodies is required to determine their effect during the acquisition of immunity to PAM. There should be further work on pregnancy immunomodulation and its effect on antibody production for peripheral *P. falciparum* antigens. Also there is the need to further investigate the effect of the ABO blood group system to the types of antibodies generated to these DBL domains and its effect on the level of protection which may be conferred in placental malaria or its clinical importance in PAM pathophysiology since the ABO system has been associated with soluble endothelial markers and adhesion molecules (Verheof *et al.,* 1999) and therefore may be a determinant factor in the levels and strength of immunoglobulins produced against these domains.

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APPENDIX

BUFFERS AND SOLUTIONS

A. IgM PURIFICATION REAGENTS PREPARATION

I. Binding Buffer for purification of IgM using MBL **pH = 7.4**

Volume: 800ml

Components	Amount
Distilled H_2O	800ml
10mM Tris	1.21 g
1.25M NaCl	73.13 g
20mM $CaCl_2$	0.3g

PROCEDURE

- Add 1.21g of 10mM Tris to 73.13g of 1.25M NaCl and 0.3g of 20mM $CaCl_2$.
- Fill up to 800ml with distilled water
- Measure the pH of the solution. Adjust if necessary

II. Elution Buffer for IgM Purification **pH 7.4; Volume: 500ml**

Components	Amount
10mM Tris of	1.21g
1.25M NaCl	73.13g
20mM $CaCl_2$	0.3g
Distilled H_2O	500 ml

PROCEDURE

- Add 1.21g of 10mM Tris to 73.13g of 1.25M NaCl and 0.3g of mM $CaCl_2$
- Dilute with distilled water
- Adjust pH to 7.4
- Bring volume to 500ml with dH_20.

III. PHYSIOLOGICAL BUFFER

PBS: Volume: 500ml

Components	Amount
PBS	1 tablet
De-ioinized	500 ml H_2O

PROCEDURE

- Dissolve 1 tablet of PBS in 500 ml of distilled water
- Place the flask on the magnetic stirrer without heating until all is in solution

IV. PHYSIOLOGICAL pH BUFFER; $NaHCO_3$

Volume: 1000ml and Concentration of 150mM

Components	Amount
$NaHCO_3$	12.6g
De-ioinized	1000 ml H_2O

PROCEDURE

- Dissolve 12.6g of $NaHCO_3$ in 1000 ml of distilled water
- Stir till all is in solution

B. REAGENTS PREPARATION FOR IgG PURIFICATION

I. Washing Buffer for purification of IgG using Dynabeads Protein G **Citrate Phosphate Buffer (pH 5.0); Volume: 1 litre**

a.

Components	Amount
Distilled H_2O	1 litre
Citric Acid	4.7 g
Dibasic Sodium Phosphate (Na_2HPO_4)dehydrate	9.2 g

PROCEDURE

- Add 9.2g Dibasic Sodium Phosphate (Na_2HPO_4)dehydrate (MW=178) to 4.7g of Citric Acid (MW 192)
- Fill up to 1 litre with distilled water
- Measure the pH of the solution. Adjust if necessary

b. By preparing 0.2M dibasic sodium phosphate and 0.1M Citric acid

Components	Amount	Amount
0.1 M Citric Acid	2.6g 250ml	24.3ml
Distilled H_2O		
0.2M Dibasic Sodium Phosphate (Na_2HPO_4)	7.1 g	25.7ml
Distilled H_2O	250ml	

PROCEDURE:

- Measure 2.6g of Citric Acid and dissolve in 250ml of distilled water to make 0.1M Citric Acid solution.
- Measure and dissolve 7.1g of Dibasic Sodium Phosphate (Na_2HPO_4) in 250ml distilled water to make 0.2M solution
- Add 24.3ml of Citric Acid to 25.7ml of Dibasic Sodium Phosphate (Na_2HPO_4) to make a Citrate Phosphate Buffer with pH of 5.0 and confirming with pH meter

II. Elution Buffer for Purification **0.1M Citrate (pH 2-3); Volume: 1L**

a.

Components	Amount
Citric acid	9.802g
Sodium citrate-$2H_2O_2$	14.4g
30% H_2O_2	1ml
Distilled H_2O	1000ml

PROCEDURE

- Add 9.802 g citric acid to 14.4 g sodium citrate-2H_20
- Adjust pH to 3 and add 1 mL 30% H_2O_2 □ Bring volume to 1L with dH_20.

b. By preparing 0.2M dibasic sodium phosphate and 0.1M Citric acid

Components	Amount	Amount
0.1M Citric Acid	2.6g 250ml	
Distilled H_2O		46.5ml
0.1M Sodium Citrate	6.5 g	
Distilled H_2O	250ml	3.5ml

PROCEDURE:

- Measure 2.6g of Citric Acid and dissolve in 250ml of distilled water to make 0.1M Citric Acid solution.
- Measure and dissolve 6.5g of Sodium Citrate in 250ml distilled water to make 0.1M solution
- Add 46.5ml of Citric Acid to 3.5ml of Sodium Citrate to make 0.1M Citrate Buffer with pH (2-3) and confirming with pH meter

III. Storage buffer for Dynabeads Purification

0.1M Na-phosphate buffer; Volume: 1L

Components	Amount
sodium phosphate monobasic	13.9 g
sodium phosphate dibasic heptahydrate	53.65 g
De-ioinized H_2O 1L	

PROCEDURE:

- Add 13.9 g sodium phosphate monobasic to 500 mL dH20 to prepare 0.2 M Monobasic Stock
- Add 53.65 g sodium phosphate dibasic heptahydrate to 1 L dH20 to prepare 0.2 M Dibasic Stock
- Add 15.9 ml of monobasic stock to 284.1 of dibasic stock to prepare

  0.1 M Buffer and dilute with distilled water to 1L.

C. REAGENTS PREPARATION FOR ELISA

I. Diluent for ELISA

PBS pH 7.2; Volume: 500ml

Components	Amount
PBS	1 tablet
Distilled H_2O	500 ml

PROCEDURE

- Dissolve 1 tablet of PBS in 500 ml distilled water
- Place the flask on the magnetic stirrer without heating until all PBS is dissolved in the solution
- Measure the pH of PBS solution. Adjust the pH to 7.2 as necessary, by adding drop wise 3M HCL or 3M NaOH whilst stirring.

V. Wash Buffer for ELISA

1 X PBS with 0.1% Tween 20

Volume: 5L

Components	Amount
PBS	10 tablets
Tween 20	5 ml
distilled H_2O	5000 ml

PROCEDURE

- Add 10 tablets of PBS to a beaker containing 5000 ml deionized water
- Place the flask on the magnetic stirrer without heating until all PBS is dissolved in the solution
- Measure the pH of PBS solution. Adjust the pH to 7.2 as necessary, by adding drop wise 3M HCL or 3M NaOH whilst stirring.
- Add 5 ml of Tween 20 and continue stirring until all is in solution.

VI. Blocking solution for ELISA

5% Non-Fat Dry Milk in 1 X PBS

Volume: 500ml

Components	Amount
PBS	1 tablet
Non-Fat Dry Milk	25g
Distilled H_2O	500 ml

PROCEDURE

- Dissolve 1 tablet of PBS in 500 ml distilled water
- Place the flask on the magnetic stirrer without heating until all is dissolved in the solution

Measure the pH of PBS solution. Adjust the pH to 7.2 as necessary, by adding drop wise 3M HCL or 3M NaOH whilst stirring.