Prevalence of anti–*Plasmodium Falciparum* Merozoite Surface Protein-1_{19} (PfMSP1-$_{19}$) Antibodies in Sudanese Pregnant Women at Umdurman Maternity Hospital.

Submitted by

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Dedication

To those who gave me the meaning of life

My parents

My Husband & children

My brothers & sister

And,

My friends

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ABSTRACT

The polymorphic merozoite surface protein (MSP-1) of *Plasmodium falciparum* is a major asexual blood-stage malaria vaccine candidate. Immunological mechanisms responsible for eliminating malaria parasites remain unclear, but antibodies to the carboxyl-terminal 19-kDa segment of the MSP1-$_{19}$ have been implicated. This study aimed to determine the prevalence of anti-MSP-1$_{19}$ antibodies in pregnant women.

In a prospective, case-control study, antibody levels in pregnant and non-pregnant women were compared using bulk ELISA technique. Results showed that the antibodies levels to MSP-1$_{19}$ in pregnant women were lower compared to non-pregnant. Furthermore, multigravidae have higher anti- MSP1-$_{19}$ antibodies levels compared to primigravidae. On the other hand, increasing prevalence of anti-MSP-1$_{19}$ antibodies with the increase in age in pregnant women could be detected.

In conclusion: low anti- MSP1-$_{19}$ could be a possible explanation for the increased pregnancy -associated malaria in pregnant women especially primigravidae.

Arabic Abstract

البروتين السطحى للميروزيت ((merozoite surface protein (MSP-1) المتعدد الاشكال لطفيل الملاريا الخبيثة هو الطور اللاجنسى فى الدم الرئسى المرشح كمصل للملاريا. والاليات المتعلقة بعلم المناعة المسئولة عن ازالة طفيليات الملاريا تظل غير واضحة، ولكن الاجسام المضادة الموجهة للكاريوكسيل النهائى فى قطعة الميروزويت ($MSP1_{-19}$) لها دخل فى ذلك. استهدفت هذه الدراسة تحديد انتشار الاجسام المضادة ل($MSP1_{-19}$) فى النساء الحوامل.

فى دراسة محتملة للحالة/ المنظم ، قورنت مستويات الاجسام المضادة فى النساء الحوامل و النساء غير الحوامل باستعمال (ELISA technique)، واظهرت النتائج ان مستويات الاجسام المضادة ل ($MSP1_{-19}$) فى النساء الحوامل اقل بالمقارنة بغير الحوامل. بالاضافة الى ذلك فأن الحامل متعددةالولادة (multigravidae) لها مستوى اعلى من الاجسام المضادة مقارنة بالحامل لاول مرة (primigravidae) من ناحية اخرى، زيادة انتشار الاجسام المضادة ل($MSP1_{-19}$) مع زيادة العمر فى النساء الحوامل يمكن ان تكتشف.

ختاما، فأن انخفاض الاجسام المضادة ل($MSP1_{-19}$) يكون تفسير ممكن لزيادة الملاريا المصاحبة للحمل في النساء الحوامل خاصة الحوامل لاول مرة .

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Introduction

Malaria is one of the major health problems in Sudan and pregnant women are at greater risk for developing malaria. Merozoite surface proteins-1_{19} of *P.falciparum* are important malaria vaccine candidates, and collection of information about humoral responses to this antigen will help in choosing candidate vaccines. The studies of prevalence of anti-MSP1_{19} antibodies in pregnant women will also help in setting future control programs, especially vaccination.

The objective of the study is to determine the prevalence of anti-*P.falciparum* merozoite surface protein (PfMSP-1_{19}) antibodies in pregnant women using bulk ELISA technique.

CHAPTER ONE

LITERATURE REVIEW

1.1 The Malaria problem:

Malaria infection is endemic across the tropics and subtropical regions; it affects people in more than 90 countries, causing 300–500 million infections/year and is estimated to lead to approximately 1 million deaths each year (Breman, 2001; Murphy & Breman, 2001). Most infections and most severe morbidity and mortality are caused by *Plasmodium falciparum*. The other three human malaria parasites (*P.vivax*, *P.malariae* and *P.ovale*) contribute to fewer infections and to moderate disease and relatively few deaths (Mendis, *et al.*, 2001). Most *P.falciparum* infections and consequences are in sub-Saharan Africa, but Asia, Southeast Asia and the Americas are also sites of transmission for this parasite. Although a few reports of adverse consequences of *P.vivax* in pregnancy exist (Nosten, *et a.,* 1999). *P.falciparum* is the only human malaria parasite that is more common in pregnant than in non-pregnant women and is the only human parasite that has substantial adverse effects on pregnancy, and pregnancy outcome (Brabin, 1991; Steketee, *et al.,* 2001).

1.2 The Burden of Malaria in Sudan:

Malaria is endemic throughout the Sudan. Endemecity varies from hypo-endemic in the North to hyper-to-holo endemic in the South. The disease is caused in > 90% of cases by *Plasmodium falciparum*. Annually, malaria is estimated to lead to 7.5 million cases and 35,000 deaths. (WHO/EMRO, 2001).

Also according to the annual statistical report of the Federal Ministry of Health 17.4 - 44.8% of all out-patient clinic visits are due to malaria. Between 9.6 – 36.3% of all hospital

admissions are a consequence of malaria and malaria is a leading cause of reported deaths, contributing to 10–15% of total deaths that occur at hospital level (FMOH, 2003).

1.3 Malaria in pregnancy:

Malaria infection during pregnancy is a major public health problem in tropical and subtropical regions throughout the world. In most endemic areas of the world, pregnant women are the main adult risk group for malaria. Malaria during pregnancy has been most widely evaluated in Africa south of the Sahara where ninety percent (90%) of the global malaria burden occurs. The burden of malaria infection during pregnancy is caused chiefly by *Plasmodium falciparum*, the most common malaria species in Africa. Every year at least 30 million pregnancies occur among women, in malarious areas of Africa, most of who reside in area of relatively stable malaria transmission (WHO/EMRO, 2001). In Africa perinatal mortality due to malaria is at about 1500/day. In areas where malaria is endemic, 20-40% of all babies born may have a low birth weight (Kakkilaya, 2004).

In the Sudan each year more than 1.2 million women become pregnant, of those 750,000 are in areas with high malaria transmission: intense perennial, high seasonal transmission or in areas of irrigation (FMOH; RBM, 2004). Malaria contributed considerably to maternal morbidity. It accounted for 37.2% of all maternal deaths in Sudan at hospital level (Dafalla, *et al.,* 2003). Also it was found to be a cause of 18.1% of low birth weight (Taha, *et al.,* 1995).

1.4 The symptoms and complications of malaria during pregnancy:

The symptoms and complication of malaria during pregnancy differ with the intensity of malaria transmission and thus with the level of immunity the pregnant women has acquired. According to the Malaria and Pregnancy Network (2000) malaria causes up to fifteen percent of maternal anaemia and about thirty five percent of preventable low birth weight. Low birth weight is a leading cause of neonatal mortality.

The clinical features of *Plasmodium falciparum* malaria in pregnancy depend to a large extent on the immune status of the women. In pregnant women with little or no pre-existing immunity, such as women from non-endemic areas or travelers to malarious area, infection is associated with high risks of severe disease with maternal and perinatal mortality. These women are at particular risk of cerebral malaria, hypoglycaemia, pulmonary oedema and severe haemolytic anaemia. Fetal and perinatal loss has been documented to be as high as 60-70% in non-immune women with malaria (Shulman and Dorman, 2003).

Pregnancy-associated malaria is characterized by the sequestration of *P. falciparum*-infected erythrocytes in placental intervillous spaces. Placental parasites appear to express a specific phenotype defined by the adherence of *P.falciparum*- infected erythrocytes to the glycosaminoglycans Chondroitin sulfate A (CSA) and hyaluronic acid, expressed by the syncytiotrophoblast (Fried and Duffy, 1996; Beeson, *et al.,* 2000). Parasite adhesion to CSA is mediated by *P.falciparum*-variant surface antigens (VSAs) expressed on the surface of infected erythrocytes (Buffet, *et al.,* 1999; Reeder*, et al.,* 1999) Placental parasites bind to CSA and not to other VSAs ligands, such as CD36 and ICAM-1, whereas the opposite is true for most parasite isolates from non-pregnant individuals (Fried and Duffy, 1996; Beeson, *et al.,* 2000; Maubert , *et al.,* 2000).

1.5 Malaria Antigens For MalariaVaccine Development:

The complex life cycle of the malaria parasite has complicated vaccine development efforts. Each parasite stage has different antigens (table 1) that lead to protective immunity and immune responses effective against one stage (for example, sporozoites), but that generally have been ineffective against other parasite stages (such as the asexual and sexual stages). This has resulted in a rich diversity of approaches to malaria vaccine development and to multiple current vaccine candidates (Hoffman, 1996). These antigens are classified as the follows:

1.5.1 PRE-ERYTHROCYTIC PHASE ANTIGENS:

1.5.1.1 Circumsporozoite Protein (CS or CSP)

The circumsporozoite protein is the main sporozoite coat protein. It has a central area consisting of repeat sequences that are highly immunogenic and present in the differing malaria species. Studies reveal that the central repeat region can elicit a B-cell immune response, producing antibodies that block sporozoites in culture. Induces CD8+ and CD4+ CTL response in mice (Zevering, *et al.,* 1998).

1.5.1.2 Sporozoite Surface Protein 2 (SSP2)

SSP2 is a protein found on the surface of sporozoites from the murine malaria parasite *P. yoelii*. The equivalent protein found in *P. falciparum* is known as thrombospondin-related

anonymous protein (TRAP). It possesses a sequence homologous to the conserved region II of the CS protein. Antibody raised to recombinant *P. falciparum* TRAP was demonstrated to inhibit sporozoite invasion of hepatoma cells *in vitro* (Nardin and Nussenzweig, 1999).

1.5.1.3 Liver Stage-Specific Antigen (LSA-1)

LSA-1 is first expressed in infected liver cells. It has a 17 amino acid repeat and is immunogenic it causes CTL response in Africans that gives protection to severe malaria (Hollingdale, 1998).

1.5.2 ERYTHROCYTIC PHASE ANTIGENS: (Table A)

1.5.2.1 Merozoite Surface Protein 1 (MSP-1/MSA-1) (Fig. 2)

Several merozoite surface proteins have been described. The best characterized is merozoite surface protein-1 (MSP-1). It is the major surface antigen of merozoites and is the best-studied protein (Reeder, 2001). *Pf*MSP-1 is synthesized as a polypeptide of ~ 195 KD, which is subjected to several proteolytic cleavages during maturation of the merozoite (Stafford, *et al.,* 1994). This proteolytic processing is coincident with merozoite maturation and invasion (Cooper, 1993). During the first phase, it is cleaved to yield four peptide fragments, of which the 42 kDa C-terminal fragment (MSP1-42) remains fixed to the merozoite membrane. During the second phase, occurring at the moment of erythrocyte invasion, MSP1-42 itself is cleaved to yield a polypeptide of molecular weight 19 kDa (MSP1-19). The latter cleavage is essential for erythrocyte invasion (Pizarro, *et al*., 2003).

The highly conserved 19-kDa C-terminal processing fragment (MSP-1_{19}) contains epitopes targeted by antibodies that inhibit erythrocytic invasion. This 19-kDa antigenic domain is a potential malaria vaccine candidate (Qari, *et al.,* 1998).

1.5.2.2 Merozoite Surface Protein 2 (MSP-2)

MSP-2 is a glycoprotein with a molecular size of 45-kD. It has a central repeat region that has significant variation among isolates while the N- and C- terminal regions are well conserved. The antibody response is directed almost completely towards variant regions of MSP-2. The conserved regions are rarely recognized (Taylor, *et al*., 1998).

1.5.2.3 Apical membrane antigen I (AMA-1)

Apical Merozoite Antigen 1 is a membrane protein produced in the apical organelles of the merozoite. During invasion of erythrocytes, AMA1 redistributes over the entire surface of the parasite, and like MSP1, is proteolytically cleaved. The sequence of AMA-1 is relatively conserved with greater than 50% amino acid conservation among the different *Plasmodium* species, is a leading malaria vaccine candidate. Known to stimulate humoral response in patients with chronic malaria. It also seems to stimulate T-cell response in *vitro* (Amante, 1997).

1.5.2. 4 Erythrocytic-Binding Antigen (EBA-175):

EBA-175 is a transmembrane protein characterized by two conserved cysteine-rich domains and is expressed on the surface of merozoites. It thought to be involved in attaching merozoites to erythrocyte receptors via sialic acid during invasion. Immunizing rabbits with this protein can give blocking effect to merozoite invasion (Clough, 1996).

1.5.2.5 Ring-Infected Erythrocytic Surface Antigen (RESA):

RESA is expressed on erythrocytes infected with ring stage parasites. Causes humoral immune response. In *vitro* RESA inhibits IL-2 levels. Evidence of intrauterine protection passed to fetuses via this Ag (Chaba, *et al.*1998).

1.5.2.6 Other Antigens:

Other antigens include serine repeat antigen (SERA) or serine- rich protein (SERP), glycophorin binding protein (GBP-130), histidine rich protein 2 (HRP-2), rhoptry-associated proteins (RAP-1 and RAP-2), Pfs230 and Pfs40 **(**Staalso**,** *et al.,* 1998).

1.5.3 ANTI-DISEASE ANTIGENS:

1.5.3.1 Erythrocyte Membrane Proteins 1 (PfEMP1) (Fig.1):

After invading the erythrocyte, *Plasmodium falciparum* expresses the virulence factor *Pf*EMP1, which is transported to the surface of the red blood cell (Pizarro, *et al*., 2003). *Pf*EMP1 is a member of the *var gene* family. The 40-50 *var* genes exhibit a high degree of variability, but have a similar overall structure (Figure 1). *Pf*EMP1 has a large extracellular N-terminal domain, a transmembrane region and a C-terminal intracellular domain. The C-terminal region is conserved between members of the *var* family and is believed to anchor *Pf*EMP1 to the erythrocyte submembrane cytoskeleton. (Smith, *el al*., 2001). *Pf*EMP1 probably functions as a ligand that enables the infected-RBC to cytoadhere to various host cell receptors, including the scavenger receptor CD36, an 88-kDa intergral membrane protein found on monocytes, platelets and endothelial cells. Another receptor is intracellular adhesion molecule-1 (ICAM1). ICAM1 is a member of the immunoglobulin superfamily and functions

in cell-cell adhesion. Chondroitin sulfate A (CSA) has been implicated in the cytoadherence within the placenta and may contribute to the adverse affects of *P. falciparum* during pregnancy (Beeson and Brown, 2002). An other receptors are platelet endothelial cell adhesion molecule-1 (PECAM-1), thrombospondin (TSP) (Shulman *et al*., 2003). These adhesions assist the parasite in escaping splenic clearance and promote sequestration of parasites in vital organs such as brain and placenta. (Beeson and Brown, 2002).

*Pf*EMP1 is an adhesin molecule, which confers on the infected erythrocyte the capacity to auto-agglutinate, to adhere to uninfected red blood cells or to be sequestered on vascular endothelial cells in diverse tissues. Agglutination and sequestration of infected erythrocytes are correlated with many of the pathogenic effects of malaria (Pizarro, *et al*., 2003) Antibodies to variable regions of these proteins, measured by agglutination, correlates with clinical protection against *falciparum* malaria (Staalso**,** *et al.,* 1998).

1.5.3.2 Sequestrin

Sequestrin is a 270-kDa surface knob protein that is expressed on the surface of infected erythrocytes and it is a ligand for CD36. This surface protein is also a good vaccine candidate except that it shows a high rate of antigenic variation (Sharma, 1997).

1.5.3.3 Others

Two glycoproteins that are released upon schizonts rupture are currently being studied. They are shown to induce release of tumor necrosis factor (TNF-β), which may be a factor in the onset of malaria related illness (Tolle, *et al.,* 1993).

1.5.4 Transmission Phase Antigens:

1.5.4.1 Pfs25

Pfs25 is found on the surface of *P. facliparum* zygotes and ookinetes. Inside the mosquito, monoclonal antibodies to the Pfs25 antigen can block development of the sexual stages, thus inhibiting the production of sporozoites. Pfs25 seems to be the most promising sexual blood stage antigen that may someday be used in a vaccine (Staalso***,*** *et al.,* 1998).

1.6 Immunity to malaria and antigenic variability of *P. falciparum:*

P.falciparum parasites show a remarkably high degree of polymorphism at the various stages of their life cycle, which has important implications for the efficacy of parasite-neutralizing immune responses (Lockyer, *et al.*, 1989; Bull, *et al*., 1998; Miller, *et al*., 1993; Fenton, *et al*., 1991; Konate, *et al*., 1999). Antigenic diversity in field populations of *P.falciparum* parasites may delay acquisition of protective immunity to malaria, the development of which may thus require repeated exposure to many different antigenic types or strains circulating in a given locality. The antigenic diversity reflects polymorphisms in allelic gene products while polymorphisms in many antigens are caused by variations in the sequence of the short tandem repeats, which is a characteristic of many malaria antigens and which frequently constitute immunodominant regions. MSP1 and MSP2 *genes* are the best-studied with regard to allelic polymorphisms (Snounou *et al*., 1999).

Antigenic variation is a process by which a clonal parasite population can switch its antigenic phenotype (Gardner, *et al*., 2002). In *P.falciparum* the variant antigens are expressed at the surface of infected erythrocytes and the expression of these antigens can be modulated in a given parasite population either by immune pressure or transfer from intact to splenectomized

animals. Antigenic variation is usually considered as a mechanism that allows parasite survival in an immune-competent host. However, antigenic variation of *Pf*EMP-1 can also occur *in vitro* in the absence of immune pressure (Biggs, *et al*., 1991; Roberts, *et al*., 1992).

Although resistance to infection with malaria parasites and to the disease itself increases with age in individuals living in endemic areas, it can take more than a decade to reach substantial protection, and never reaches completion. The age at which maximal protection is reached also correlates inversely with the intensity of parasite transmission (mosquito-human); therefore, these observations could support the hypothesis that the malaria antigens that induce protective immune responses might be poorly immunogenic (weak inducers of immunity). Alternatively, these antigens might be very polymorphic (varying between different strains of *P.falciparum* - antigenic diversity) or variable (changing with time within individual strains-antigenic variation), therefore requiring the accumulation of immunological memory (acquired immunity) to a large number of different antigenic epitopes before substantial protection is achieved. Indeed, a number of recent studies point to the latter possibility (Taylor and Robinson, 1998).

1.7 Immune responses to maternal malaria:

The immune response to malaria is not well understood. There has been some recent research into the unique immune response offered by pregnant women against malaria.

One popular model, emerging in the last decade, to explain the unique susceptibility of pregnant women to malaria is that of pregnancy-associated immunosuppression. According to this model, pregnancy induces a state of general immunosuppression in the body sustained by elevated levels of serum cortisol. This immunosuppression prevents fetal rejection and

thus, renders pregnant women susceptible to infection. However, this theory does not explain the diminished susceptibility to malaria experienced by multigravid women. (Meeusen, *et al.,* 2001) showed that Pregnancy is characterised by a transient depression of cell-mediated immunity of the woman that allows foetal allograft retention, but also interferes with resistance of the mother to various infectious diseases. Cellular immune responses to *P. falciparum* antigens are depressed in pregnant women compared to non-pregnant controls (Fievet, *et al.,* 1995; Riley, *et al*., 1998). This pregnancy-induced immunosuppression may persist several months after delivery, but generally restoration of immune responses to *P. falciparum* antigens is being observed within six months after delivery (Fievet, *et al.,* 1997; Diagne, *et al*., 2000).

Studies in pregnant women have reported that anti-*P. falciparum* antibodies to three asexual-stage antigens appear to be associated with protective immunity. Two studies have shown that pregnant women who lack antibodies to the ring-infected stage antigen (RESA) are more susceptible to *P.falciparum* infection (Astagneau, *et al.,* 1994; Mvondo, *et al.,* 1992). However, two other studies have not found this association (Deloron, *et al.,* 1989; Fievet, *et al.,* 1995). The ability of anti-RESA antibodies to reduce placental parasitemia has not been investigated. In 1996, Fried and Duffy reported that parasites sequestered in the placenta express a ligand that binds specifically to chondroitin sulfate A (CSA) (Fried and Duffy, 1996). The ligand, CSA-L, is thought to be a variant of *P. falciparum* erythrocyte membrane protein 1 (Fried and Duffy, 1996; Fried, *et al.,* 1998). Since antibodies inhibit the binding of IRBC to CSA in vitro (Alkhalil, *et al.,* 2000; Fried *et al.,* 1998; Maubert, *et al.,* 1999; O'Neil-Dunne, *et al.,* 2001; Ricke, *et al.,* 2000), they are likely to be protective in vivo. Branch, *et al.,* in 2000, reported that placental parasite densities were significantly lower in Kenyan

mothers who had immunoglobulin G (IgG) antibodies to the carboxyl-terminal 19-kDa segment of the merozoite surface protein 1 (MSP-1_{19}) than mothers who did not.

Recently, it has been shown that almost all pregnant women lack anti-CSA-L antibodies at conception (O'Neil-Dunne, *et al.,* 2001). In a study from Burundi, it was shown that multigravid women begin producing significant anti-adhesion antibodies during the fourth month of gestation, whereas primigravidae do not begin producing these antibodies until around 6 months (O'Neil-Dunne, *et al.,* 2001).

A longitudinal study in Africa, demonstrated that pregnant women were more susceptible to malaria during first pregnancies than second pregnancies (Fievet, *et al.,* 1997). Bull and colleagues recently reported that disease protection is dependent on a variant-specific immune protection directed against a variety of members of the *Pf*EMP1 family (Bull, *et al.,* 1998). Antibodies to MSP-1_{19} can block merozoite invasion, either by inhibiting the binding of merozoites to erythrocytes by blocking processing of MSP-1 or by other mechanisms (Blackman, *et al.,* 1994; Guevara, *et al.,* 1997; Holder, *et al.,* 1999).

1.8 Malaria in pregnancy and the role of chondroitin sulfate A:

Women living in endemic areas who are resistant to malaria before their pregnancy tend to lose this protection when they become pregnant (Brabin, 1983). This observation has often been interpreted as a consequence of the immunosuppression that is necessary to protect the fetus from being rejected by the mother's immune system (Weinberg, 1984). However, in malaria-endemic areas this increased susceptibility to malaria (which can cause severe

disease in the mother as well as abortion, stillbirth and low birth weight of the offspring) is disproportionately higher in women undergoing a first pregnancy (primigravidae) (McGregor, 1987). These women have not been exposed to and have no immunity to such parasites before becoming pregnant. The first pregnancy enables them to develop specific immunity, which protects them during subsequent pregnancies. This hypothesis is supported by the fact that multigravid pregnant women in endemic areas have antibodies to placental parasites acquired during previous malaria-infected pregnancies, (Maubert, *et al*., 1999). These antibodies inhibit the cytoadherence of placental parasites to human syncyliotrophoblast (Ricke, *et al.,* 2000).

The parasitized RBCs found in the placentas of women experiencing their first pregnancy show a remarkable preference to bind to chondroitin sulfate A (CSA), which is a ligand that is present on the placental syncytiotrophoblast but is not readily accessible on cells elsewhere in the body (Fried and Duffy, 1996; Maubert, *et al.,* 1997). The molecule in the parasite that mediates binding to CSA appears to be a particular variant of *Pf*EMP1 (Reeder, *et al.,* 1999). The high frequency of parasites found in the placenta that bind CSA can explain the susceptibility of primigravidae to clinical malaria and points to the importance to protection of immunity that is specific for parasite variants (Ricke, *et al.,* 2000). These adhesion specificities are eliminated from a non-pregnant individual, owing to a lack of suitable adhesion receptors on the host cells, and presumably before they have induced appreciable levels of antibodies to the CSA-specific *Pf*EMP1 variant. In contrast, because CSA becomes available in the developing placentas of primigravidae, parasites that are able to bind CSA and are present in the blood can suddenly multiply unhindered. With successive pregnancies, the levels of the antibodies that are directed against *Pf*EMP1 variant molecules that can bind

CSA increase, and are therefore able to limit the multiplication of CSA-binding parasites (Ricke, *et al.,* 2000). Protection against pregnancy-associated malaria can be gradually acquired in this way. It has recently been discovered that most *P.falciparum* isolates from infected placentas can also bind to hyaluronic acid, a second receptor for parasite adhesion that is present on the placental lining (Beeson, *et al*., 2000) these findings are changing our understanding of the mediation of placental parasite accumulation, there is still much to discover about the reasons for the increased susceptibility of pregnant women to malaria and the pathogenesis of placental malaria (Maubert, *et al.,* 1997; Beeson, *et al*., 2000).

1.9 Vaccination against malaria:

Despite long and intensive research efforts, no vaccine against malaria in humans is yet available; this is in spite of recent advances particularly with sporozoite vaccines (Stoute, *et al*., 1997; Seder and Hill, 2000). Several different approaches, both in terms of antigens and technologies, are being taken by various research groups (Taylor and Robinson, 2000), but they are all focused on relatively conserved parasite antigens. If variant-specific responses are as important to the natural acquisition of protective immunity as some of the data might suggest, it follows that a strategy of artificial immunization based on responses to conserved parasite antigens must induce immune responses that are very different to those induced by natural exposure to *P. falciparum* in endemic areas. One hope lies with *Pf*EMP1, antibodies to which show significant correlation with development of clinical immunity (Bull, *et al*., 1998; Bull, *et al*., 2000). Unfortunately, the variant nature of *Pf*EMP1 remains a major obstacle for vaccine design, as the immune response to it is highly variant-specific. However, the recent demonstration that monoclonal antibodies recognize cross-reactive epitopes of the

cysteine-rich interdomain region 1 (CIDR1) of *Pf*EMP1. These epitopes are functionally conserved for binding to the CD36 ligand expressed by host epithelial cells (***Fig.1)***, provides credence for development of effective vaccines against this variant antigen. Whether this will be possible remains to be seen (Gamain, *et al*., 2001).

Although a general malaria vaccine appears to be a distant possibility, there is much hope for a vaccine against placental malaria. As aforementioned, Fried and Duffy have proven that multigravid women are able to mount an effective antibody response against parasite sequestration on the placental surface. Presently, two possibilities have been explored with respect to parasite adhesion to the chondroitin sulfate A receptor. First, the administration of excessive soluble CSA to pregnant women has proven to drastically reduce parasite adhesion; however, in excess levels, this soluble protein is severely nephrotoxic. Second, studies have demonstrated that the administration of chondroitinase AC can effectively reduce parasite adhesion by 95%. This preliminary data is being further tested in combination with therapeutic use of monoclonal antibodies to CSA (Fried and Duffy, 1998).

CHAPTER TWO

Materials and Methods

2.1 The Study Area:

This study was conducted at Omdurman Maternity Hospital, which is a central referral maternity hospital that was established in 1957. Since then the hospital has continued to provide obstetric and paediatric care to an increasing population, which has reached 2.5 million in its catchments area. The hospital has 134 beds, 2 labour wards, 3 surgical theatres, a blood bank, and a laboratory. In 2003 deliveries at the hospital reached 17,288. Attached to the hospital is the First School of Midwifery in Sudan.

Malaria transmission is seasonal malaria related to rain fall, *P. falciparum* is the predominant species, and accounts for 95% of all malaria cases in the hospital catchments area. *Anopheles arabiensis* is the main vector (FMOH; RBM, 2004).

2.2 The study design:

A longitudinal, prospective study was carried out at Omdurman Maternity Hospital, and Institute of Endemic Diseases, from December 2003 to October 2004.

2.2.1 Study population:

The study population consisted of 100 women, of whom 67 were pregnant women (first, second, and third trimester) and 33 were non pregnant. Each woman received a unique index number. Following informed consent, all recruited women were clinically examined and screened for malaria at the beginning of the study. All women were interviewed in a special

questionnaire form that contained demographic data, number of pregnancies, gestational age, and past history of malaria attacks.

2.2.2 Blood sampling:

Venous blood samples were collected [2-5ml] in vacutainers from all women, for parasitological (smears), haematological and serological (ELISA) investigations. .

2.2.3 The health team:

The health (investigating) team consisted of: a medical doctor, microscopist, and two assistants.

2.3 The laboratory work:

The laboratory work was carried out at the medical laboratory in Omdurman and the Institute of Endemic Diseases, University of Khartoum.

2.3.1 Preparations of thick and thin films:

Air-dried thick and thin blood films were stained with Giemsa (10%) for ten minutes and then examined for parasitaemia by trained technicians.

2.3.2 Serum preparations:

Serum was collected in plain tubes after the clotted whole blood was centrifuged for fifteen minutes at 1000 rpm and stored frozen at -20 °C for later anti-body estimation.

Sera of non-exposed donors were used as negative controls. While sera from individuals having high anti-body level (or showed a high clear positive anti-bodies response to the different fragments of the merozoite surface proteins) were used as positive controls.

2.3.3 Enzyme-linked immunosorbent assays (ELISA) for the detection of human antibodies using recombinant MSP-1_{19}:

ELISA technique used is known of its ability to recognize the recombinant $MSP1_{19}$ as described by Cavanagh (2001).

Recombinant antigen fragments (*Pf*$MSP1_{19}$) were used in the assay. The GST (Glutathion-s-transferase) control protein was included on all plates in order that the Optical Density (OD) reading specific for PfMSP1, could be calculated. Sera were examined in duplicates. This involved subtracting the average OD of antibodies against GST from the average OD of the antibodies directed against duplicate wells coated with the recombinant antigen, thus obtaining the specific anti-$MSP1_{19}$ recombinant antigen OD.

Ninety six wells microtitre plates were coated with 50 ng/well of recombinant antigens (MSP-1_{19}) in 100 µl of coating buffer (15mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.3), and incubated in the dark for three days at 4^oC. The wells were emptied, and washed three times with the washing buffer (0.05% Tween 20 in PBS). Unoccupied protein binding sites were blocked with 200 µl/well blocking buffer (1% [w/v] skimmed milk powder in washing buffer) for 5 hours at room temperature and washed again three times by washing buffer.

Hundred µl per well of the human sera (diluted 1:500 in the blocking buffer) were added to duplicate antigen-coated wells and incubated overnight at 4^oC. After three washes with washing buffer, the wells were incubated for 3 hours with 100 µl/well of the horseradish peroxidase-congugated rabbit anti-human IgG (1:5000 in the blocking buffer), and washed three times. Then incubating for up to 15 minutes at room temperature with 100 µl/well of

substrate (0.1 mg ml^{-1} O-phenylene-diamine (OPD) [Sigma] and 0.12% H_2O_2) in development buffer (24.5 mM citric acid monohydrate and 52 mM Na_2HPO_4, pH 5.0).

Finally the reaction was stopped by addition of 20 µl/well of sulphuric acid (2M H_2SO_4). Optical densities (OD) were measured at 490 nm using the plate reader. The corrected OD value for each sera sample was involved subtracting the average OD of antibodies against GST from the average OD of the antibodies directed against duplicate wells coated with the recombinant MSP-1_{19} antigen.

2.4 Control sera:

Negative control plasma samples were collected from healthy donors who had not been exposed to malaria. A positive control from a malaria endemic area was used for comparison with the cohort of samples tested. The cut-off point was calculated as:

Cut-off point = Mean OD of the negative controls readings + 3 SDs.

The cut off point was calculated as 0.13 OD

All the titration which were above the cut-off point were considered as positive plasma, and all those under the cut-of point were considered as negative sera.

CHAPTER THREE

RESULTS

3.1 Population and the history of malaria: -

The total number of the population of the study was hundred. Pregnant women were 67% of the study population, (aged between 19-40years), while non-pregnant constituted 33% (ages 19-45 years). Forty four % (30/67) were primigravidae, and 55.2% (37/67) were multigravidae.

Past history of malaria was more frequent among non-pregnant women (table 1), where it was reported more frequently in women over 30 years of age, while in pregnant women previous malaria attacks were more common among women aged between 20-30 years (table 2). Also was reported more frequent among multigravid women (table 6).

Parasitologically, 5/67 (7.5%) pregnant women tested positive, while only 1/33 from non-pregnant women tested positive (table 1).

3.2 Prevalence of anti-MSP-1_{19} antibodies: -

The hundred women who participated in this study were screened to determine the prevalence of anti-MSP-1 antibodies. The number and percentages of prevalence of MSP-1_{19} antibody are shown in (table-1).

Antibody levels were categorized into two groups, those with levels <0.13 (Negative) and those with levels >0.13 (Positive). Although malaria was more prevalent in pregnant women, the percentage of women with positive levels for anti-MSP1-$_{19}$ antibody were more in non-pregnant women (table 1).

Pregnant women ≤ 30 year had higher levels of anti-MSP1_{19}, while in non-pregnant women, higher levels of anti-MSP-1_{19} antibodies were found > 30years of age (table 3). Multigravid women had higher levels of anti-MSP-1_{19} antibodies (table 5).

Table (1): History of malaria attacks & anti-MSP1-$_{19}$ bodies levels in the study population:

	Numbers	***Previous history of malaria***		***Parasitology for malaria***		***Level of anti-MSP-1$_{19}$***	
		Positive	Negative	Positive	Negative	Positive	Negative
Pregnant	**67**	**49 (73.1%)**	**18 (26.9%)**	**5 (7.5%)**	**62 (92.5%)**	**50 (75.8%)**	**16 (24.2%)**
Non-pregnant	**33**	**26 (78.8%)**	**7 (21.2%)**	**1 (3.0%)**	**32 (97.0%)**	**28 (84.8%)**	**5 (15.2)**

Table (2): Distribution of previous history of malaria according to age-groups:

Age Group	*Pregnant*			*Non-pregnant*		
	Total number	*+ve*	*-ve*	*TN*	*+ve*	*-ve*
<21	12(17.9%)	8(11.9%)	4(6.0%)	1(3.0%)	1(3.0%)	_
21-30	35(52.2%)	25(37.3%)	10(14.9%)	11(33.3%)	8(24.2%)	3(9.1%)
31-40	20(29.9%)	16(23.9%)	4(6.0%)	18(54.5%)	15(45.5%)	3(9.1%)
>40	_	_	_	3(9.1%)	2(6.1%)	1(3.0%)
Total	***67(100%)***	***49(73.1%)***	***18(26.9%)***	***33(100%)***	***26(78.8%)***	***7(21.2%)***

+ve: positive

-ve: Negative

Table (3): Anti-MSP-1_{19} antibody levels in different age-groups in the study population:

	Age-groups											
	< 21			***21-30***			***31-40***			***> 40***		
	Number	***Positive***	***Negative***	***Number***	***Positive***	***Negative***	***Number***	***Positive***	***Negative***	***Number***	***Positive***	***Negative***
Pregnant	12 (18.2%)	10 (15.2%)	2 (3.0%)	34 (51.5%)	22 (33.3%)	12 (18.2%)	20 (30.3%)	18 (27.3%)	2 (3.0%)	–	–	–
Non pregnant	1 (3.0%)	1 (3.0%)	–	11 (33.3%)	8 (24.2%)	3 (9.1%)	18 (54.5%)	17 (51.5%)	1 (3.0%)	3 (9.1%)	2 (6.1%)	1 (3.0%)

Table (4): Distribution of parity in different age-groups in the study population:

Age-group	Primigravidae	Multigravidae	Total
< 21	*8(11.9%)*	*4(06.0%)*	*12(17.9%)*
21-30	*20(29.9%)*	*15(22.4%)*	*35(52.2%)*
31-40	*2(03.0%)*	*18(26.9%)*	*20(29.9%)*
Total	*30(44.8%)*	*37(55.2%)*	*67(100.0%)*

Table (5): The distribution of anti-MSP1-$_{19}$ antibody levels according to parity

	Number	***Negative***	***Positive***
Primigravidae	30 (44.8%)	12(17.9%)	18(26.9%)
Multigravidae	37 (55.2%)	4(06.0%)	32(47.8%)

Positive= above a designated cut off level.
Negative= below a designated cut off level

Table (6): Comparison of previous history of malaria and antibody levels according to parity

	Number	*Previous history of malaria*		*Antibody levels*	
		Positive	*Negative*	*Positive*	*Negative*
Preimigravidae	***30(44.8%)***	***23(76.7%)***	***7(23.3%)***	***18(26.9%)***	***12(17.9%)***
Multigravidae	***37(55.2%)***	***26(70.3%)***	***11(29.7%)***	***32(47.8%)***	***4(06.0%)***

CHAPTER FOUR

Discussion

It was clear from this study that anti-MSP-1_{19} antibodies were low in pregnant women compared to non-pregnant controls. This is generally in line with the immunosuppressive theory of pregnancy (McGregor, 1987). The relatively high levels in multigravid women was probably due to more previous exposures of these women compared to primigravidae as those also tended to be younger in the study group. This agrees with Oralee *et al*., (2000), who suggested that the MSP1-$_{19}$ antibody response develops with age, not with multiple experiences with parasitemia. These antibodies are important in the development of cumulative protective immunity to malaria. This finding also agree with Meeusen *et al*., (2001) who discussed immune response to maternal malaria and reported that, the sustained immune-suppression during pregnancy prevents fetal rejection, but does not explain the diminished susceptibility to malaria experienced by multigravid women. However, earlier findings reported that, multigravid pregnant women in endemic areas have antibodies to placental parasites acquired during previous malaria-infected pregnancies (Maubert, *et al.,* 1999). These antibodies were claimed to inhibit the cytoadherence of placental parasites to human syncyliotrophoblast. (Ricke*, et al.,* 2000). It is not clearly understood whether the antigen molecules that induced antibodies production play a role in long-term immune responses and protection against malaria. *P. falciprum*.

Antibodies estimated in the present work, were known to be produced against the MSP1-$_{19}$ antigenic domain which is a potential vaccine candidate. Binding of these antibodies to their epitope inhibits erythrocytic invasion by the merozoites (Qari *et al.,* 1998).

In this study, the prevalence of the positive anti- MSP1-$_{19}$ among the study group was 84.8% in non-pregnant women compared to only 75.8% in pregnant ones (Table 1). This finding seems to agree with a previously reported observation by Brabin, (1983) who showed that women living in endemic areas were resistant to malaria before their pregnancy and tended to lose this protection when they become pregnant.

The immune suppressive theory of pregnancy was further supported by Fievet *et al*., (1995) and Riley, *et al*., (1998) who reported that cellular immune responses to *P. falciparum* antigens were depressed in pregnant women in comparison with non-pregnant controls This observation has also often been interpreted as a consequence of the immunosuppression that is necessary to protect the fetus from being rejected by the mother's immune system (Weinberg, 1984).

The prevalence of positive anti- MSP1-$_{19}$ in multigravidae women in the present study was two times the positive cases in primigravidae group (Table 5). This finding may corroborated previous findings reported by Fried, *et al.,* (1998) and Maubert, *et at*., (1999) who claimed that reduced incidence of malaria infections in multigravid women could be due to antibodies directed against pregnancy-associated parasites acquired during the first infected pregnancy that inhibit the cytoadherence of placental parasites to the human syncytiotrophoblast. Although some studies reported the absence of antibodies in primigravidae, other studies have shown that primigravidae do produce anti-adhesion antibodies. Most studies have reported increased levels of antibodies with increasing gravidities (Gysin,*et al.,*1999; Maubert, *et al.,* 1999; Ricke*, et al.,* 2000). This was suggested also by O'Neil-Dunne, *et al.,* (2001), who reported that multigravid women begin producing significant anti-adhesion antibodies during the fourth month of

gestation, whereas primigravidae do not begin producing these antibodies until around 6 months. On the other hand, Bull, *et al.,* (1998) claimed that disease protection is dependent on a variant-specific immune protection directed against a variety of members of the *Pf*EMP1 family.

An important finding from the present study is that it demonstrated that there was clear association between antibody levels and age in pregnant women, (Table 3) that the percentage of the positive cases in the age group of less than 21 years old is only half the percentages in age groups of 21-30 and that of 31-40 years old. This could be another manifestation supporting the findings of Oralee, *et al.,* (2000), who suggested that the anti- MSP1-$_{19}$ antibody response develops with age, consequently he suggested that an anti-malaria vaccine strategy for pregnant mothers could delay infants first parasitemias until they are more capable of mounting a favorable anti- MSP1-$_{19}$ response.

In conclusion: low anti- MSP1-$_{19}$ could be a possible explanation for the increased pregnancy -associated malaria in pregnant women especially primigravidae.

Large scales longitudinal studies are needed to elucidate the role of reduce anti-malaria antibodies in the increased frequency of malaria attacks in pregnant women.

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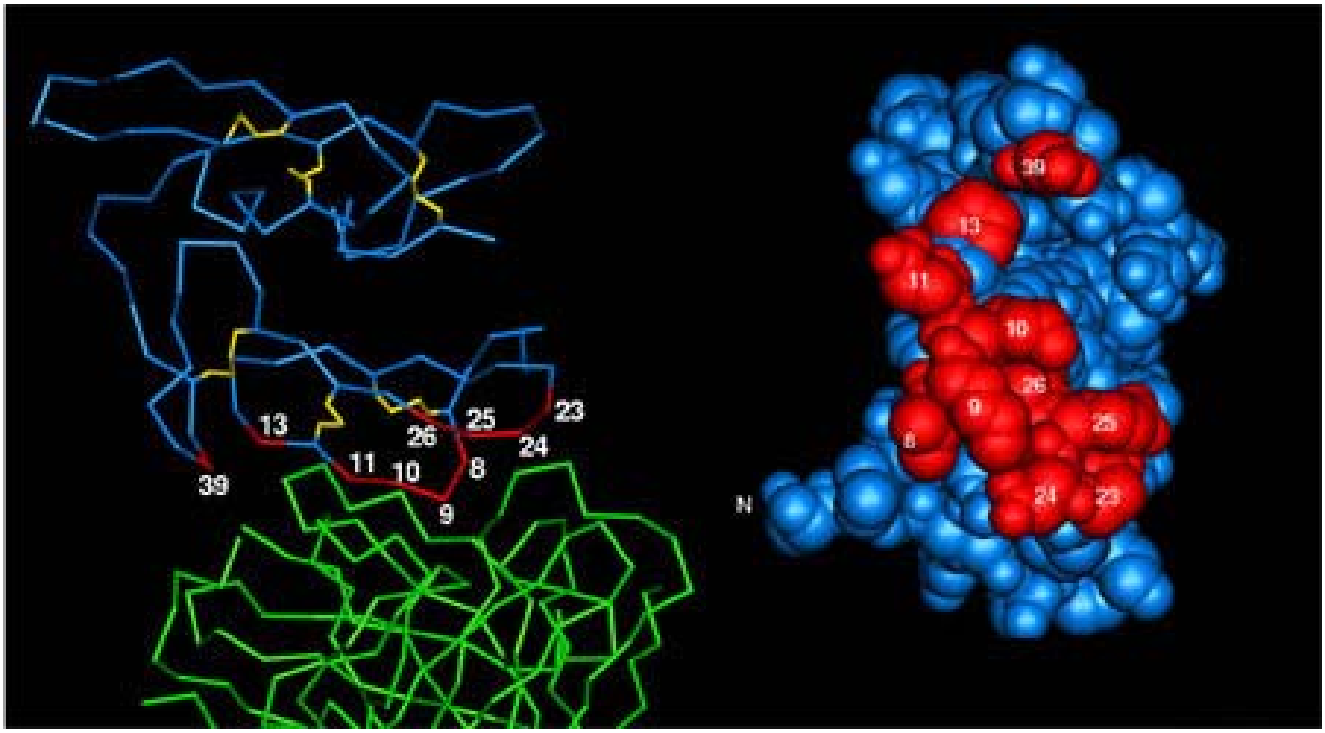
Figure 1: The epitope of MSP-1_{19} of *Plasmodium falciparum*



The epitope of MSP-1_{19} of *Plasmodium falciparum* is recognised by a specific monoclonal antibody. MSP-1_{19} is shown in blue with the epitope indicated in red. The antibody is shown in green).

Table A: <u>Common Malaria parasite antigens:</u>

ANTIGEN	DISEASE PHASE FOUND IN	LOCATION	IMMUNE RESPONSE
Circumsporozoite protein (CS or CSP)	Pre-erythrocytic	Main sporozoite coat protein	Central area of repeat sequences. Promotes strong B-cell response that produces Abs that block sporozoites in culture. Induces CD8+ and CD4+ CTL response in mice.
Sporozoite surface protein 2 (SSP2)	Pre-erythrocytic	Found on sporozoites of P. yoeli	Mice immunized with CS and SSP2 expressing cells were protected by CD8+ CTLs.
Liver stage-specific antigen 1 (LSA- 1)	Pre-erythrocytic	Infected liver cells	17 A.A. repeat causes CTL response in Africans that gave protection to severe malaria.
Glycoproteins released during schizont rupture	Pre-erythrocytic	Released by the destruction of infected hepatocyte	Induces the release of TNF which may cause malaria related illness.
Merozoite surface protein 1 (MSP-1 or MSA- 1)	Erythrocytic	Major surface antigen of merozoites	195-kDa protein breaks down to 19-kDa. Abs to 19-kDA reduces efficiency erythrocyte invasion.
Merozoite surface protein 2 (MSP-2 or MSA- 2)	Erythrocytic	Second merozoite surface antigen unrelated to MSP-1.	Has variable interior repeat region, but N and C ends are conserved. No immunogenicity known.
Erythrocyte binding antigen 175 (EBA-175)	Erythrocytic	Surface of merozoites. Thought to be involved in attaching merozoite to erythrocyte.	Immunizing rabbits with this protein can give blocking effect to merozoite invasion.
Ring-infected erythrocyte surface antigen (RESA)	Erythrocytic	Expressed on erythrocytes infected with ring stage parasites.	Causes humoral immune response. In vitro RESA inhibits IL-2 levels. Evidence of intrauterine protection passed to fetuses via this Ag. vitro RESA inhibits IL-2 levels.
Serine repeat antigen (SERA)	Erythrocytic	Antigen on merozoite involved with binding to the erythrocyte.	Monkeys immunized with SERA and Freund's adjuvant had high Ab titers to SERA

			and resistance to *P. falciparum*.
Glycophorin binding protein (GBP- 130)	Erythrocytic	Merozoite	Causes humoral immune response in rhesus monkeys, but this immunity is not transferable with the monkey's serum.
Apical merozoite antigen (AMA- 1)	Erythrocytic	Apical region of the merozoite. May be involved in erythrocyte invasion.	Known to stimulate humoral response in patients with chronic malaria. Also seems to stimulate T-cell response in vitro.
Histidine rich protein 2 (HRP- 2)	Erythrocytic	Merozoite	Humoral response used to test presence of merozoites in patient's bloodstream.
Rhoptry-associated proteins (RAP-1 and RAP-2)	Erythrocytic	Associated with the apical region of the merozoite.	Cause T-cell proliferation, and are involved in upregulating IL-6 and TNF production. Immunization with these proteins inhibits parasite growth in monkeys.
Erythrocyte membrane proteins 1 (PfEMP1)	Erythrocytic	Infected erythrocytes	Involved in sequestration of infected erythrocytes.
Sequestrin	Erythrocytic	Infected erythrocytes	Ligand for CD36, a recognition protein expressed by vascular epithelium.
Pfs25	Transmission	Surface of P. falciparum zygotes and ookinetes	Can block development of sexual stages and sporozoite development.