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Cortisol, Prolactin, Cytokines and susceptibility of Pregnant Sudanese Women to *Plasmodium falciparum* Malaria

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Dedicated

To

The Soul of My Father,

My Greatest Mother,

My Dear Husband,

My Beloved Sister,

And Sweet Daughter & Son

LIST OF PUBLICATIONS

This thesis is based on the work presented in the following papers:

1. **Bayoumi NK**, Bakhet KH, Mohmmed AA, Eltom AM, Elbashir MI, Mavoungou E, Adam I**.** Cytokine Profiles in Peripheral, Placental and Cord Blood in an Area of Unstable Malaria Transmission in Eastern Sudan. Journal of Tropical Pediatrics 2009;55(4):233-7.

2. **Bayoumi NK,** Elhassan EM, Elbashir MI, Adam I**.** Cortisol, prolactin, cytokines and the susceptibility of pregnant Sudanese women to *Plasmodium falciparum* malaria. Annals of Tropical Medicine & Parasitology 2009; 103(2): 111–117.

3. Bakhet KH, **Bayoumi NK,** Adam I**.** Peripheral, Placental and Cord Cytokines Profile in Spontaneous Labor and Elective Caesarean Section. Iran J Immunol 2008; 5(3): 185-188.

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ABSTRACT

Introduction: Globally, 200 million pregnant women infected with malaria each year. Pregnant women are more susceptible to malaria infection than nonpregnant ones. It has been proposed that nonspecific immunosuppression may be caused by pregnancy-associated hormones. Cortisol and prolactin are among the most important candidates which affect maternal immunity to *Plasmodium falciparum* malaria. Understanding the hormonal and cytokine interactions that underlie susceptibility to the disease should be helpful in elucidating the pathogenesis of *Plasmodium falciparum* malaria during pregnancy.

Objectives: The current study was conducted in Wad Medani and New Halfa, areas characterized by unstable malarial transmission in central and eastern Sudan, respectively. Its aims were to investigate the role of and interactions between cortisol, prolactin, interferon-γ (IFN-γ), interleukin-4 (IL-4) and interleukin-10 (IL-10) in pregnant women with *Plasmodium falciparum* malaria and to investigate the cytokine profiles in peripheral, placental and cord blood in parturient women in these areas.

Methods: In Wad Medani, the 82 pregnant subjects who were enrolled either had uncomplicated, *Plasmodium falciparum* malaria (the 45 cases) or were apparently uninfected and healthy women (the 37 controls) who were well matched to the cases. Five ml of venous blood were withdrawn in plain tube, centrifuged and kept at -20 until processed in the laboratory for cortisol, prolactin and cytokine analysis. Total serum cortisol concentrations were determined with the ^{125}I-F RIA cortisol test kit, whereas[125]I-PRL IRMA prolactin test kit was used to determine serum prolactin concentrations. Enzyme-linked immunosorbent assay was used to measure the concentrations of the three cytokines, IFN-γ, IL-4 and IL-10. In New Halfa, 5 mL of maternal, placental and cord blood was collected immediately after delivery, quickly withdrawn in plain tube and centrifuged and kept at -20 until processed. Enzyme-linked immunosorbent assay was used to measure the concentrations of the three cytokines, IFN-γ, IL-4 and IL-10, in the sera from peripheral, placental and cord blood of 87 parturient women (53 were found to have past placental malaria infections).

Results: Wad Medani, compared with the controls, the cases were found to have significantly higher serum concentrations of total cortisol and IL-10 and significantly lower levels of prolactin and IFN-γ (but similar concentrations of IL-4). The hormone and cytokine concentrations measured in the infected primigravidae were similar to those recorded in the infected multigravidae. Among the cases, there was a significant positive correlation between serum cortisol and IL-10 (r=0.188; P=0.025) and significant negative correlations between prolactin and both IL-4 (r=20.175; P=0.038) and IL-10 (r=20.186; P=0.027) but no significant correlation between prolactin and cortisol. In New Halfa, The concentrations of these cytokines were significantly higher in peripheral and placental sera from uninfected women than in sera from infected women. IFN-γ concentrations were higher in the cord sera from uninfected women in comparison to the infected ones too. The levels of these cytokines were not significantly different between the primigravidae and multigravidae. Cord sera in all the groups had the lower levels of these cytokines. Strong positive correlations were observed between peripheral and placental cytokines.

Conclusions: In conclusion, it appears that, irrespective of parity, cortisol, prolactin and certain cytokines are key mediators in the host response to *Plasmodium falciparum* infection during pregnancy in women living in central Sudan, where malarial transmission is unstable. In eastern Sudan, the patterns of the immune responses that occur in placental, peripheral and cord blood were influenced by the malaria infections, irrespective to the parity. Additionally, immune response during *Plasmodium falciparum* infection is not different in the peripheral and placental compartments.

المستخلص

المقدمة: عالمياً تصاب حوالي 200 مليون إمرأة حامل بمرض الملاريا سنوياً. فالنساء الحوامل هن الأكثر عرضة للإصابة بمرض الملاريا من النساء غير الحوامل. وقد أقترح أنه من الممكن أن يتسبب الحمل المرتبط بالهرمونات في كبت المناعة غير المحدد. الكورتزول والبرولاكتين هي من ضمن أهم المرشحات التي تؤثر في مناعة الأم للملاريا من النوع بلازميديوم فالسبارم. فهم التفاعلات الهرمونية والسايتوكاين التي ترتكز علي القابلية للمرض هي من الأشياء التي تساعد في شرح وتوضيح تولد ومنشأ مرض الملاريا من النوع بلازميديوم فالسبارم أثناء الحمل.

الأهداف: تم إجراء الدراسة الحالية بودمدني وحلفا الجديدة، وهي مناطق تتميز بانتقال مرض الملاريا غير المستقر بوسط وشرق السودان. وتتمثل أهداف هذه الدراسة في البحث عن الدور والتفاعلات بين الكورتزول والبرولاكتين وانترفيرون-قاما (IFN-γ) وانترليوكين-4 (IL-4) و انترليوكين-10 (IL-10) لدي المرأة الحامل المصابة بالملاريا من النوع بلازميديوم فالسبارم وكذلك التقصي عن سيمات السايتوكاين في دم الأجزاء الطرفية و الحبل السري والمشيمة لدي المرأة عند المخاض في هذه المناطق.

المنهجية: تم تسجيل 82 من النساء الحوامل بودمدني اللائي إما يعانين من مرض الملاريا من النوع بلازميديوم فالسبارم غير المعقدة (45 حالة) أو اللائي يبدو عليهن أنهن ظاهريا غير مصابات وبصحة جيدة (37 مجموعة ضابطة) و اللائي تمت مماثلتهن مع الحالات. تم سحب 5 مللتر من الدم الوريدي في أنبوب إختبار نظيف ثم تمت له عملية الطرد المركزي وتم حفظه عند 20- حتي تمت المعالجة بالمختبر لتحليل الكورتزول والبرولاكتين والسايتوكاين. تم تحديد تركيزات الكورتيزول في مصل الدم بإستخدام أدوات إختبار الكورتيزول (I-F RIA ^{125}) بينما تم إستخدام أدوات إختبار البرولاكتين (I-PRL IRMA ^{125}) لتحديد تركيزات البرولاكتين في مصل الدم. تم إستخدام Enzyme-linked immunosorbent assay لقياس تركيزات السايتوكلاينات الثلاثة، IFN-γ, IL-4 and IL-10. بحلفا الجديدة، تم أخذ 5 مللتر من دم الأم المحيطي ودم الحبل السري والمشيمة مباشرة بعد الوضع و تم وضعه حالاً في أنبوب أختبار نظيف ثم إخضاعة لعملية الطرد المركزي وحفظه عند 20- حتي تمت معالجته. تم إستخدام Enzyme-linked immunosorbent assay لقياس تركيزات السايتوكلانات الثلاثة IFN-γ, IL-4 and IL-10 في الأمصال من الدم الحبلي والمشيمي والطرفي من النساء عند المخاض البالغ عددهن 87 (وجد أن 37 منهن كانت لديهن اصابات سابقة بمرض الملاريا المشيمي).

النتائج: فى ود مدني، مقارنة بالمجموعة الضابطة، وجد أن الحالات لديها تركيزات عالية للكورتيزول و (IL-10) في مصل الدم و مستويات أقل للبرولاكتين و INF-γ (لكن نفس تركيزات IL-4) تركيزات الهورمون والسايتوكاين

التي تم قياسها عند المرأة المصابة ذات الحمل للمرة الأولى كانت مشابهة لتلك التي تم تدوينها عند المرأة المصابة المتكررة الحمل. من بين الحالات، هنالك علاقة إرتباط موجبة ذات دلالة بين تركيزات الكورتيزول وIL-10 في مصل الدم ($r=0.188; P=0.025$) وعلاقة ارتباط ذات دلالة سالبة بين تركيزات البرولاكتين وكل من IL-4 (r=20.175; P=0.038) و IL-10 (r=20.186; P=0.027) إلا أنه ليس هنالك علاقة ذات دلالة بين تركيزات البرولاكتين والكورتيزول. في حلفا الجديدة، تركيزات هذه السايتوكاينات أعلي في مصل الدم المحيطي و المشيمة عند المرأة غير المصابة مقارنة بالنساء المصابات. تركيزات IFN-γ كانت أعلي في أمصال الدم من الحبل السري عند المرأة غير المصابة مقارنة بالنساء المصابات أيضاً. وجد أن مستويات هذه السايتوكاينات غير مختلفة بصورة كبيرة بين المرأة ذات الحمل للمرة الاولي والمرأة متكررت الحمل. أمصال الدم من الحبل السري في كل المجموعات لديها مستويات أقل من هذه السايتوكاينات. وتمت ملاحظة علاقات قوية موجبة بين السايتوكاينات في الأجزاء الطرفية والمشيمية.

الخلاصة: يبدو أنه بغض النظر عن الإنجابية، الكورتيزول والبرولاكتين و بعض السايتوكاينات هي الوسائط الرئيسية لإستجابة العائل للإصابة بالملاريا من النوع بلازميديوم فالسبارم أثناء الحمل لدي النساء اللائي يقطن في وسط السودان حيث انتقال الملاريا غير المستقر. وفي شرق السودان وجد أن أنماط الإستجابة المناعية التي تحدث في الدم المشيمي والطرفي والحبل السري تتأثر بالإصابة بمرض الملاريا بغض النظر عن الإنجابية. إضافةٌ الي ذلك، فإن الإستجابة المناعية أثناء الإصابة بالبلازميديوم فالسبارم لا تختلف في الأجزاء الطرفية والمشيمية.

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CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

1.1. Global Malaria Situation

Malaria is a major cause of illness and death globally and it is a common and serious tropical parasitic disease. It is caused by parasites of the genus *Plasmodium* of which four species are known to infect humans' namely *P. falciparum*, *P. vivax, P. ovale*, and *P. malariae* (1).

3.3 billion people live under the threat of malaria (50% of the world population) with a worldwide incidence of 247 million cases per year (86% in Africa) (2). It kills over a million each year – mostly children. Over 90 % of malaria death occurs in Africa (2) where around 66% of the populations are thought to be at risk. In contrast, less than 15% of the global total of malaria death occurs in Asia (including Eastern Europe), despite the fact that an estimated 49% of the people in this region are living under threat from the disease. In the Americas 14% of the population are at risk (1).

1.2. Malaria in Sudan

Malaria is a leading cause of morbidity and mortality in Sudan. It contributes an estimated 50% of all malaria cases in the region, an estimated 7.5 million cases and 35,000 death annually reported by World Health Organization (3). Never the less Abdalla SI *et al*. (2007) reported that the incidence of malaria in Sudan was estimated to be about 9 million episodes in 2002 and the number of deaths due to malaria was about 44,000. Children under five years of age had the highest burden. Males had the highest incidence

and mortality (4). Symptomatic malaria accounts for 20-40% of outpatient clinic visits and approximately 30% of hospital admissions (5,6).

The entire population of Sudan is at risk of malaria, although this occurs with different degrees. In the northern and western states malaria is mainly low to moderate with predominantly seasonal transmission and epidemic outbreaks. In southern Sudan, malaria is moderate to high or highly intense, generally with perennial transmission (6). In eastern Sudan, transmission and intensity of malaria is perennial and moderate rather than low (7). And there was a significant positive correlation between malaria cases and rainfall in the area, and epidemic malaria was found to associate with heavy rains (8).

The predominant parasite species is *Plasmodium falciparum*, whereas *Plasmodium ovale* is sporadically distributed. *Plasmodium malariae* is particularly considered with Southern Sudan, while in Eastern Sudan *Plasmodium vivax* is widely spread and close to the Ethiopian border it may reach up to 20 % (5,9). Nevertheless, Himeidan and collages proved that in eastern Sudan *Plasmodium falciparum* accounts of about 95% of malaria cases, whereas *Plasmodium vivax* and *Plasmodium ovale* accounts of 3% and 2% of malaria cases respectively (7). In New Halfa, eastern Sudan, cerebral malaria is more frequent during adolescence and early adulthood, and it is the major cause of malaria mortality (10). Uniquely in this area, cerebral malaria may be associated with latent parasitaemia in partially immune adults (11).

1.3. Malaria Parasite Life Cycle

Malaria parasites are transmitted by Anopheline mosquitoes. While feeding on its host, the mosquito releases the sporozoite forms of the parasite into the bloodstream, and within minutes the sporozoites invade hepatocytes. Over the ensuing week, the parasite multiplies 10,000-fold within the liver cell, which then ruptures, releasing merozoite forms of the parasite that rapidly invade red blood cells. The parasite matures and divides within the erythrocyte for 48-72 h (depending on the *Plasmodium* species), then causes the cell to rupture and release a new broad of merozoites that invade fresh red cells and
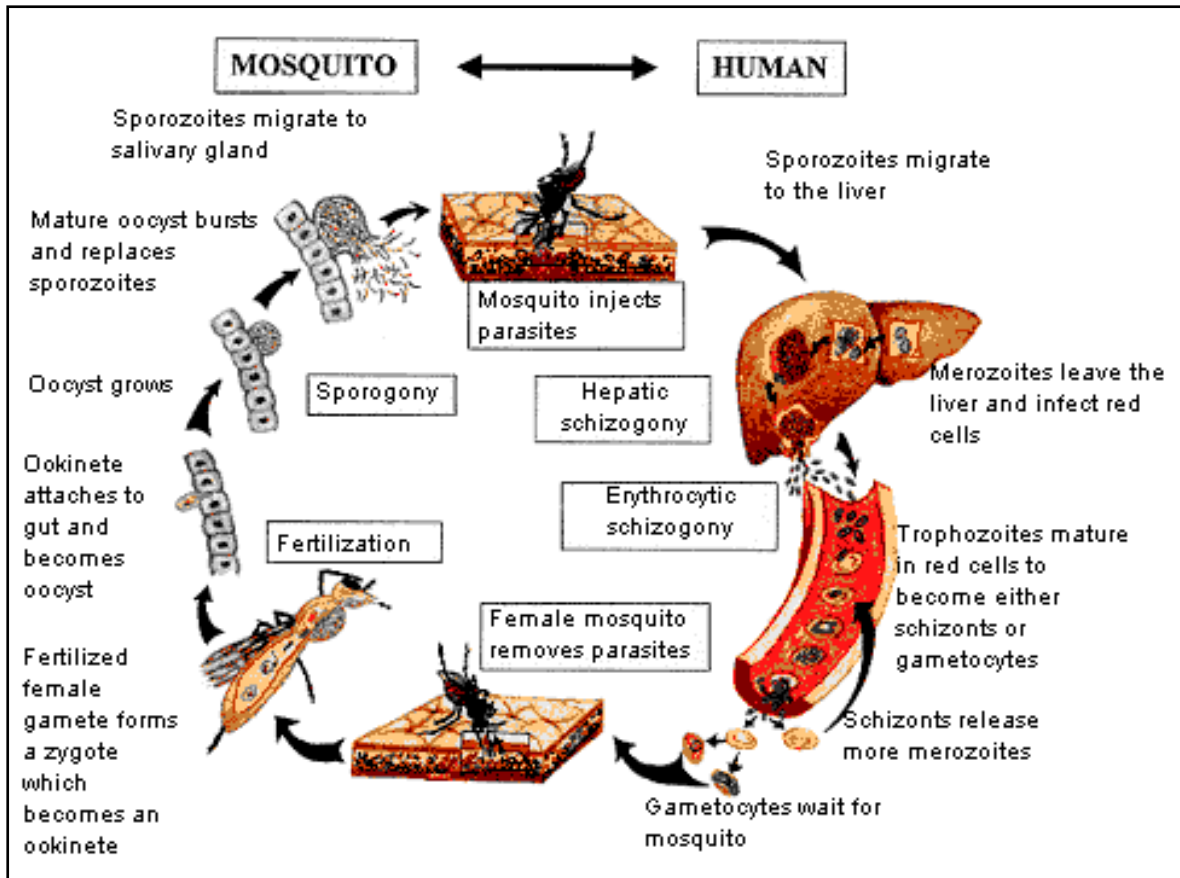
Figure 1.1: Malaria parasite life cycle

http://www.emro.who.int/rbm/AboutMalaria-QuickOverview.htm

resume the cycle (figure 1.1). Symptoms accompany the rupture of the red cells which explain the periodicity of malaria fevers.

1.4. Malaria in Pregnancy

Malaria is a public health problem throughout the world. More than 90% of the cases occur in sub-Saharan Africa where 25 million pregnant women are at risk of *Plasmodium falciparum* infection every year, and one in four women has evidence of placental infection at the time of delivery (12). It affects millions in developing countries, principally young children and pregnant women. Pregnant women are more likely to become infected than non-pregnant ones, and they are more susceptible in their first or second pregnancies (13,14,15).

In Africa, 30 million women living in malaria-endemic areas become pregnant each year. Malaria infection during pregnancy poses substantial risk to the mother, the fetus, and the neonate. It results in about 200,000 newborn deaths each year (12,16).

In malaria low transmission areas, levels of acquired immunity are low and pregnant women are susceptible to episodes of severe malaria, which can result in stillbirths or spontaneous abortion or in mother or fetus death (12). In high transmission areas where levels of acquired immunity tend to be high, women present an asymptomatic infection, which was more common in primigravidae (17). Nevertheless, pregnant women in high transmission areas suffer from substantial malaria-related morbidity and mortality, especially among low parity women. The association of susceptibility to malaria in pregnant women to parity suggests that protective immunity to this type of malaria can be developed (18).

In New Halfa, eastern Sudan, an area of unstable malaria transmission, Adam I *et al.* (2005) reported that the prevalence of *Plasmodium falciparum* malaria is considerable in pregnant women and severe cases occur (19). Pregnant women with blood group O were at higher risk of past-chronic placental malaria infection in the area (20) and maternal death due to severe pulmonary oedema caused by *Plasmodium falciparum* malaria was

recorded (21). Moreover it has been shown that primigravidae and all parities were infected with *falciparum* malaria, different severity manifestations were observed, and there were higher perinatal mortalities recorded in the area (22). Further study in the same area illustrated that there was a high prevalence of anaemia and folate deficiency and that ferritin, serum folate and vitamin B_{12} levels were not significantly associated with anaemia (23). In Gadarif area of eastern Sudan, placental malaria infections have been found to affect pregnant women irrespective of their age or parity (24). In Wad Medani, central Sudan, more than 50% of the women were parous and different forms of clinical presentations of severe malaria were observed, including cerebral malaria and hyperpyrexia (25).

Malaria during pregnancy increases the chance of anaemia in mothers (26) which, if severe, can increase the risk of maternal death. In Africa, anaemia due to malaria may cause as many as 10,000 maternal deaths every year (13). In Sudan, it has been shown that maternal anaemia was the most important and frequent complication of malaria during pregnancy (27). Similar results were observed in Malawi (28), Ethiopia (29), Thailand (30), Gabon (15), and the Republic of Yemen (31).

During pregnancy, malaria parasites in the placenta can interfere with the transfer of oxygen and nutrients from the mother to the unborn baby. Malaria infection in the mother, therefore, increases the risk of spontaneous abortion, stillbirth, preterm birth, and low birthweight (32). Shulman and Dorman (2003) reported that in malaria endemic areas pregnant women may not present with a high fever but are at high risk of severe anaemia and of delivering a low birthweight baby (33). In Africa about 5-14% of all low birthweight babies are born to mothers infected with malaria, and an estimated 3-5% of all infant deaths can also be traced to malaria infection in mothers. In some cases, malaria parasites can cross from the placenta into the baby's blood and cause anaemia in the baby (9). In Tanzania nearly 1 in 5 children born had a low birth weight (LBW), and >20% of these children were born prematurely (34). In Uganda it has been reported that malaria is an important cause of stillbirth and LBW (35). In Sudan LBW associated with malaria infection has been observed in central (36) and eastern (37) parts of the country.

1.5. Malaria Immunity

Humans with no previous experience of malaria infection immutably become ill on their first exposure to the parasite. They develop a febrile illness, which may become severe and, in some cases, may lead to death (38). Repeated or prolonged exposure to malaria infection ultimately leads to the development of clinical immunity such that, despite remaining susceptible to infection, parasite replication is controlled and the infection is eliminated without the development of clinical signs and symptoms (39).

Effective immunity to malaria has been clearly demonstrated among individuals naturally exposed to malaria (40), it is complex, and is essentially both species and stage specific. It is regulated by the synchronized action of the innate and adaptive immune systems in addition to environmental factors although the relative importance of each remains unclear (40,41). The cellular arm of the immune system is considered more important in controlling liver-stage infections, although antibodies contribute to protection; humoral immune mechanisms may be more important in controlling the blood stages (42). Immunity to malaria is only partial and it is rarely sterile, but it is associated with low-grade parasites via an episode of clinical disease through life or at least as long as the individual remains continuously in the endemic area.

In endemic areas, children born to immune mothers are protected by the passive transfer of maternal immunity for the first 6 months of life. As this attenuated, there is a progressive increase of acquired immunity with age but this is predisposed by 1 or 2 years of increased susceptibility to malaria before acquisition of active immunity. It was shown that the risk of becoming infected with malaria increased significantly at about the age of 18 weeks, indicating that children under the age of 18 weeks had a lower risk of becoming infected than children above that age and the vast majority of malaria infections in children under 5 months of age are of very low density and completely asymptomatic (43). In such areas, young children are particularly susceptible to malaria infection, and it has been estimated that a quarter of all childhood deaths are due to malaria. However, with continuous exposure, older children and adults ultimately develop complete protection from severe illness and death, although as mentioned above

sterile immunity is probably never achieved (38). Generally, acquisition of active immunity is slow and needs repeated parasite exposure to be maintained.

1.5.1. Malaria Immunity During Pregnancy

Pregnancy causes a number of physiological changes that affect the way the *Plasmodium* parasite invade its host. During pregnancy, an immune adaptation (Down regulation of normal maternal immune response) occurs to prevent the rejection of the fetus (44). However, in spite of this depression, the maternal immune system continues to respond to the parasite and antibodies preventing *Plasmodium falciparum*'s attachment to the placenta can be produced and associated with better outcomes for the fetus (45). Additionally, it has been shown that there were a significant variations in risk and severity of infection between primigravides and multigravides with risk and severity decreasing in proportion to the number of pregnancies (44,46). This suggests that immune build-up is achieved after several pregnancies and infections (47).

Cell mediated immunity is particularly suppressed during pregnancy, and the mother is increasingly reliant on humoral immunity. This immunosuppression was believed to account for pregnant women increased risk of infection, including malaria (47).

During pregnancy infection with *Plasmodium falciparum* malaria is associated with poor birth outcomes, including low birth weight (LBW) due to preterm delivery and intrauterine growth retardation, particularly among primigravidae (48). Malaria parasites sequester in the placenta during pregnancy (49). Placental parasite infection is associated with local immune responses, including elevated proinflammatory cytokine levels (50,51) and monocyte infiltration into the placental intervillous space (52).

In the endemic countries of Africa, children under the age of five years and pregnant women bear the brunt of the burden of malaria disease (16). This is because they have lower immunity to the disease compared to other people in the same environment. In pregnant women this may be due to the transient depression of their cell-mediated immunity that occurs to allow retention of the fetal allograft (53). This was supported by

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the finding that cellular immune responses to *Plasmodium falciparum* are depressed in pregnant women in comparison with non-pregnant control ones (48,54).

Susceptibility to pregnancy-associated malaria probably represents a combination of immunological and hormonal changes associated with pregnancy, in addition to the unique ability of a subset of infected erythrocytes to sequester in the placenta (55,56). The observation that infected erythrocytes accumulate in the maternal vascular area of the placenta (the intervillous space) in much higher densities than in the peripheral circulation is central to the pathogenesis of *Plasmodium falciparum* infection in pregnancy (57). Also sequester in the placenta are the trophozoite and schizont stages which are absent from peripheral blood (58). Additionally, Walter *et al.* (1982) observed that in pregnancy-associated malaria there is an increased numbers of maternal phagocytic cells especially monocytes in the intervillous space (49).

As we mentioned before malaria immunity is regulated by the synchronized action of the innate and adaptive immune systems in addition to environmental factors.

1.5.2. Innate Immunity to Malaria

Innate immunity to malaria which is the defense first line, can limit the peak of parasitaemia, prevent severe pathology and reduce the load of circulating infected cells (59). But it fails to eliminate the infection completely, leading to persistent low-grade parasitaemia, which might frequently fall below the limit of detection by microscopy, but it might persist for many months or years (60). However some individuals are naturally resistant to malaria infection while others are less likely to develop a severe form of the disease. The rupture of erythrocytic schizonts is usually accompanied by bouts of fever, nausea, headaches and other symptoms of a systemic proinflammatory cytokine response, much of which is now believed to be produced by cells of the innate immune system (61).

Dendritic cells, macrophages, natural killer (NK) cells, NK T cells, and γδ T cells help establishing the nature of the adaptive immune response to malaria. Early production of

immunoregulatory cytokines by these cells and antigen presentation by dendritic cells are probably important determinants of response to infection. In the placenta, macrophages aid in parasite elimination by phagocytosis and release of reactive oxygen intermediates as well as by enhancing innate responses through cytokines (62).

Innate immune response against *Plasmodium falciparum* is the result of several thousand years of co-evolution between the parasite and his host. An early IFN-γ production during infection is associated with a better evolution of the disease. Natural killer (NK) cells are among the first cells in peripheral blood to produce IFN-γ in response to *Plasmodium falciparum*-infected erythrocytes (63). IFN-γ is a parasiticidal macrophage activator and this may be of greater importance for innate malaria immunity. NK cells increase in number early in malaria infection and they are able to lyse *Plasmodium falciparum*-infected erythrocytes *in vitro*. They are found in blood, in secondary lymphoid organs as well as in peripheral non-lymphoid tissues (41,63). Activation of human NK cells by *Plasmodium falciparum* iRBCs (infected red blood cells) produce an early burst of IFN-γ and it requires two signals: 1.The first one is dependent upon contact between the NK cell and iRBC and 2. The second one is cytokine mediated and likely dependent upon interactions between iRBCs and dendritic cells or monocyte-macrophages. It has been shown that human NK cells form stable conjugates with iRBC but not with uninfected RBC and that production of IFN-γ is dependent upon direct contact between the NK cell and the iRBC. NK cells respond to iRBC only in the presence of a source of IL-12/IL-18 and there is heterogeneity in the ability between donors to respond to iRBC (39).

Innate resistance to *Plasmodium falciparum* malaria infection is usually partial, and may be associated with two factors: First the fact that malaria parasite fail to invade certain types of human RBCs as in the case of Melanesian Ovalocytosis an erythrocyte membrane defect, which generate ovalocytic cells that are resistant to invasion by all human malaria parasites (64). Also the Duffy negative cells are resistant to invasion by *Plasmodium vivax* only, because the receptor for the *Plasmodium vivax* merozoites on the red blood cell is associated with antigens of the Duffy blood groups. Second: the fact that certain host erythrocytes have a reduced ability to maintain parasites growth as in the case of individuals with G6PD (Glucose-6 phosphate dehydrogenase) deficiency or with

certain abnormalities of haemoglobin, such as sickle cell anaemia or β thalassemia (53). Bayoumi *et al.* (1990) supported the hypotheses that the sickle cell trait protects individuals from *Plasmodium falciparum* infections, at least in part, by modulating the immune response. Furthermore, they mentioned that lymphocytes of individuals carrying the HbAS genotype present higher reactivity to malaria antigens in proliferative assay than lymphocytes from HbAA controls living in the same area (65).

Malaria infection gives rise to highly increased concentration of non-malaria-specific immunoglobulin. This is also true for the $CD4^+$ T cells from malaria-naïve donors responding by *in vitro* proliferation and cytokine production upon exposure to malaria antigens (41).

1.5.3. Acquired Immunity to Malaria

Plasmodium falciparum infection can lead to substantial protective immunity to malaria. Naturally acquired immunity to *falciparum* malaria protects millions of people routinely exposed to *Plasmodium falciparum* infection from severe disease and death and available evidence suggests that acquisition of protection against some severe malaria syndromes can be fairly rapid. Acquisition of protection following natural parasite exposure is a slow process that may take years or decades to develop and probably sterile immunity never results from it (66). However Doolan *et al.* (2009) mentioned that naturally acquired immunity should be appreciated as being virtually 100% effective against severe disease and death among heavily exposed adults. Even in exposed infants the immunity that occurs may exceed 90% effectiveness. Among high-risk infants in sub-Saharan Africa, the induction of an adult-like immune status would greatly diminish disease and death caused by *Plasmodium falciparum* infection (67). Children who start building immunity after the age of 1 year do so at a slower rate than those who start in infancy (68). The age at which acquired immunity becomes evident varies depending on the level of exposure, with higher exposure resulting in earlier development of clinical immunity. Children living in areas of high endemicity experience less frequent episodes of malaria after the

age of 5 years, while people in areas of hypoendemicity may never develop clinical immunity (69).

There are two types of adaptive immune response, called humoral immunity and cell mediated immunity, which are mediated by different components of the immune system and function to eliminate the infection.

1.5.3.1. Humoral Immunity

The humoral immunity is the aspect of immunity that is mediated by secreted antibodies produced in the cells of the B lymphocytes (B cell). These secreted antibodies bind to antigens on the surfaces of invading parasites, which flags them for destruction.

In endemic areas, *Plasmodium falciparum* infection lead to a potent humoral immune responses, involving the production of immunoglobulins (Igs, antibodies Ab) by B lymphocytes, of which IgM and IgG predominates. It was found that both anti-malarial IgG and IgM antibodies were increased after malaria outbreaks (70). Moreover, a potential role for protection is provided by IgG isotypes, both in animal models and in humans (71). Roussilhon *et al*. (2007) proved that IgG3 antibodies can naturally develop along with protection against *Plasmodium falciparum* infection in young children, and that these antibodies have been found to achieve parasite killing under *in vitro* and *in vivo* conditions, and they can be readily elicited by immunization in naïve volunteers (72). In Brazil endemic areas highest levels of IgG, IgG1, IgG2 and IgG3 antibodies were observed in individuals with asymptomatic and uncomplicated malaria, while highest levels of IgG4, IgE and IgM antibodies were predominant among individuals with complicated malaria. A predominance of IgG1, IgG2 and IgG3 antibodies were found in individuals reporting more than five previous clinical malaria attacks, while IgM, IgA and IgE antibodies predominated among individuals reporting five or less previous clinical malaria attacks. It has been elucidated that there was differential regulation in the anti- *Plasmodium falciparum* antibody pattern in different clinical expressions of malaria

and that even in unstable transmission areas, protective immunity against malaria can be observed, when the appropriated antibodies are produced (73).

The role of antibodies in clinical protection against malaria erythrocytic stages has long been recognized by *in vivo* transfer of antibodies from protected adults to nonprotected individuals infected with *Plasmodium falciparum* malaria (74,75). Additionally, the induction of these antibodies might differ according to the nature of the malaria parasite antigen and the level of malaria transmission (76).

Large proportion of these immunoglobulins is not malaria-specific, however 5% or more is species, and stage specific reacting with a wide variety of the parasite antigens (41). Passive transfer of IgG from immune donors indicates that antibodies may be protective by decreasing parasitaemia and clinical disease (75). Edozein *et al.* (1962) observed that the treatment of children having acute *Plasmodium falciparum* malaria with γ-globulin result in a constant fall in the trophozoite-counts by the 4th day, and that their blood was found to be negative by the 8th day (77).

Crucial to the acquired protective immunity to *Plasmodium falciparum* malaria is the cooperation between monocytes and antibodies. The antidodies produced in response to *Plasmodium falciparum* infection are of particular importance, since certain isotypes known as cytophilic antidodies can cooperate with monocytes via FcγRI and FcγRII receptors in opsonization and phagocytosis or participate in both antibody-dependent cellular inhibition (ADCI), as well as antibody-dependent cellular cytotoxicity (ADCC) (78-80). FcγR (fragment crystalline gamma receptor) is a specific cell surface receptor for IgG molecules. They are largely expressed by neutrophils, monocytes and macrophages, natural killer (NK) cells, platelets, eosinophils, basophils, mast cells and B cells. Some of these cells express only one or two of the three possible FcγR receptors, the high affinity FcγR-I, and the low affinity FcγR-II and FcγR-III (81).

Guitard *et al*. (2008) reported that primigravidae infected during pregnancy present higher level of IgG3 at delivery than at enrolment. This suggests that this IgG subtype is predominantly implicated in the protection and acquisition of immunity against a placental infection (82).

In Kenya, antibodies that inhibit *Plasmodium falciparum* adhesion to the placental receptor chondroitin sulfate A (CSA) are found to be associated with a reduced risk of placental malaria. Furthermore, these antibodies to placental parasites are associated with reduced levels of placental parasitemia and increased birth weights and gestational ages of newborns (45). Consequently, antibodies inhibiting CSA-specific parasite sequestration in the placenta are considered to be important in acquisition of protection against PAM (Pregnancy-associated malaria) (83). Moreover, it is proved that the isotype/subtype profile of anti-VSA antibodies (anti-variant surface antigens antibodies) IgG1 and IgG3 does not alter with age, gravidity, or repeated infection (84).

It has been suggested that there is no effect of transmission intensity in the protective effect of antibodies inhibit *Plasmodium falciparum* adhesion to the placental receptor chondroitin sulfate A (45). In contrast, it has been reported that the anti-VSA IgG levels depend on transmission intensity (85).

1.5.3.2. Cell Mediated Immunity

Cell-mediated immunity is an immune response that mediated by cells known as T lymphocytes. It involves the activation of macrophages, natural killer cells (NK), antigen-specific cytotoxic T lymphocytes, and the release of various cytokines in response to an antigen. T lymphocytes can be distinguished from other lymphocyte types, such as B cells and natural killer cells by the presence of a special receptor on their cell surface called T cell receptors (TCR).

T lymphocytes, are further subdivided into functionally distinct populations, the best defined of which are helper T cells (Th) and cytotoxic T cells. Helper (CD4$^+$) and cytotoxic (CD8$^+$) T cells recognize antigens presented by the major histocompatibility complex (MHC) on the surfaces of other cells. The CD4$^+$ or T helper (Th) cells play a central role in most immune reactions, they comprise at least two functionally distinct cell types Th1 and Th2 distinguished on the basis of their cytokine production. Th1 cells produce proinflammatory cytokines such as interferon-γ (IFN-γ) and mediate cellular

immunity; in contrast, Th2 cells produce cytokines (interleukin-4 (IL-4), IL-5, IL-9, IL-10, IL-13, and so on) which regulate B-cell proliferation and antibody class-switching and consequently regulate humoral immunity (86).

Cellular immune mechanisms regulate immunity to malaria acute infections by mechanisms possibly augmented by cytophilic Abs, which are absolutely dependent on the macrophage-activating cytokine IFN-γ. Cell-mediated immune effector mechanisms to *Plasmodium falciparum* infection, involve macrophage activation by NK cell-, γδT cell- or Th1-derived IFN-γ for enhanced phagocytosis and killing of parasitized erythrocytes (87), and inhibition of parasite growth and development inside hepatocytes by CD8^{+} cytotoxic cells (88). Nitric oxide (NO), produced by macrophages in response to parasitic components and T cell production, can have antiparasitic effects (89). It has been shown to kill *Plasmodium falciparum* parasites *in vitro* at high concentrations (90).

T cells from different subsets play a major role in protective immunity against pre-erythrocytic stages of malaria parasites. CD4^{+} T cells play a triple role in protective immunity against the liver stages of the malaria parasite. CD4^{+} T cells: 1- help B cells to induce a high level of antimalaria humoral response; 2- assist in the induction of CD8^{+} T cell responses; and 3- directly inhibit the development of liver-stage parasites. Additionally exposure of humans and animals to malaria sporozoites induces αβ CD8^{+} and CD4^{+} T cells specific for antigens expressed in pre-erythrocytic stages of the parasite. These T cells inhibit parasite development in the liver (88). MHC class-I-restricted CD8^{+} cytotoxic T cells have a great significance in pre-erythroctic immunity and contribute to protection against severe malaria. It has been proposed that CD8^{+} T cells may be involved in immunosuppression activity in acute malaria and down modulate inflammatory response. CD8^{+} cytotoxic T lymphocytes have no effect on human erythrocytes because they do not express MHC class I molecules. In contrast to the CD8^{+} T cells, CD4^{+} cells have an important regulatory and effector function (91,92).

During pregnancy, the immune system may be biased toward type 2 humoral defense mechanisms rather than towards type 1 cellular responses, this may be fundamental for fetal wellbeing (93). This was convinced by the finding that Th2 type responses were

associated with successful pregnancy, whereas Th1 type responses were associated with some forms of pregnancy failure (55). The balance between cytokines produced by different cell types is critical for the course of infection (92).

1.6. Cytokines

Cytokines are regulatory polypeptides or glycoproteins that can be produced by virtually every nucleated cell type in the body and have pleiotropic regulatory effects on hematopoietic, endocrine, neural and many other cell types (94). Cytokines usually act close to where they are produced, either on the same cell that secrets the cytokine (autocrine) or on a nearby cell (paracrine). When occasionally produced in large amounts, cytokines spilling over into the circulation to act at a distance from the site of production as endocrine mediators (95).

In the immune system, $CD4^+$ T cells play a central role because they produce large amounts of cytokines and regulate a variety of immune functions (86). As mentioned above, $CD4^+$ T cells can be classified into Th1 and Th2 types according to their pattern of cytokine production (96).

The Th1 type cells are associated with cytotoxic T cell functions, whereas Th2 cells are involved in the antibody production response (97). These T cell subsets reciprocally regulate one another since the proliferation and functions of Th2 cells were inhibited by IFN-γ, one of the Th1 products, whereas the Th2 products, IL-4 and IL-10, suppress cytokine production by Th1 cells (98,99). The balance between these subsets is critical for the outcome of an infection (100).

Angulo and Fresno (2002) in their review concluded that each cytokine has a different role at different stages of the infectious process (101). Significant to parasite clearance in *Plasmodium falciparum* malaria is the secretion of proinflammatory cytokines; however these inflammatory cytokines must be downregulated at the appropriate point in the infection to prevent pathology. The timing and possibly the site of cytokine release and the relative concentrations of the counteractive groups of cytokines, contribute to

successful resolution of the infection (102). It is conceivable that an early production of Th1 proinflammatory cytokines such as IL-12, IFN-γ and IL-18 is crucial to a better response and resolution of malaria infection (103), nevertheless it leads to up-regulation of TNF-α, which is believed to be the principle mediator of malaria pathology (102).

During the acute phase of uncomplicated *Plasmodium falciparum* malaria increase of the Th1 cytokine IFN-γ may play a role in limiting progression from uncomplicated malaria to severe and life-threatening complications (92). It has been shown that CD4$^+$ T cells, of either Th1 or Th2 type have regulatory functions in human *Plasmodium falciparum* malaria. Both Th1 and Th2 responses seem to be required for the control of infection, but they need to be sufficiently harmonized in intensity and time (92,100).

Manifestations of malaria disease varied and appear to be regulated by age and the acquisition of immunity, host and parasite genetic polymorphisms, and regional variation (104). It has been mentioned that an early proinflammatory cytokine response mediates protective immunity, whereas a late response contributes to pathology (105). Absolute levels and ratios of proinflammatory and anti-inflammatory cytokines influence susceptibility to infection, clinical disease, and anaemia. High ratios of proinflammatory to anti-inflammatory cytokines were proved to be associated with increased risk of fever (102). Inflammatory cytokines play a significant role in human immune responses to malaria, inspite of the fact that the balance between pro- and anti-inflammatory cytokines and the pathogenic effects that can result from dysregulation are poorly understood. Many studies proved that the severity of malarial disease is affected by the balance of proinflammatory to anti-inflammatory cytokines in plasma (104,106-111).

Pregnancy is an event of immunologic tolerance, whereby a woman accepts the implantation of the fetal allograft in her uterus (112). The implantation process of the human placenta associated with a release of cytokines at and around the site of implantation (113). It is believed that these cytokines may play an important role in the development of pregnancy (114). During pregnancy, the immune system may be biased toward type 2 humoral defense mechanisms rather than towards type 1 cellular responses, this may be fundamental for fetal wellbeing (99). The systemic suppression of

proinflammatory responses from T helper 1 (Th1) cells, i.e., decreased circulating levels of IFN-γ and tumor necrosis factor α, along with increased local expression of anti-inflammatory cytokines such as interleukin (IL)-4, IL-6, and IL-10, has been reported (112).

Placental malaria is associated with cell mediated inflammatory responses and alters the cytokine balance in favor of Th1 types (e.g. proinflammatory) (113,114). The placental production of chemokines may be an important trigger for monocyte accumulation in the placenta (115). IFN-γ appears to play a key role in protecting against placental parasitemia, as does IL-10, in preventing pathogenesis (116). Additionally, it has been shown that placental cytokine changes are associated with poor pregnancy outcomes in humans (50). Alterations in cytokine levels may contribute to preterm deliveries (PTDs) through the induction of anaemia and/or altering to cellular immune responses required for eliminating placental parasites. In pregnant women, malarial parasites sequester in the placenta and stimulate the accumulation of activated macrophages in the intervillous space where they secrete large amounts of TNF-α. In response to this inflammatory challenge, maternal and fetal cells secrete IL-10 to limit pathology and to protect the fetal allograft. Although IL-10 is important in modulating the possible deleterious effects of inflammatory cytokines, its over-expression may have detrimental consequences resulting in PTD by enhancing maternal anaemia and suppressing anti-parasite inflammatory responses leading to persistent placental parasitemia (117).

1.6.1. Interferon Gamma (IFN-γ)

IFN-γ serves critical functions in innate and cell-mediated immunity. It is the principle macrophage-activating cytokine. It is produced by CD4$^+$ T helper cell type 1 (Th1) lymphocytes, CD8$^+$ cytotoxic lymphocytes, and NK cells (95). However, there is now evidence that other cells, such as B cells, NKT cells, and professional antigen-presenting cells (APCs) secrete IFN-γ (118). Recently, it has been proved that IFN-γ production can also occur in other cell types, including monocyte/macrophages (119). Furthermore, production of IFN-γ by professional antigen presenting cells (APCs) such as

monocyte/macrophage, dendritic cells (DCs) acting locally, may be important in cell self-activation and activation of nearby cells (119,120). In early host defense against infection, NK cells and possibly professional APCs is likely to be important producers of IFN-γ, whereas, in the adaptive immune response, T lymphocytes become the major source of it (120,121).

IFN-γ, or type II interferon, is an important cytokine in cell-mediated immunity against intracellular microbes. It enhances the microbicidal function of macrophages by inducing the synthesis of reactive oxygen intermediates and nitric oxide in addition to stimulating expression of class I and class II MHC molecules and costimulators on APCs. Moreover, the differentiation of naïve CD4$^+$ T cells to the Th1 subset and the inhibition of the proliferation of Th2 cells were found to be promoted by IFN-γ. Furthermore, IFN-γ control IgG class switching in B cells, activates neutrophils, and stimulates the cytolytic activity of NK cells (95).

It has been reported that, cytokines secreted by APCs control IFN-γ production, most notably interleukin IL-12 and IL-18. These cytokines serve as a bridge to link IFN-γ production with infection in the innate immune response (122-128). The recognition of many pathogens by macrophages induces secretion of IL-12 and Chemokines. These chemokines causes attraction of NK cells to the site of inflammation, and IL-12 promotes IFN-γ synthesis in these cells (129,130).

Upon appropriate activation by pathogens, macrophages are known to be important producers of IL-12 or IL-18. Combined stimulation of macrophages with IL-12 and IL-18 promote macrophages to secrete high levels of IFN-γ leading to autocrine macrophage activation (125,131). Although T cells are the major source of IFN-γ, IL-18 and IL-12 act synergistically to increase IFN-γ production from murine bone marrow–derived macrophages. Moreover several studies proved that, the combined action of IL-18 plus IL-12 was far more effective in inducing IFN-γ production from these macrophagic cells than either cytokine alone (125,128). It has been shown that, IL-12 is needed for IL-18–induced IFN-γ production and that IL-18 induces IFN-γ production only when its receptor is unregulated by IL-12 (128). Production of IFN-γ is increased by IL-1, IL-2,

growth factors, estrogen, and IFN-γ itself and is inhibited by glucocorticoids, transforming growth factor-β (TGF-β), IL-4 and IL-10 (121,131). Nevertheless, Fukao *et al.* (2000) reported that IL-4 and IL-18 increasing IFN-γ production by denderitic cells (DCs) (122).

In humans, serum IFN-γ levels have been correlated with protection and resistance to re-infection with *Plasmodium falciparum* (132). In pre-erythrocytic immunity, IFN-γ is clearly associated with protective immunity. In Africa, IFN-γ production by CD4^{+} T cells to specific erythrocytic antigens is associated with protection against malaria re-infection. In murine malaria, T cell clones secreting IFN-γ have protected role possibly mediated by macrophages and neutrophils. Cytophilic IgG blood-stage-specific antibodies production may be induced by IFN-γ which may also assist in antibody-dependent cellular inhibitory mechanisms (133).

IFN-γ was associated with protection from high-density infection but not from low-density infection. The predominant sources of early IFN-γ were γδ T and αβ T cells, suggesting that IFN-γ –associated protection is mediated, in part, by γδ T and αβ T cells. The αβ T cells seem to be malaria-specific memory T cells, while on the other hand the γδ T cells may be previously unprimed cells, memory cells, or both (134).

On the other hand, IFN-γ has been clearly linked to the onset of pathology in mice as well as in humans. The detrimental effects of IFN-γ are believed to be due to its ability to activate macrophages which, in turn, produce endogenous pyrogens such as TNF-α, IL-1 and IL-6, leading to an inflammatory cascade (108,135). Moreover, the role of IFN-γ as an endogenous pyrogen is consolidated by the findings of Harpaz *et al.* (1992). (136).

Thus, developing clinical immunity may depend on the ability to down-regulate the nonprotective cross-reactive T cell response, leaving the innate response and protective T cells specifically primed by malaria infection to control parasitemia. As a consequence of the dual role of IFNγ, its production needs to be tightly regulated in order to achieve clearance of infection while on the contrary avoiding detrimental effects, a state which is characteristic of clinical immunity. This indicates that the cellular sources of IFN-γ and the balance between innate and adaptive sources of IFN-γ may change depending on

level of immunity, which may in turn influence the absolute levels that are produced (137).

1.6.2. Interleukin-10 (IL-10)

IL-10 is a pleiotropic cytokine produced by monocytes, macrophages, and lymphocytes. It has been implicated as an important regulator of the functions of lymphoid and myeloid cells. It is a potent suppressor of the effector functions of macrophages, T cells, and NK cells as a consequence of its ability to block activation of cytokine synthesis and several accessory cell functions of macrophages. Additionally, IL-10 seems to contribute to the regulation of proliferation and differentiation of B cells, mast cells, and thymocytes (138). It is also has a role in the downregulation of class II MHC expression and in the inhibition of the production of proinflammatory cytokines by monocytes (139).

Pregnancy is proposed to be a Th2 phenomenon, where Th2 cytokines inhibit Th1 responses to allow foetal survival. The significance of IL-10 as an immunomodulatory cytokine produced by Th2 cells, in the maintenance of normal pregnancy is becoming increasingly evident (140). It has been reported that IL-10 characterizes normal human pregnancy and is believed to prevent inflammatory responses that might interfere with the integrity of the materno-fetal placental barrier (93,141). During placental malaria, in spite of the placental shift toward Th1-type cytokines, IL-10 concentrations are elevated compared with healthy placentas (50,117,140). Nevertheless, in Kenya, normal placentas showed a bias toward type 2 cytokines; type 1 cytokines IFN-γ and IL-2 were absent in placentas not exposed to malaria but present in a large proportion of placentas from a holoendemic area. TNF-α and TGF-β (transforming growth factor-β) concentrations were significantly higher, and IL-10 concentrations significantly lower, in placentas from the holoendemic area. Consequently, maternal malaria decreases IL-10 concentrations and elicits IFN-γ, IL-2, and TNF-α in the placenta, shifting the balance toward type 1 cytokines. Among primigravidas, elevations of placental IFN-γ at all stages of infection, in association with elevated concentrations of TNF-α and possibly IL-2, are associated with poor outcomes for both mother and child (50).

In *Plasmodium falciparum* malaria, the balance between Th1 cytokines such as TNF-α, IFN-γ and Th2 cytokines such as IL-10, IL-4 may be critical in the development of severe malaria. It has been reported that higher plasma IL-10 concentrations over TNF-α levels might provide protection against severe malarial anaemia by down-regulating the severe pathologic effects of TNF-α. Consequently, higher levels of IL-10 versus TNF-α may prevent development of malaria anaemia by controlling the extreme inflammatory activities of TNF-α (107). Moreover, in experimental cerebral malaria, IL-10 was found to play a protective role (142).

Additionally, IL-10 was found in the cord blood serum, and its levels were conversely correlated with gestational age (143). The presence of IL-10 during T cell priming, further suppresses the generation of a Th1 response by down-regulating MHC class I and II expression, thus reducing APC function (139,144,145). Moreover, IL-10 may promote T cells differentiation into regulatory, IL-10-secreting T cells which suppress Ag-specific (antigen specific) effector responses (146-150). It has been shown that IL-10 production was suppressed by IFN-γ and that IFN-γ, and IL-10 antagonizes each other's production and function (151). Nevertheless, IL-10 was found to be produced by human uterine natural killer cells but does not affect their production of IFN-γ (152).

Peyron F *et al.* (1994) found an association between circulating IL-10 and the presence of clinical symptoms. Elevated levels of circulating IL-10 has been observed in patients with mild malaria, even much less than observed in patients with severe disease. Nevertheless, there was no correlation between the levels of IL-10 and fever or parasitaemia (153). Moreover, in rodent malaria IL-10 as a regulatory cytokine is found to be associated with disease exacerbation (154). Nevertheless, a defect in interleukin-10 leads to enhanced malarial disease in *Plasmodium chabaudi chabaudi* infection in mice (155).

The balance between proinflammatory and regulatory immune responses is fundamental to the outcome of malaria infection. Failure to develop an effective proinflammatory response can result in unrestricted parasite replication, while failure of the regulation of this response results in the development of severe immunopathology. IL-10 and TGF-β

are reported as important components of the regulatory response. IL-10 is considered to control inflammation during malaria infections and thus protect against immuno-pathology, but, on the other hand, it reduces the effectiveness of other immune mechanisms which remove the parasites. During malaria infection the major source of IL-10 is adaptive regulatory CD4$^+$ T cells. IL-10 produced by these adaptive CD4$^+$ T cells prevents hepatic immunopathology but also suppresses the effector T cell response, preventing parasite clearance (156).

1.6.3. Interleukin-4 (IL-4)

IL-4 is a highly pleiotropic, anti-inflammatory cytokine (157). It is the major stimulus for the production of IgE antibodies (95). γδ T cells are seemed to be essential for inducing IL-4-dependent IgE and IgG1 responses (158). IL-4 was found to be the essential differentiation factor for Th2 cells development from naïve CD4$^+$ T helper cells and at the same time it acts as a potent inhibitor of the development of Th1 induced by IFN-γ and IL-12. On the other hand, it has been suggested that a combination of IL-4 and TGF-β may provide an alternative IL-12-independent pathway of Th1 development (159). Additionally, IL-4 is a critical regulator of the commitment of CD4$^+$ T cells to the production of IL-4 and to the inhibition of their production of IFN-γ (160). Furthermore, IL-4 is a major inducer and mediator of allergic and parasitic immune responses. Promoting the differentiation of naïve CD4$^+$ T cells into Th2 cells is a major mechanism through which it mediates this function (160-164). Chen *et al*. (2004) suggested that IL-4 is a potent directing factor for bone marrow progenitor cells to differentiate into Th2 cytokine-producing eosinophils (165). Additionally, tissue basophils are known to be the principle source of *in vivo* IL-4 production in parasitic infections which induces a Th2-type response causes their accumulation (166).

IL-4 is found to be produced by many types of cells include cells of the mast cell/basophil lineage, eosinophils, NK1.1$^+$, CD4$^+$ T cells, NK T cells, γδ T cells, and conventional CD4$^+$ T cells (167). Initial IL-4 producers are of great importance because they are thought to produce the first burst of IL-4 to prime naïve CD4$^+$ T cells into Th2

cells and results in the initiation of the Th2 response. It has been shown that a small percent of CD4^{+} T cells that have no previous experience of antigenic stimulation might produce IL-4 which initiate Th2 immune response (168). Thus, autocrine IL-4 production by naïve CD4 T cells can drive the appearance of Th2 cells (167). IL-4 antagonize the IFN-γ macrophage-activating effects and consequently inhibits cell-mediated immune reactions and this is considered as one of the mechanisms by which Th2 cells acts as inhibitors of immune inflammation (95).

During malaria infection, IL-4 secreted by CD4^{+} T cells was found to be crucial to the development of CD8^{+} T cell responses against hepatocytes infected with malaria parasites. CD8^{+} T cell can inhibit the development of malaria liver stages. The main function of IL-4-secreting CD4^{+} T cells may be to maintain the proliferative activity of recently activated CD8 cells, to prevent their death after activation, or both (169). In West Africa, it has been reported that the malaria protected Fulani had significantly higher serum levels of anti-malaria IgG and IgE antibodies and higher proportions of malaria specific IL-4 and IFN-γ producing cells, when compared to their sympatric ethnic neighbors, the Dogon. This association of higher anti-malarial IgE and IgG antibodies and increased numbers of specific IL-4- and IFN-γ-producing cells may assist in explaining the difference in the antibody responses observed between the two study groups and the lower susceptibility to malaria observed in the Fulani. Additionally, the higher proportions of malaria specific IL-4 and IFN-γ producing cells was explained by the role of CD1- restricted NKT cells in immune protection (170). Studies in *Plasmodium chabaudi* IL-4-deficient mice showed that IL-4 per se is not required for parasite elimination and limitation of cytokine-induced tissue damage (171,172).

As mentioned before, during pregnancy, the immune system may be biased toward type 2 humoral defense mechanisms rather than towards type 1 cellular responses, this may be fundamental for fetal wellbeing (99). This is supported by the findings of Fried *et al.* (1998) who proved the predominance of type 2 cytokines (IL-4, IL-6, and IL-10) and the absence of type 1 cytokines (IFN-γ and IL-2) in placentas from Nairobi, Kenya (50). Moreover, it has been evident that maternal T lymphocytes at foeto–maternal interface play an important role in the fetal development and survival. Additionally, cells from the

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cumulus oophorus were found to constitutively produce IL-4 but the mechanisms responsible for this production are still unknown (167). It has been reported that progesterone which is highly produced by the *cumulus oophorus*/oocyte complex, up-regulates the production of LIF (leukemia inhibitory factor) by T cells (of the *cumulus oophorus*) and that the progesterone-induced LIF production is mediated by IL-4. Progesterone produced by cumulus granulosa cells may favor IL-4 production by T cells, which in turn can produce LIF. As a consequence of the role of LIF in the enhancement of the *in vitro* growth and development of mammalian embryos, it has been suggested that T cells present in the cumulus oophorus produce cytokines that may provide a microenvironment suitable for pre-implantation development of the mammalian embryo (173). T cells in the cumulus oophorus were found to produce higher levels of IL-4 than the T cells of peripheral blood from the same women. Additionally, the development and function of Th1 cells and macrophages can be inhibited by IL-4 and IL-10 and this will consequently lead to prevention of the allograft rejection (167).

1.7. Hormones

The word hormone is derived from the Greek hormao meaning 'I excite or arouse'. They are chemicals released by cells that affect cells in other parts of the body. Only a small amount of hormone is required to alter cell metabolism. It is essentially a chemical messenger that transports a signal from one cell to another. All multicellular organisms produce hormones. Hormones communicate their effect by their unique chemical structures recognized by specific receptors on their target cells, by their patterns of secretion and their concentrations in the general or localized circulation. A single hormone may affect more than one function and each function may be controlled by several hormones.

Endocrine hormone molecules are secreted directly into the bloodstream, while exocrine hormones (or ectohormones) are secreted directly into a duct, and from the duct they either flow into the bloodstream or they flow from cell to cell by diffusion in a process known as paracrine signaling (174,175).

1.7.1. Hormones and cytokines

Accumulated information illustrates that the immune system have an influence on the endocrine system and vice versa. It has been proved that cytokines interact and modulate steroidogenesis at the levels of the adrenal glands, testes, and ovaries, affecting their function and development (176). The modulation action of the immune system on the endocrine system was shown by the use of antigenic stimuli or cytokines in experimental animals resulting in an obvious alteration in the hypothalamus–pituitary–adrenal (HPA) axis activity. This was consolidated by results from human studies after administration of IL-6 or TNF. Additionally, synthesis of steroid hormones by cells of the endocrine system was conspicuously affected by certain cytokines such as TNF and TGFβ1. On the other hand, the endocrine system hormones have a modulatory effect on the functions of the immune system exemplified by the inhibition of cytokines by cortisol, estrogens, testosterone, and dehydroepiandrosterone. This illustrates that the functions of the two systems are closely linked together (177).

1.7.2. Pregnancy associated hormones, cytokines and susceptibility to malaria

Pregnant women are more susceptible to *Plasmodium falciparum* malaria than nonpregnant ones. The mechanisms responsible for their incremented susceptibility to asymptomatic infection, elevated parasitaemia and clinical episodes are unknown. Nevertheless, pregnancy-associated hormones are regarded to have a role since they down-regulate innate and acquired immune responses.

Cytokines play a significant role in the modulation of immune responses. Studies on cytokines produced at the maternal–fetal interface and their regulation illustrates that a local shift in the cytokine pattern from Th1 towards Th2 associated with successful pregnancy (99).

Naïve CD4$^+$ T cells are referred to as precursors of Th cells, their development into type1 Th1 and type 2 Th2 effector cells can be affected by many factors including hormones (178). Some evidence suggests that the differentiation of Th cells into polarized Th1 or

Th2 cells is influenced by steroidal and non-steroidal hormones (178,179,180). At the placental level progesteron production may be responsible in part, for increased production of Th2-type cytokines which have been involved in survival of the fetal allograft and maintenance of successful pregnancy (179). The production of IL-4 cytokine was found to be promoted by progesterone, while the production of IFN-γ by T cells was promoted by relaxin. Leukemia inhibitory factor (LIF) which is important in embryo implantation is up-regulated by IL-4 and progesterone. Furthermore, the production by decidual T cells of LIF and/or Th2 cytokines participate in the retention of pregnancy (178). Both IL-4 and IL-10 can inhibit the development and function of Th1 cells and macrophages and consequently prevent the rejection of the fetal allograft. A defect in the integrity of the hormonal-cytokine network at the maternofetal interface can result in fetal loss (181). Piccinni and collages proved that during pregnancy progesterone levels were higher than physiological concentrations excreting a positive modulatory effect on the production of Th2-type cytokines (e.g. IL-4). Moreover, they showed that relaxin may counterbalance this effect on Th2 excerted by progesterone, and it protects the mother against intracellular pathogens by promoting a Th1 response whenever it is required (178,181). Cellular immune functions, including the cytotoxic activity of natural killer cells has been found to be affected by many hormones. Cortisol an adrenocortical hormone in humans, and prolactin a 24 kDa single chain hormone secreted by the anterior pituitary gland, are among the most important nominators which affect maternal immunity to *Plasmodium falciparum* malaria during pregnancy (182,183).

1.7.2.1. Cortisol

Cortisol is a corticosteroid hormone or glucocorticoid produced by the adrenal cortex, which is part of the adrenal gland. It is usually referred to as the stress hormone as it is involved in response to stress and anxiety (184). Any type of stress either physical or neurogenic results in an immediate and remarkable elevation in ACTH (adrenocorticotropic hormone) by the anterior pituitary gland, which stimulate a greatly increased production of cortisol. Cortisol has an anti-inflammatory effect. Secretion or

injection of large amounts of cortisol has two fundamental anti-inflammatory effects: 1. it can block the early stages of inflammation even before its beginning, 2. if inflammation was already begins, it causes rapid resolution of inflammation and promote rapid healing (185).

The anti-inflammatory effects of cortisol results from its ability to stabilizes the lysosomal membranes, decreases the permeability of the capillaries, decreases both migration of white blood cells into the site of inflammation and phagocytosis of the damaged cells, suppresses the immune system causing a remarkable decrease in lymphocyte reproduction, and lowers fever as a consequence to its ability to reduce the release of IL-1 from the white blood cells (185). Furthermore, it increases blood pressure and blood sugar (184).

1.7.2.2. Prolactin

Prolactin is a pleiotropic hormone produced by the anterior pituitary gland. It is primarily associated with lactation but it has also been considered to play a role in the regulation of immune functions. Prolactin levels increases steadily from the 5th week of pregnancy till delivery, when its concentrations reach 10 to 20 times the normal nonpregnant level. Few weeks after delivery, prolactin levels return to normal nonpregnant level. Nevertheless, 10 to 20- fold surges in prolactin secretion occur each time the mother nurses her baby and lasts for about one hour (185). Prolactin is known to have a potent proinflammatory effects and proinflammatory cytokines is clearly influences its secretion. These cytokines can stimulate or depress prolactin secretion depending on the animal species studied and the severity of inflammation. Prolactin differ between genders, it is higher in women than in men (186). In spite of the association of prolactin with the development of mammary glands and initiation and maintenance of lactation, its receptors were found to be widely distributed among many different tissues (187).

1.7.2.3. Cortisol, Prolactin and susceptibility to malaria

There are indications that changes in the serum concentrations of cortisol and prolactin may be associated with the loss of antimalarial immunity observed during pregnancy (182,183). Cortisol has a role in the regulation of malaria immunity during pregnancy. It has considerable immunosuppressive activities in man, and it has been shown that its level was higher in primigravidae than in multigravidae. During pregnancy increased cortisol levels was found to be associated with suppression in cellular immune reactivity in order to prevent destruction of the fetal allograft in addition to promoting the maternal immune system to reinforce or to induce other immune reactions such as humoral responses (188). Moreover, cortisol has been found to reduce the adherence of infected erythrocytes to monocytes (182).

In Sudan Adam I, and collages reported that cortisol levels were not significantly different between pregnant women infected with *Plasmodium falciparum* malaria and noninfected ones and between infected primigravidae and infected multigravidae. Moreover, they showed that there was no significant difference in prolactin levels between *Plasmodium falciparum* infected and noninfected pregnant women, or between infected and noninfected primigravidas and multigravidas. They mentioned that prolactin levels increased with pregnancy duration but there were no significant correlations between cortisol levels and pregnancy duration observed (189).

In Gambia, it has been reported that cortisol concentration in primigravidae is conversely related to mononuclear cell proliferation in response to malarial antigens (182). In Tanzania, the serum concentration of total cortisol was found to be significantly higher in women with clinical malaria than in women without recorded malaria during pregnancy (188). These findings agree with that of another study in a holoendemic area in Kenya (183). Ordi *et al.* (2001) reported that there was a selective absence of NK cells in maternal malaria and they presumed that this absence may have a role in the hindrance of parasite clearance during the course of the infection (190). Moreover, increased maternal cortisol has also been found to be associated with increased risk of spontaneous abortion within the first 3 weeks of pregnancy (191).

Pearson insinuated to the increased pulsatile levels of prolactin which is initiated at the second trimester and continued through to the postpartum period positing a possible effect of prolactin on NK cells in maternal malaria (192,193). Citing various studies, he pointed out that higher cortisol levels and lower prolactin levels are found during normal labor in primiparous women (194).

The "cortisol hypothesis" of McGregor (195) and Vluegels *et al.* (188) has been revived by Bouyou-Akotet *et al.* (196) who measured NK cell cytotoxicity and cortisol and prolactin concentrations in peripheral venous blood samples obtained from pregnant Gabonese women at the time of delivery. Cortisol concentrations were found to be significantly higher in primigravidae than in multigravidae, and prolactin concentrations were significantly lower. They found that NK cell-mediated cytotoxicity against *Plasmodium falciparum*-infected erythrocytes *in vitro* was lower in samples obtained from primigravidae than in multigravidae ones. Also there was an inverse correlation between the magnitude of the NK cell cytolytic effect and cortisol production, while a positive correlation was found between this effect and prolactin production. Consequently, they reported that depressed NK cell cytotoxic activity against *Plasmodium falciparum*–infected erythrocytes is correlated with high cortisol concentrations and may contribute to increased susceptibility to malaria during pregnancy.

These findings were discussed and refuted by Pearson (194) who supported the prolactin hypothesis but he mentioned that delivery was not an appropriate time to assay for prolactin levels because it naturally fall 24 hours preceding the onset of delivery and Bouyou-Akotet *et al.* (197) have conceded this fact. However, they recently reported that cortisol and prolactin concentrations increase during pregnancy, regardless of parity and that primigravidae showed increased plasma cortisol concentration than multigravidae from the second trimester of pregnancy onwards. Inversely, they reported that plasma concentration of prolactin was higher in multigravidae throughout pregnancy. Synchronized increase of cortisol and prolactin concentrations with the period of pregnancy proposing that a sustained increase in cortisol level causes increased susceptibility of pregnant women to malaria, specifically in primigravidae. They observed a strong association between cortisol concentration and *Plasmodium falciparum*

infection. Cortisol concentrations were found to be higher in *Plasmodium falciparum*-infected primigravidae than in uninfected primigravidae throughout pregnancy and at the time of delivery. In contrast, *Plasmodium falciparum* state did not affect prolactin concentration. Furthermore, cortisol has been found to affect parasite load and this is clearly explained by the significant positive correlation observed between cortisol concentration and parasite load in *Plasmodium falciparum*-infected primigravidae (182).

Furthermore, it has been elucidated that the surface expression and function of the triggering receptors (e.g. NKp46 and NKp30) responsible for NK-mediated recognition and killing of susceptible target cells is regulated by hormones (182,193). Prolactin up-regulates and cortisol down-regulates the surface expression of NKp46 and NKp30. These findings are important because the action of NKp30 together with NKp46 lead to the induction of cytotoxic activity against a variety of target cells. These results are significantly important for the understanding of the involvement of NK cells in the susceptibility of pregnant women to *Plasmodium falciparum* malaria (198).

Eventually, Mavoungou (199) in his review concluded that, natural killer cells are important cells of the immune system and that their functions in infectious diseases and pregnancy are tightly regulated by several activating and inhibitory receptors which control cell proliferation, cytotoxicity and cytokine production. He explained that the production of hormones and other pregnancy regulatory factors in primigravidae may alter cell function, thereby conferring an advantage for malaria infection. Furthermore, he shed light upon the causal relationship between high cortisol levels and depressed NK cell cytotoxicity against *Plasmodium falciparum*-parasitized erythrocytes and susceptibility to malaria. Additionally he considered that *Plasmodium falciparum*–infected erythrocytes become sensitive to NK cytolysis, and prolactin, and cortisol serum levels were related with NK cells cytolytic activity.

It seems that there is few information exists on the relationship between the cytokine interactions that underlie both control and disease, pathogenesis of malaria during pregnancy, susceptibility to malaria and pregnancy associated hormones particularly cortisol and prolactin. Glucocorticoids can induce an *in vitro* shift in cytokine balance

toward a predominant type 2 immune response. It has been illustrated that they decrease IFN-γ and increase IL-4 and IL-10 production (200,201). Suguitan Jr. *et al*. reviewed that cortisol inhibits cell-mediated immune responses, lymphocyte proliferation and the production of IL-2, IFN-γ and TNF-α by macrphages and T cells. In contrast they reviewed that prolactin increases T cell activation and the production of IL-2, IFN-γ and TNF-α (202). Moreover, Lina Matera in her review mentioned that prolactin when occur in high concentrations has been found to increase IL-4 and IL-10 production (203). On the other hand TNF-α was proved to have a suppressive effect on the synthesis of cortisol (204).

Justifications

The immunosuppression associated with pregnancy leads to an increased risk of infection, including malaria. Various hormones have been found to produce a nonspecific immunosuppression. Cortisol and prolactin are among the most important candidates which affect maternal immunity to *Plasmodium falciparum* malaria.

In Sudan, malaria in pregnancy has received relatively little attention and this result in a very limited available data. Accordingly, this study will be achieved to provide critically needed data in this field.

THE OBJECTIVES

General Objectives

Its aim was to asses the role of the interactions between hormones and cytokines in pathogenesis of *Plasmodium falciparum* malaria during pregnancy and to investigate the cytokine profiles in peripheral, placental and cord blood in parturient women of eastern Sudan.

Specific Objectives

1. To measure the concentrations of cortisol and prolactin in pregnant Sudanese women with *Plasmodium falciparum* malaria at the time of delivery in Wad Medani.

2. To investigate the relationship between the concentrations of cortisol, prolactin and cytokines during pregnancy and to compare cytokine concentrations at the time of delivery, in infected and uninfected pregnant women in Wad Medani and New Halfa respectively.

3. To investigate the relationship between the concentrations of cortisol, prolactin and cytokines during pregnancy and to compare cytokine concentrations at the time of delivery, in primigravidae and multigravidae in Wad Medani and New Halfa respectively.

4. To describe the cytokine profile in peripheral, placental and cord blood in parturient Sudanese women of eastern Sudan.

5. To identify the patterns of the immune response during *Plasmodium falciparum* infection in the peripheral and placental compartments.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Study site and duration

This study was carried out in the period between October 2006 through December 2007. Part of the study was conducted at the antenatal clinic of Wad Medani hospital, central Sudan. Wad Medani, a city lies on the west bank of the Blue Nile, at an altitude of about 411 meters; 136 km southeast of the capital, Khartoum, Gezira State, east-central Sudan. The region is mesoendemic for *Plasmodium falciparum* malaria and it is characterized by unstable malaria transmission. The predominant malaria parasite species is *Plasmodium falciparum* (205).

The reminder of the study was conducted at the labour ward of New Halfa teaching hospital, eastern Sudan. Eastern Sudan is an area that is characterized by unstable malaria transmission. It is mesoendemic for *Plasmodium falciparum* malaria which is the predominant malaria species in the area. *Anopheles arabiensis* was the main vector (99.9%) found in the area and it is perennial rather than seasonal. The New Halfa area is located in the semi-arid belt of the Sudan approximately 500 km east of Khartoum in the middle of an agricultural scheme [altitude 450 m, 15° $19^{'}$ N & 35° $36^{'}$ E] (206).

2.2. Ethical considerations

Witnessed, written informed consent was obtained from all patients participating in the study. The study received ethical clearance from the Research Board of the University of Khartoum's Faculty of Medicine.

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2.3. Study Design

The case control part of the study was carried out to asses the role of the interactions between hormones and cytokines in pathogenesis of *Plasmodium falciparum* malaria during pregnancy and the cross sectional part was conducted to investigate the cytokine profiles in peripheral, placental and cord blood in parturient women of eastern Sudan.

For endocrine study pregnant women attending the delivery unit, of Wad Madni hospital were approached to participate in the study in the period between October through December 2007. Healthy pregnant women from the same area and from the same age group were recruited as controls.

Pregnant women with a singleton baby attending the delivery unit of New Halfa Teaching Hospital, were approached to participate in the cytokine profile study in the period between October 2006 and March 2007. Those with antepartum haemorrhage, hypertensive disorder of pregnancy (diastolic blood pressure > 90 mm Hg) and diabetes mellitus were excluded.

A structured questionnaire was administered to collect information about maternal socio–demographic characters and medical history, and the results of the clinical and haematological examinations data.

2.4. Study population

2.4.1. Patients

A total of ninety eight pregnant women infected with *Plasmodium falciparum* malaria were enrolled in this study. Forty five were involved in the assessment of the role of interactions between hormones and cytokines in the pathogenesis of *Plasmodium falciparum* malaria during pregnancy. Fifty three were involved in the study of cytokine profile.

2.4.2. Controls

A total of thirty seven healthy pregnant women (free of malaria) that matched for age, parity, weight, haemoglobin and gestational age were taken as controls. They were included in the assessment of the role of interactions between hormones and cytokines in the pathogenesis of *Plasmodium falciparum* malaria during pregnancy.

2.5. Malaria diagnosis

Thick and thin blood films were prepared from a finger prick, stained with Giemsa and examined by light microscopy under an oil-immersion objective, at × 1000 magnification. Parasite density was calculated by counting the number of asexual parasites per 300 white blood cells, assuming a mean white blood cell count of 6,000/µL. All blood smears were examined by two independent microscopists. If there was a difference in species diagnosis or if the parasite density differed by 50% between the two, a third microscopist re-examine the smears for a final species diagnosis. The final parasite density was the mean of the counts of the two initial microscopists or an average of the two closest counts. Maternal haemoglobin concentrations were estimated by Hemocue haemoglobinometer (HemoCue AB, Angelhom, Sweden).

2.6. Histopathology

Full thickness placental blocks of around 2-3 cm were taken from the placentae, kept in neutral buffer formalin for histopathology examinations. The presence of placental malaria infection was based on the pathological classification of Bulmer *et al.* (207); uninfected (no parasites or pigment), acute (parasites in intervillous spaces), chronic (parasites in maternal erythrocytes and pigment in fibrin or cells within fibrin and/or chorionic villous syncytiotrophoblast or stroma), past (no parasites but pigment confined to fibrin or cells within fibrin).

2.7. Hormonal analysis

2.7.1. Sample collection

Five ml of venous blood were withdrawn in plain tube, centrifuged and kept at -20 until processed in the laboratory for cortisol and prolactin analysis.

2.7.2. Hormonal Measurements

2.7.2.1. Cortisol Measurement

Total serum cortisol concentrations were determined with the ^{125}I-F RIA cortisol test kit (IMK-484). The test consists of a quantitative radioimmunoassay for the determination of cortisol in human serum, using ^{125}I-F (radio labeled iodine-cortisol) as tracer. The sensitivity of this radioimmunoassay was 0.8 ng/ml and the range of curve was 10-600 ng/ml.

Reagents and samples were equilibrated to room temperature before use. Test tubes were labeled in duplicates for total counts (T), nonspecific binding (NSB), standards (S_A-S_G) and samples. Standards were A: 0, B: 10, C: 30, D: 75, E: 150, F: 800 and G: 600 ng/ml. 0.5 ml of distilled water was added to each vial of standards except standard A (S_A) to which 1.0 ml of distilled water was added. All reagents and samples were homogenized by gentle mixing avoiding foaming. Starting with S_A 50μl of each standard (S_A-S_G) was pipetted into the appropriately labeled tubes. 50μl of S_A was pipetted into the nonspecific binding (NSB) labeled tubes. 50μl of each sample was pipetted into the appropriately labeled tubes. 200μl of ^{125}I-F was pipetted to all tubes. 100μl of F antibody (F = cortisol) to the standards and samples labeled tubes. 100μl of normal saline was added to the NSB labeled tubes. 200μl of magnetic second antibody was pipetted to all tubes except tubes labeled for total counts. All tubes were vortexed, mixed, covered and incubated for 1 hour at 37°C. Tubes were centrifuged at 2000X for 20 minutes. After centrifugation, tubes were placed carefully into suitable decantation racks, and then the supernatants were discarded. Then the tubes were inverted and placed on a pad of absorbent tissues

and allowed to drain for 5 minutes. Then the radioactivity of each tube was counted (60 sec/tube) using a γ counter (STRATEC Biomedical Systems, Birkenfeld, Germany). The average count of each set of duplicate tubes was calculated. A standard curve was generated and used for the calculation of the final concentration per tube value of the samples.

2.7.2.2. Prolactin Measurement

Total serum prolactin concentrations were determined with the ^{125}I-PRL IRMA prolactin test kit. The test consists of a quantitative immunoradiometric assay system for the direct quantitative in vitro determination of human prolactin in human serum using ^{125}I-Anti-PRL-Ab (radio labeled iodine-anti-prolactin-antibody) as tracer.

Reagents and samples were equilibrated to room temperature before use. PRL-Ab precoated tubes were labeled in duplicates for total counts (T), standards (S_0-S_6) and samples. Standards were S_0: 0, S_1: 50, S_2: 125, S_3: 300, S_4: 800, S_5: 2000 and S_6: 4000 μIU/ml. 0.5 ml of distilled water was added to each vial of standards except standard zero (S_0) 30 minutes before use. All reagents and samples were homogenized by gentle mixing avoiding foaming. Starting with S_0 50μl of each standard (S_0-S_6) was pipetted into the appropriately labeled tubes. 50μl of each sample was pipetted into the appropriately labeled tubes. 200μl of ^{125}I-Anti-PRL-Ab was pipetted to all tubes. All tubes were vortexed, mixed, covered and incubated for 2 hour at 37°C. Fluid was decanted from all tubes except the total and blot on an absorbent pad. Then all tubes except the total were washed two times with 2 ml distilled water. Then the radioactivity of each tube was counted (60 sec/tube) using a γ counter (STRATEC Biomedical Systems, Birkenfeld, Germany). The average count of each set of duplicate tubes was calculated. A standard curve was generated and used for the calculation of the final concentration per tube value of the samples.

2.8. Cytokine Analysis

2.8.1. Sample collection

Immediately after delivery, 5 mL of maternal, placental and cord blood was collected (using the biopsy-pool method for the placental cytokine analysis). Briefly, a block of tissue (5 cm × 5 cm × 5 cm) was excised from the basal side of the placenta, resulting in the formation of a large pool of intervillous blood at the excision site. Blood was quickly withdrawn in plain tube and centrifuged and kept at -20 until processed in the laboratory for cytokines.

2.8.2. Cytokines measurement

Sera samples obtained at delivery were analyzed by standard sandwich enzyme-linked immunosorbent assay (ELISA) for interferon gamma (IFN-γ), interleukin-4 (IL-4) and interleukin-10 (IL-10) using pairs of cytokine-specific, monoclonal antibodies according to the manufacturer's instructions (eBioscience, Germany).

In brief NUNC Maxisorp flat bottom 96 well ELISA plates were coated with 100 µl/well of capture antibody in Coating Buffer (Coating Buffer is a dried powder formulation of phosphate buffered saline (PBS) reconstituted in 1 Liter of distilled, deionized water).

Capture antibody used was pretitrated, purified antibody: for IFN-γ clone NIB42, for IL-4 clone 8D4-8, for IL-10 clone JES3-9D7.

Plates were sealed and incubated overnight at 4°C; the wells were washed five times with >250 µl/well washing buffer (Washing Buffer is a dried powder formulation of phosphate buffered saline with 0.05% Tween-20 reconstituted in 1 Liter of distilled, deionized water). Unoccupied binding sites on the plates were blocked by 200 µl/well of 1X Assay Diluent, incubated at room temperature for 1 hour, then again the wells washed five times. Standards were diluted using 1X Assay Diluent and 100 µl/well of standard were added to the appropriate wells. Two-fold serial dilutions were performed of the top standards to make the standard curve. 100 µl/well of sera were added to the appropriate

wells and incubated over night at 4°C. After five washes, the wells were incubated for one hour at room temperature with 100 µl/well of detection antibody diluted in 1X Assay Diluent.

Detection Antibody used was pretitrated biotin conjugated antibody: clone 4S.B3 for IFN-γ, clone MP4-25D2 for IL-4 and clones JES3-12G8 for IL-10.

The plates were then washed again and incubated for 30 minutes with 100 µl/well of Avidin-HRP (Avidin-horseradish peroxidase) diluted in 1X Assay Diluent. After another washing cycle (5 times) 100 µl/well of Substrate Solution (Tetramethylbenzidine (TMB)) were added to each well and incubated at room temperature for 15 minutes. The reaction was stopped by the addition of 50 µl of stop solution (1M H_3PO_4 or 2N H_2SO_4) to each well. The optical densities were measured at 450 nm, using the ELISA reader (Labsystems Multiskan MCC/340). All samples were run in duplicates and the mean value was used in all analyses.

For IFN-γ the assay sensitivity was 4 pg/ml and the standard curve range was 4-500 pg/ml. For IL-4 assay sensitivity was 2 pg/ml, and the standard curve range was 2-200 pg/ml. For IL-10 assay sensitivity was 2 pg/ml, and the standard curve range was 2-300 pg/ml.

2.9. Statistics

Data were entered in computer using SPSS for windows and double-checked before analysis. Data were checked for normality. For the endocrine phase of the study conducted in Wad Madni, cortisol data were normally distributed and student t-test was used for comparing the mean (SD). Cytokines and prolactin were found to be not normally distributed; Mann-Whitney U test was used to determine the significance of differences between the infected and non-infected groups. Correlations between continuous variables were assessed by the Spearman rank test. $P < 0.05$ was regarded as significant.

For the second phase studying cytokine profile conducted in New Halfa, data (cytokines) were not normally distributed; Mann-Whitney U test (2-group comparisons) or Kruskal-Wallis (> 2-group comparisons) tests were used to determine the significance of differences between the variables. *Post hoc* test for multiple means comparisons was used for multivariate analysis. Correlations between continuous variables were assessed by the Spearman rank test. $P < 0.05$ was regarded as significant.

CHAPTER THREE

RESULTS

In the case control part of the study carried out in Wad Medani to asses the role of the interactions between hormones and cytokines in pathogenesis of *Plasmodium falciparum* malaria during pregnancy 45 pregnant women with uncomplicated, *Plasmodium falciparum* malaria (18 of them primigravidae) and 37 similar but apparently uninfected pregnant controls were enrolled (Table 3.1). The costs of the assay kits precluded the enrollment of one control for each case. Parasitaemias in the cases ranged from 1760 to 29,629 asexual stages/ml. The mean (S.D.) serum concentration of cortisol in the infected cases was significantly higher than that in the uninfected controls [439.4 (172.0) *v.* 318.2 (210.3) ng/ml; P=0.005] (figure 3.1). The cases also had significantly lower prolactin and IFN-γ levels and significantly higher IL-10 levels than the controls, although the cases and controls were similar in terms of their IL-4 concentrations (Table 3.2).

Among the cases, all the assay results for the primigravidae were similar to those for the multigravidae, not only in terms of the mean (S.D.) cortisol concentrations [383.8 (146.9) *v.* 458.0 (183.7) ng/ml; P>0.05] but also in terms of the median prolactin and cytokine concentrations (Table 3.3).

Also among the cases, there were significant positive correlations between the cortisol and IL-10 concentrations (r=0.188; P=0.025) (figure 3.2) and significant negative correlations between prolactin and both IL-4 (r=20.175; P=0.038) and IL-10 (r=20.186; P=0.027). There were, however, no significant correlations between cortisol and prolactin, IL-4 or IFN- γ, or between prolactin and IFN- γ. There were also significant positive correlations, among the cases, between IL-10 and IL-4 concentrations (r=0.374; P<0.001) and between IFN- γ and IL-4 concentrations (r=0.687; P<0.001), although the IL-10 and IFN- γ concentrations of the cases did not appear to be correlated.

In the cross-sectional part conducted in New Halfa to investigate the cytokine profiles in peripheral, placental and cord blood in parturient women of eastern Sudan, triplet samples, maternal peripheral, placenta and cord sera were analyzed in 87 parturient women. While 53 women had past placental malaria infections, 34 showed no infections, according to placental histopathological examinations. 33, 54 were primigravidae and multigravidae, respectively.

Table 3.4 and table 3.5 show the concentrations of IFN-γ, IL-4 and IL10 in the peripheral, placental and cord sera from all the recruited women. Cord sera contained significantly less concentrations of these cytokines than the peripheral and placental sera. The difference was not significant when the peripheral and placental sera concentrations were compared.

When comparisons were made according to the parity, similar pattern was observed. The levels of these cytokines were not different when the primigravidae were compared to multigravidae. The same findings were observed (no difference between the cytokines levels between primigravidae and multigravidae) when data of the infected women were analyzed separately.

Strong positive correlations were observed between peripheral and placental $r= 0.89$, $P < 0.000$ (figure 3.3), and the cord $r=0.82$, $P < 0.000$ (figure 3.4) and between the placental and the cord $r =0.66$ $P< 0.000$ IFN-γ (figure 3.5). Likewise strong positive correlations were observed between peripheral and placental $r=0.82$, $P < 0.000$ IL-4 (figure 3.6) and IL-10, $r= 0.15$, $P< 0.000$ (figure 3.7).

There was no correlation between peripheral and cord $r= 0.2$, $P= 0.06$ or placental and cord, $r =0.13$, $P = 0.2$ IL-4. This was true with to regard peripheral and cord $r= 0.12$, $P = 0.2$ or placental and cord, $r= 0.1$, $P =0.3$ IL-10. The same findings were observed when the data of infected women were analyzed separately.

Table 3.1: The baseline characteristics of the 82 pregnant women with and without *Plasmodium falciparum* malaria who provided blood samples.

Variables	Women with malarial infection (the cases)	Women without malarial infection (the controls)	*P*
No. of subjects	45	37	
Mean value and SD for:			
Age, years	26.8(6.9)	26.1(6.7)	0.6
Gravidity	3.1(3.5)	2.5(2.4)	0.4
Gestational age, weeks	29.5(8.6)	27.7(9.7)	0.5
Weight, kg	65.4(6.6)	67. 6(11.2)	0.3
Haemoglobin, g/dl	10.2 (1.0)	10.8(0.9)	0.6

Table 3.2: The cytokine and prolactin concentrations detected in the sera of the 82 pregnant women with and without *Plasmodium falciparum* malaria who provided blood samples.

	Median concentration and (interquartile range)		
Variable	Women with malarial infection (the cases)	Women without malarial infection (the controls)	*P*
IFN-γ (pg/ml)	150.5(142.4-412.2)	311.5 (66.1-608.1)	0.016
IL-4 (pg/ml)	49.0 (19.1-74.5)	45.3(40.3-60.0)	0.856
IL-10 (pg/ml)	506.2 (252.0-1435.2)	39.8 (10.0-112.7)	<0.001
Prolactin (mU/litre)	5365.0(4362.0-7730.0)	7130.0 (5244.0-9322.5)	0.02

Table 3.3: The cytokine and prolactin concentrations detected in the sera of the 18 primigravidae and 27 multigravidae with *Plasmodium falciparum* malaria who provided blood samples.

	Median concentration and (interquartile range)		
Variable	Primigravidae	Multigravidae	*P*
IFN-γ (pg/ml)	73.0 (72.6–168.4)	78.3 (41.7–122.4)	0.3
IL-4 (pg/ml)	43.7 (33.5–75.1)	57.9 (15.0–76.6)	0.9
IL-10 (pg/ml)	129.4 (52.1–270.2)	72.5 (33.1–107.1)	0.1
Prolactin (mU/litre)	5175.0 (4817.6–8008.7)	5480.0 (4343.7–7848.7)	0.9

Table 3.4: The median (interquartile range) of sera cytokine levels in infected ($n = 53$) and uninfected ($n = 34$) parturient Sudanese women.

Cytokines, pg/ml	Mother	Placenta	Cord	P
IFN-γ				
Total	261.2(169.6-461.7)	249.8(169.6-388.6)	123.8(66.5-192.5)	0.02
infected	215.4(112.3-375.8)	226.8 (135.2-387.2)	123.8(80.8-224.0)	0.03
non-infected	358.6 (201.1-662.4)	278.4 (203.9-470.2)	89.4(32.1-169.6)	<0.000
P	0.01	0.04	0.03	
IL-4				
Total	25.0(15.6-41.0)	26.3(15.6-39.6)	5.0(1.0-13.4)	<0.000
infected	22.3(10.3-30.3)	21.0(11.6-34.3)	5.7(2.6-13.0)	<0.000
non-infected	41.0(23.6-82.0)	33.0 (25.0-62.9)	5.0 (5.0-16.0)	<0.000
P	<0.000	0.002	0.4	
IL-10				
Total	121.4(82.3-254.3)	148.7(86.2-276.3)	54.9(34.4-101.8)	<0.000
infected	109.7 (74.5-207.4)	121.4(78.4-211.3)	60.8 (36.4-106.2)	<0.000
non-infected	162.4 (97.0-469.1)	203.5 (110.6-367.6)	54.2 (27.6-86.2)	0.1
P	0.008	0.01	0.3	

Table 3.5: The median (interquartile range) of sera cytokine levels in primigravidae ($n = 33$) and multigravidae ($n = 54$) parturient Sudanese women.

Cytokines, pg/ml	Mother	Placenta	Cord	*P*
IFN-γ				
	238.3(175.3-438.7)	272.7 (140.9-387.2)	152.4(89.4-218.3)	0.04
Primigravidae	278.4 (146.7-467.4)	249.8(181.0-415.8)	100.9(49.3-165.2)	0.03
Multigravidae				
P	0.6	0.2	0.6	
IL-4				
Primigravidae	23.0 (16.3-35.6)	23.6(12.3-37.6)	5.0 (1.0-9.6)	<0.000
Multigravidae	26.3(13.6-48.0)	27.6 (17.3-43.0	6.3 (1.0-17.0)	<0.000
P				
	0.6	0.2	0.5	
IL-10				
Primigravidae	125.3 (84.2-271.1)	172.2(86.2-334.4)	54.9(45.2-113.6)	<0.000
Multigravidae	119.4 (82.3-240.7)	137.0(85.2-234.7)	54.9(27.6-96.0)	0.0002
P	0.6	0.6	0.1	
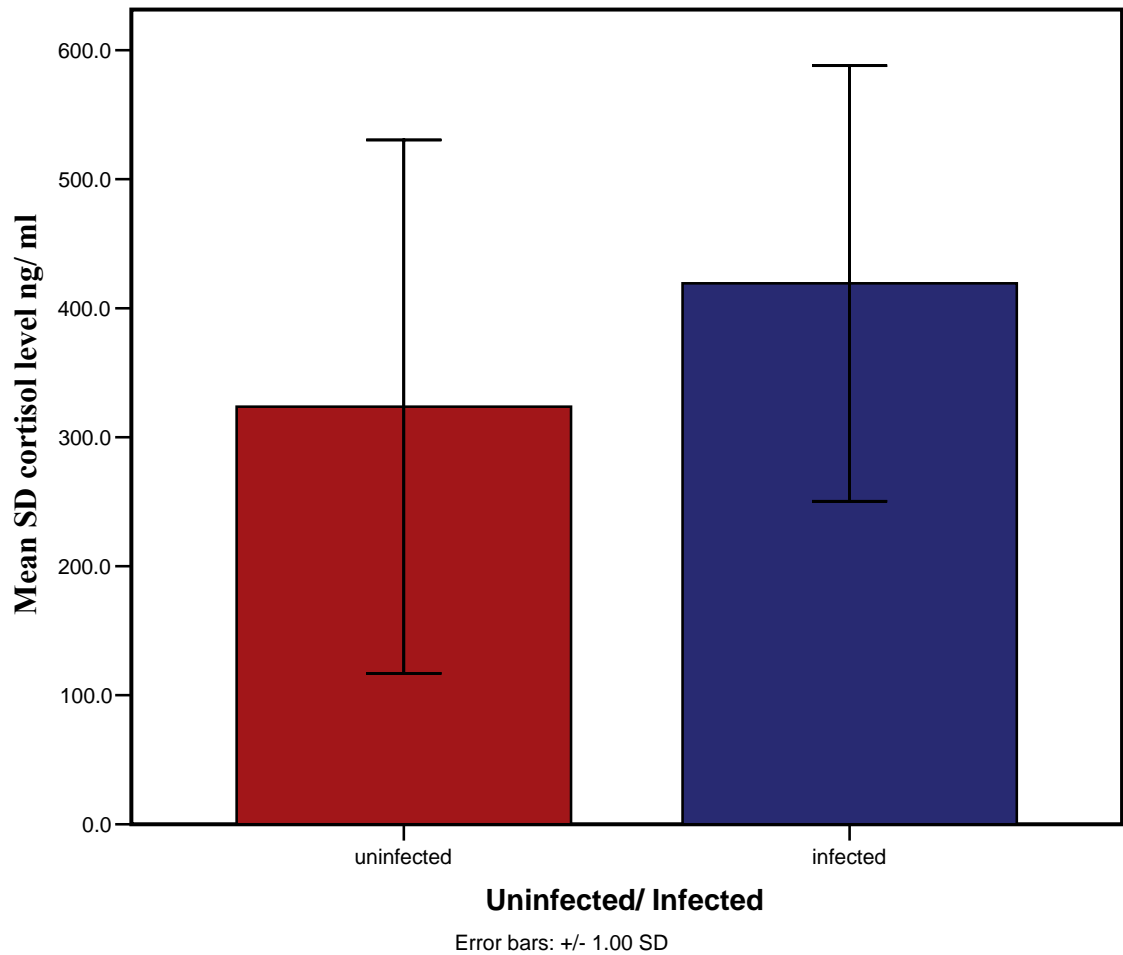
Figure 3.1: The mean (S.D.) sera concentrations of cortisol in the infected cases were significantly higher than that in the uninfected controls [439.4 (172.0) *v.* 318.2 (210.3) ng/ml; *P*=0.005].
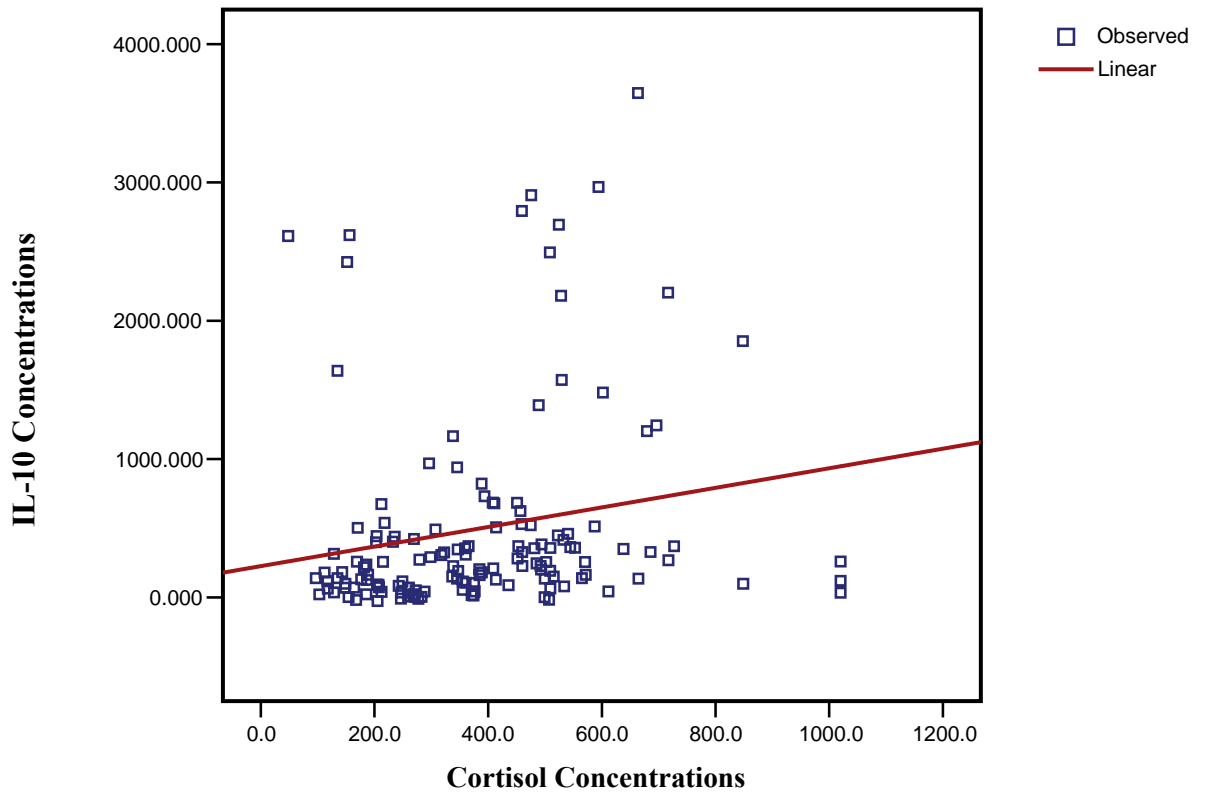
Figure 3.2: Among cases there were significant positive correlations between the cortisol and IL-10 concentrations (r=0.188; P=0.025).
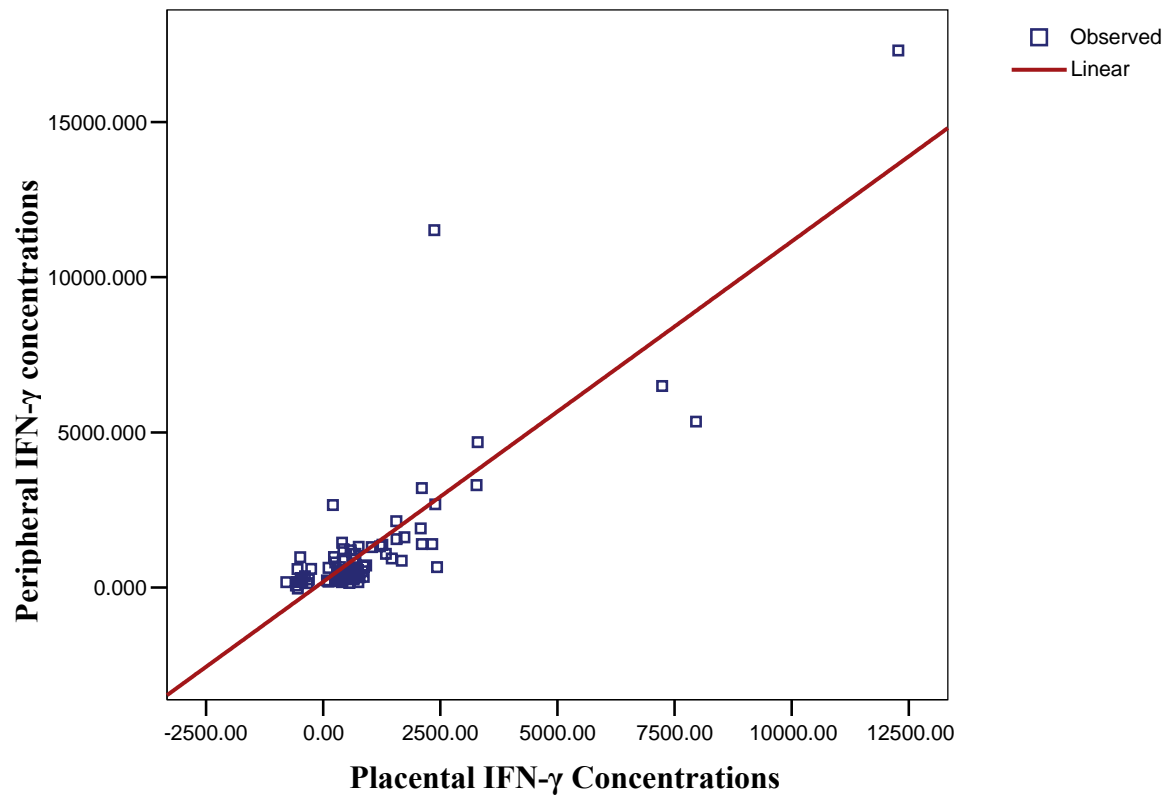
Figure 3.3: There were strong positive correlations between peripheral and placental IFN-γ concentrations $r = 0.89$, $P < 0.000$.
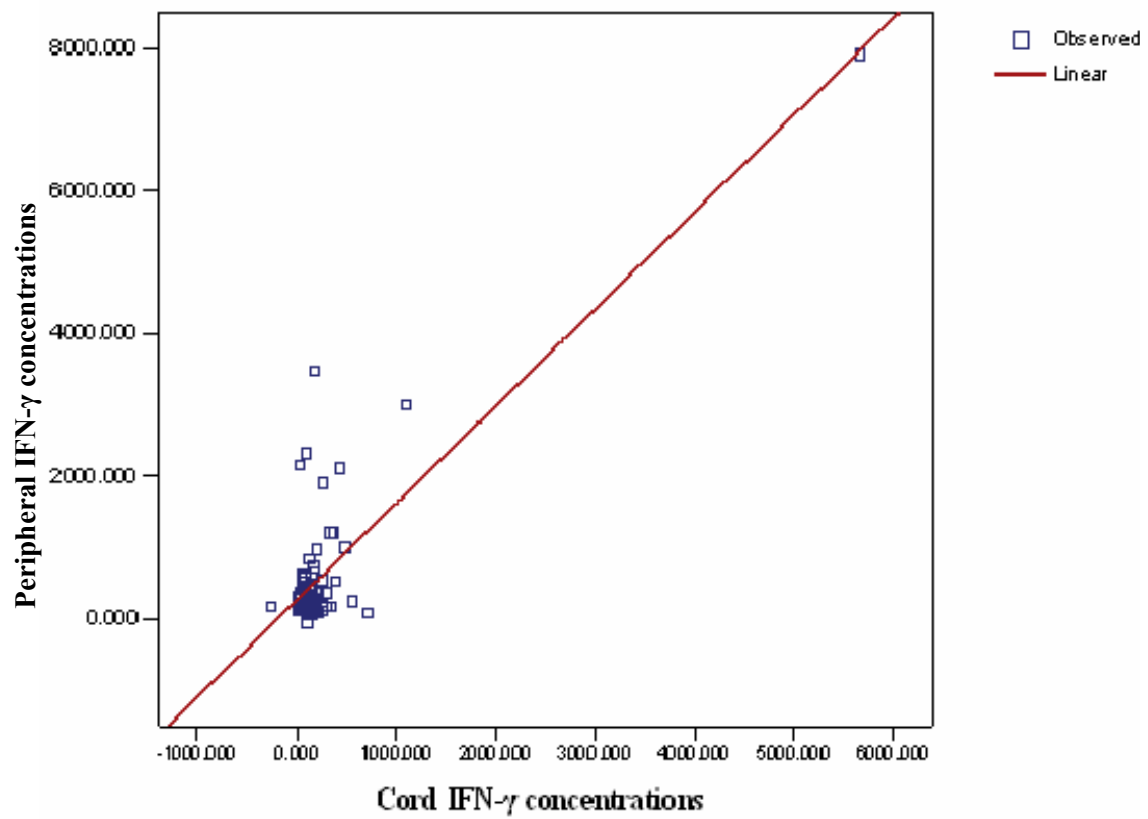
Figure 3.4: There were strong positive correlations between peripheral and cord IFN-γ concentrations *r*=0.82, *P* < 0.000.
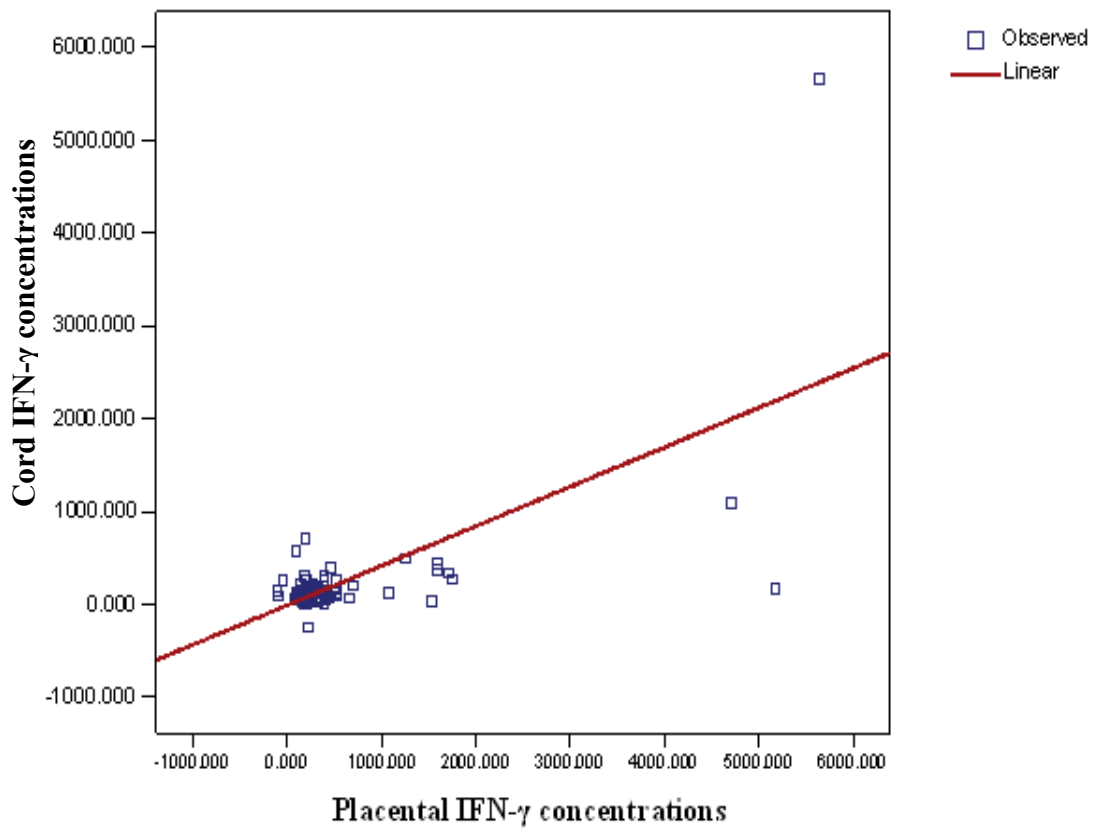
Figure 3.5: There were strong positive correlations between cord and placental IFN-γ concentrations $r = 0.66$ $P < 0.000$.
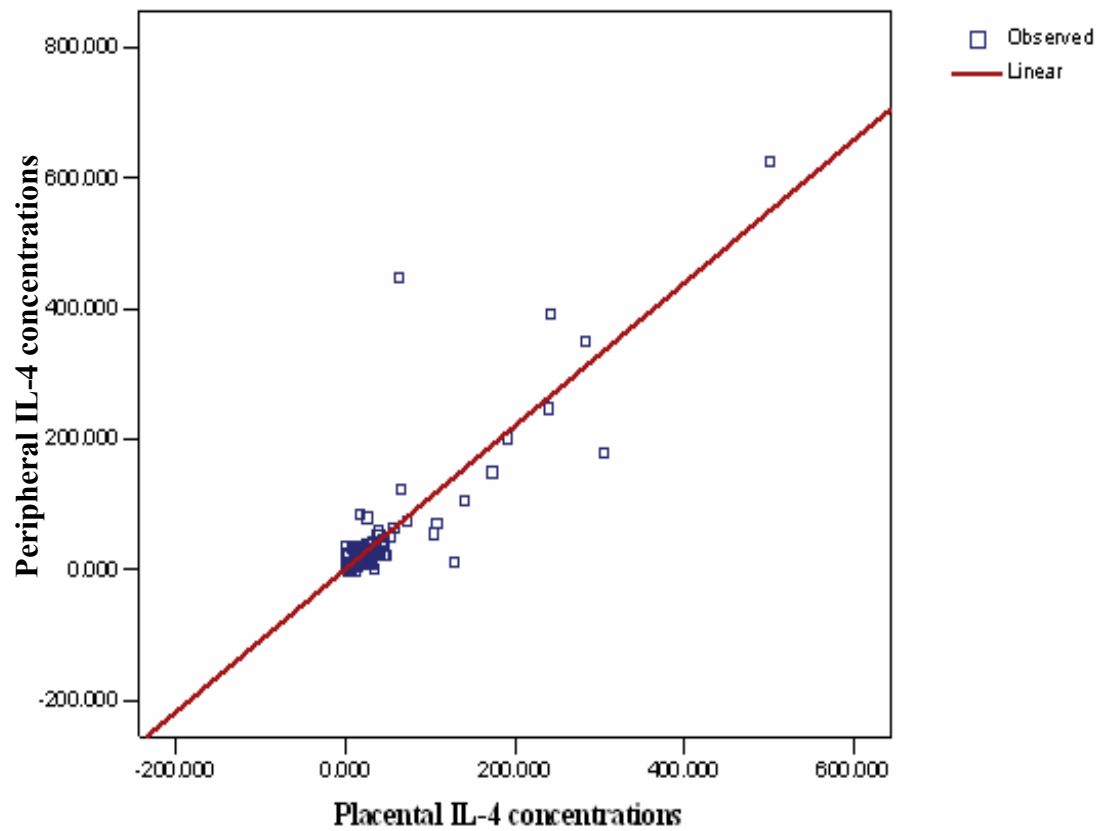
Figure 3.6: There were strong positive correlations between peripheral and placental IL-4 concentrations *r*=0.82, *P* < 0.000.
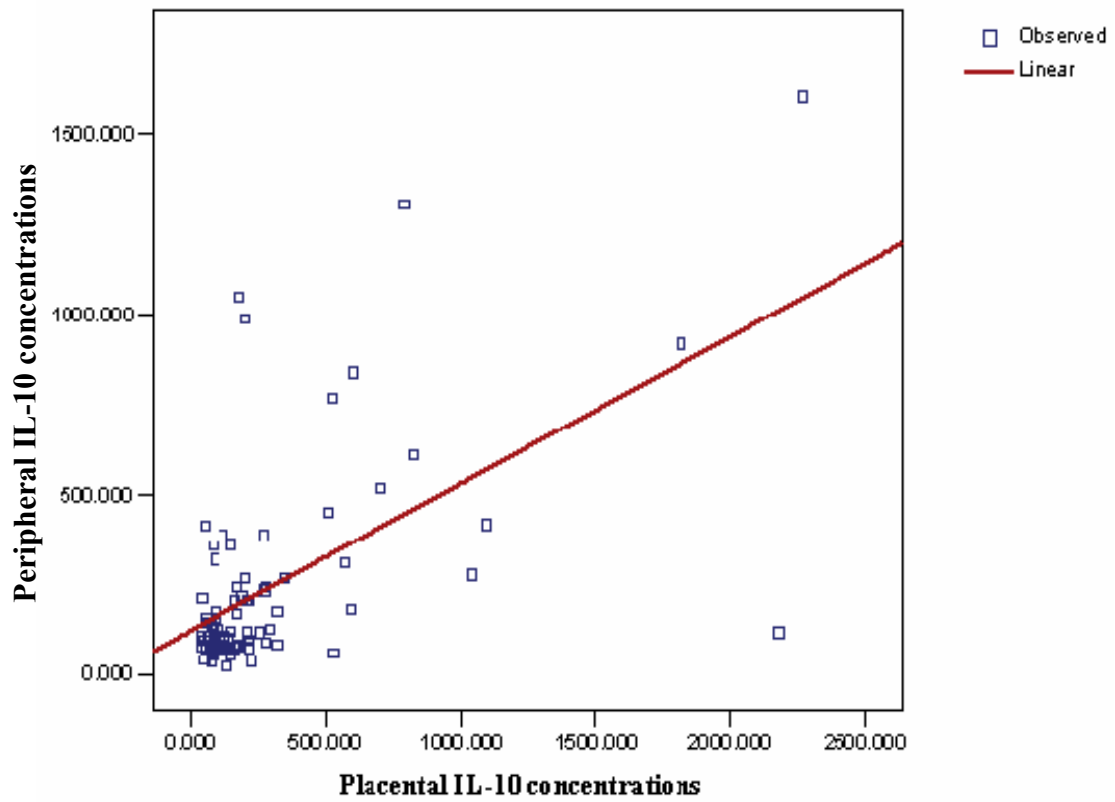
Figure 3.7: There were strong positive correlations between peripheral and placental IL-10 concentrations *r*= 0.15, *P*< 0.000.

CHAPTER FOUR

DISCUSSION

Effective immune responses against pathogens are sometimes associated with strong inflammatory reactions. To minimize damage to self, the activation of the immune system also triggers anti-inflammatory events. Both inflammatory and anti-inflammatory reactions are normal constituents of the same immune response, which coordinately fight infections while preventing immune pathology.

Pregnancy is an immunological balancing state in which the mother's immune system has to remain tolerant to the foetus in addition to maintain immune competence for defense against microorganisms (208). A complex network of hormones, cytokines and cells at the foeto–maternal interface is suggested to act collectively to maintain pregnancy. It has been proved that cytokines play a very important role in the maintenance of pregnancy by modulating immune and endocrine systems (97). In malaria, extensive evidence supports a role for cytokines in both protection from, and immunopathology of, different stages of infection (136). Moreover, malaria infections during pregnancy appear to cause significant alterations in the pattern of cytokine synthesis especially in primigravidae (51).

The current study was conducted to asses the role of — and the interactions between — cortisol, prolactin, IFN-γ, IL-4 and IL-10 in the pathogenesis of malaria during pregnancy, and to investigate the cytokine profiles in peripheral, placental and cord blood in parturient women in an area characterized by unstable malarial transmission in eastern Sudan.

The main findings of the assessment of the role of — and the interactions between — cortisol, prolactin, IFN-γ, IL-4 and IL-10 in the pathogenesis of malaria during pregnancy, were the significant differences, in the levels of both hormones investigated and IFN-γ and IL-10 (but not IL-4), observed between the pregnant women with

uncomplicated malaria and their uninfected counterparts. In terms of the hormones and cytokines investigated, the infected primigravidae were very similar to the infected multigravidae. Among the infected pregnant women, there were significant correlations between the IL-10 concentrations and both cortisol and prolactin concentrations.

The study of cytokine profile in New Halfa resulted in that, IFN-γ, IL-4 and IL-10 concentrations (mainly peripheral and placental) were higher in uninfected women than in the infected women. There were no differences in the levels of these cytokines in primigravidae and multigravidae. Furthermore, cord sera had lower levels of these cytokines in comparison to sera from placenta and maternal peripheral blood.

During pregnancy cortisol and prolactin concentrations increased regardless of parity (182). It has been reported that prolactin concentrations did not differ in *Plasmodium falciparum*–positive and –negative pregnant women (182,189,196) or between infected and noninfected primigravidas and multigravidas (189). In contrary to the previous findings, we found that prolactin concentrations were lower in pregnant women with *Plasmodium falciparum* malaria than uninfected ones. Our results are in agreement with previous studies reporting that there was no significant difference in prolactin levels between infected and noninfected primigravidas and multigravidas (189). The observation that pregnant women with *Plasmodium falciparum* malaria have higher serum levels of cortisol than their uninfected counterparts has been made several times (183,188,209). In contrast Adam I, *et al.* reported that there was no significant difference in cortisol levels between pregnant women with *Plasmodium falciparum* malaria and their uninfected counterparts (189). It has been reported that serum cortisol has a role in the regulation of malaria immunity during pregnancy. It suppresses cellular immune reactivity to prevent destruction of the fetal implant, in addition to creating the possibility for the maternal immune system to reinforce or to induce other immune reactions such as humoral responses (188).

In malaria-endemic areas, it might be expected that multigravidae, who have experienced previous foeto–maternal interactions and have, on average, presumably been more exposed to *Plasmodium falciparum* than the generally younger primigravidae in the same

area, would portray have different hormonal and cytokine pictures to those of the primigravidae. This difference would be a result of both immune cell priming, especially against malarial parasites, and the development of antibodies against the adhesion molecules that are responsible for the placental sequestration of malarial parasites (210). Moreover, the discrete pattern of cytokines seen in primigravidas may be the result of more frequent parasitaemia or could represent cellular responses occurring in the absence of established acquired immunity (50). So far, however, only Vleugels *et al.* (1986) have reported significantly higher cortisol levels in primigravidae with malaria than in multigravidae with the same disease (209).

Such gravidity-related differences may be related to the level of malarial endemicity in the area. In eastern Sudan, and possibly in other areas where malarial transmission is unstable, the epidemiology and pathogenesis of malaria during pregnancy appears the same, irrespective of parity (20,189). The pregnant women in this region appear to be particularly susceptible to *Plasmodium falciparum* malaria, irrespective of their age and parity (19), and may even develop severe forms of the disease, such as cerebral malaria (25). In the present study, the relatively high serum level of cortisol and relatively low serum level of prolactin seen in the infected women, and the correlation of the concentrations of both of these hormones with that of IL-10, indicate the effects of an integrated hypothalamus–pituitary–adrenal axis in these subjects. Activation of this axis in malaria may be the result of the release of cytokines and/or the stress generated by the disease itself. Moreover, it has been reported that cytokine concentrations are associated with the basal and peak levels of some hormones (211). As discussed above, both cortisol and prolactin appear to modulate the immune system and influence the activity of NK cells (196,212,213). Several cytokines have been reported to influence endocrine functions and, in malaria, serum concentrations of some cytokines are generally raised (135,204,214,215). In addition, IL-10 production is up-regulated by prolactin (216), perhaps via the activation of lymphocytes and macrophages (217). During malaria, it is difficult to determine accurately when circulating IL-10 becomes detectable, and cytokine secretion may be abrupt or gradual, sometimes preceding the onset of clinical manifestations (136). The low levels of IL-4 may be due to that IL-4 is a very potent cytokine that is secreted in small amounts relative to other cytokines. In addition, it is

subjected to high *in vitro* consumption due to a wide distribution of IL-4 receptors on different cell types and is therefore, hard to detect (218). Based on all these findings, we could suggest a hormone-cytokine network at foeto–maternal interface that could interact with the other immune arms and associated with better pregnancy outcomes.

The high levels of IFN-γ, IL-4 and IL-10 in sera of uninfected women in New Halfa, might suggest that these cytokines are involved in the control of parasitaemia in peripheral blood and in placenta. The low amounts of these cytokines found in *Plasmodium falciparum*-infected women further supports this idea. It has been reported that Th1 cells produce high levels of IFN-γ as a result of their activation in the presence of IL-12. Th1 cells are important effectors involved in the eradication of infectious pathogens, but its inappropriate activation can lead to immunopathology. On the contrary, IL-4 induces the development Th2 cells, which have been implicated in humoral immune responses and the eradication of helminths, but they may also result in inflammatory damage during allergic manifestations and atopy. It has been suggested that Th2 cells by their production of anti-inflammatory cytokines, such as IL-4 and IL-10, contribute to the protection of tissues and organs from autoimmune attack. This regulation is found to be achieved by alternative subsets of regulatory T cells by the production of TGF-β, which is able to inhibit both the development of Th1 and Th2 responses. Conclusively, IFN-γ and IL-4 are typical cytokines produced by Th1 and Th2 cells, respectively, and suppress the differentiation of Th2 and Th1, respectively (218-220). Our findings clearly counteracting the general opinion that IL-4 suppresses IFN-γ production. Nevertheless, it should be noted that several studies have previously reported results that are consistent with our findings. IL-4 seems to play a pro-inflammatory role in flare-up reactions of chronic arthritis. And it has been reported that treatment with anti-IL-4 was even more effective in blocking joint swelling and cell influx (157). Moreover, Ramanathan *et al*. demonstrated that IL-4 can induce the production of IFN-γ and of inflammatory cytokines under certain conditions, and illustrated that IL-4 can exert a dose-dependent differential effect on the induction of immune responses and on autoimmunity in experimental autoimmune uveoretinitis (221). Additionally, the protective antifungal responses of IL-4 induced by CD4^{+} Th1 in *Candida albicans* infections has been proved, possibly through the combined activity on cells of the innate and adaptive immune systems (222).

Our finding of higher peripheral blood IL-10 levels in *Plasmodium falciparum*-infected mothers is in agreement to the previous recent observations (223). IL-10 characterizes normal human pregnancy and is thought to prevent inflammatory responses that might damage the integrity of the materno-fetal placental barrier (50,93). During placental malaria, despite the placental shift toward Th1-type cytokines, IL-10 concentrations are elevated compared with healthy placentas (117). IL-10 has a major role in controlling inflammatory responses and preventing materno-fetal placental barrier damages (50,51,117).

During pregnancy the enhanced IL-10 and IL-4 expression, perhaps in concert with other anti-inflammatory immunomodulatory cytokines, curtails the potentially hazardous effects of Th1-related cytokine production on systemic immunity during pregnancy, thus ensuring the retention of the fetal allograft (224). Perhaps, IL-4 blocks NK activity of the decidua which may have potentially deleterious effect on the fetus like thrombosis, inflammation, and abortion (225). Furthermore, an anti-inflammatory cytokine environment is thought to be maintained during pregnancy, in part, by high progesterone levels, which induce Th0 to Th2 conversion (93,226).

In the present study, in Wad Madni, the serum concentrations of IFN-γ were lower in the infected women than in the uninfected controls (although, in the infected women, the IFN-γ concentrations were not correlated with those of cortisol or prolactin). Previously, IFN-γ has been associated with protection from malaria, with impaired IFN-γ production mooted as a cause for increased susceptibility to placental malaria (116). Although Rogerson *et al*. (2003) (227) found no detectable IFN-γ in placental biopsy specimens from Malawian primigravidae, IFN-γ was found in about 40% of placental plasma samples collected in neighboring Kenya, being associated with malarial infection and poor foetal outcome (51). The contrasting results of these two studies may again reflect differences in malarial endemicity in the study areas. The malarial infections investigated in the later study were current/ acute, not the chronic or past infections often revealed by placental histopathology.

Likewise, high levels of IL-12 have been reported in uninfected women (228). However, these studies should be compared cautiously, because of the difference in the endemicity. Furthermore, in the later study malaria infections were diagnosed by microscopy (current infections), while in our study the placental histopathology was the tool used to diagnose malaria placental infections and these were past infection.

The influence of endemicity on the results of our study is obvious as there were no significant differences in the levels of these cytokines between the primigravidae and multigravidae. Thus, in this area of eastern Sudan, the pathogenesis of malaria is the same irrespective to the parity. Previously we have observed that, pregnant women of eastern Sudan are susceptible to peripheral malaria as well as placental malaria irrespective to their age and parity (19,20).

Yet, gravidity-based differences in cytokine responses to malaria have been proposed to explain the difference in susceptibility to malaria between primigravidae and multigravidae women (116).

In contrast to the previous findings (228), our study showed that, cord sera had the lower cytokines levels. This might support the previous assumption, that because we investigated these cytokines in past malaria infections mainly. Yet, we investigated IFN-γ, IL-4 and IL-10, while the former study investigated IL-12 and IL-15 and the difference in their passage through placental barrier and neonatal antigenicity may be varied in various cytokines. Moreover, the fact that the blood within the umbilical cord is rich in primitive, undifferentiated stem cells may also clarify our results.

Unlike Bouyou-Akotet *et al*. reports (228), we found strong positive correlations between the peripheral and placenta sera concentrations of cytokines, suggesting that anti-malaria immune responses occurring in the placenta are influenced by the cytokines from mother's blood, and that the immune response during *Plasmodium falciparum* infection is not different in the peripheral and placental compartments.

4.1. Conclusions

In conclusion, it appears that, irrespective of parity, cortisol, prolactin and certain cytokines are key mediators in the host response to *Plasmodium falciparum* infection during pregnancy in women living in central Sudan, where malarial transmission is unstable. It remains possible, however, that the hormonal and cytokine perturbations seen in the infected pregnant women living in this region are simply the result of malarial infection, and not factors in the etiology of the malaria.

In eastern Sudan, the patterns of the immune responses that occur in placental, peripheral and cord blood were influenced by the malaria infections, irrespective to the parity. IFN-γ, IL-4 and IL10 are key mediators in the host response to *Plasmodium falciparum* infection during pregnancy in women living in unstable malaria transmission. Immune response during *Plasmodium falciparum* infection is not different in the peripheral and placental compartments.

4.2. Recommendation

- The strong correlation between the peripheral and placental sera concentrations of cytokines, need further biochemical explanation.
- Study of the antibody classes and if possible subclasses.
- Study of the effect of the ethnic background on the cytokine profile.
- The very complex nature of malaria during pregnancy needs further research to be elucidated.

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Cytokine Profiles in Peripheral, Placental and Cord Blood in an Area of Unstable Malaria Transmission in Eastern Sudan

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Abstract

Background: understanding the cytokine interactions that underlie both control and disease should be helpful when investigating the pathogenesis of malaria during pregnancy, as the levels of some cytokine are associated with poor pregnancy outcomes.

Aim: the study aimed to investigate the cytokine profiles in peripheral, placental and cord blood in parturient women of eastern Sudan, which is characterized by unstable malaria transmission.

Methods: enzyme-linked immunosorbent assay was used to measure the concentrations of three pro-inflammatory cytokines, interferon gamma (IFN-γ), interleukin-4 (IL-4) and interleukin-10 (IL-10), in sera from peripheral, placental and cord blood of eighty- seven Sudanese women.

Results: the concentrations of these cytokines were significantly higher in peripheral, placental sera from uninfected women than in sera from infected women. IFN-γ concentrations were higher in the cord sera from uninfected women in comparison to the infected ones too. The levels of these cytokines were not significantly different between the primiparae and multipare. Cord sera in all the groups had the lower levels of these cytokines. Strong positive correlations were observed between peripheral and placental cytokines.

Conclusion: the immune responses that occur in placental, peripheral and cord blood were influenced by the malaria infections, irrespective of the parity. Immune response during *P. falciparum* infection is not different in the peripheral and placental compartments, further studies are required.

Introduction

It has been estimated that 90% of the global malaria burden occurs in Sub-Saharan Africa, where during pregnancy 40% women are exposed to malaria infections [1]. Malaria during pregnancy poses a substantial risk to the mother, her fetus and the neonate [2]. Malaria during pregnancy is a major health problem in Sudan, where it has been reported to be associated with maternal anaemia, low birth weight infants and as the main cause of maternal mortality [3-6].

During pregnancy, the immune system may be biased toward type 2 humoral defense mechanisms rather than towards type 1 cellular responses, this may be fundamental for fetal wellbeing [7]. The systemic suppression of pro-inflammatory responses from T helper 1 (Th1) cells, i.e., increased circulating levels of IFN-γ and tumor necrosis factor α, along with increased local expression of anti-inflammatory cytokines such as interleukin (IL)-4, IL-6, and IL-10, has been reported [8].

Placental malaria is associated with cell mediated inflammatory responses and alters the cytokine balance in favor of Th1 types (i.e., pro-inflammatory) [9, 10]. The placental

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production of chemokines, may be an important trigger for monocyte accumulation in the placenta [11]. Understanding the cytokine interactions that underlie both control and disease should be helpful when investigating the pathogenesis of malaria during pregnancy.

The current study was conducted an area that is characterized by unstable malaria transmission in eastern Sudan [12], where malaria is substantial burden affecting pregnant women irrespective to their age or parity [3]. The study aimed to investigate the cytokine profiles in peripheral, placental and cord blood in parturient women so as to add to on-going data on the pathogenesis of malaria during pregnancy in the area [13, 14].

Methods

Patients

The study was conducted between October 2006 and March 2007 at the labour ward of New Halfa teaching hospital, eastern Sudan. The details of the study design have been mentioned elsewhere [14]. In summary, after taking an informed consent, women with a singleton baby were approached to participate in the study. Those with antepartum haemorrhage, hypertensive disorder of pregnancy (diastolic blood pressure > 90 mm Hg) and diabetes mellitus were excluded.

A structured questionnaire was administered to collect information about socio-demographic characteristics and parity.

Haematology.

Maternal, placental and cord blood films were prepared, the slides were Giemsa stained and the number of asexual P. falciparum parasites per 200 white blood cells were counted and double checked blindly by an expert microscopist. Maternal haemoglobin concentrations were estimated by Hemocue haemoglobinometer (HemoCue AB, Angelhom, Sweden).

Sample collection

Immediately after delivery, 5 mL of maternal, placental and cord blood was collected using the biopsy-pool method (for the placental) cytokine analysis. Briefly, a block of tissue (5 cm × 5 cm × 5 cm) was excised from the basal side of the placenta, resulting in the formation of a large pool of intervillous blood at the excision site. Blood was quickly withdrawn in plan tube and centrifuged and kept at -20 until processed in the laboratory for cytokines.

Histopathology

Full thickness placental blocks of around 2-3 cm were taken from the placentae, kept in neutral buffer formalin for histopathology examinations. The presence of placental malaria infection was based on the pathological classification of Bulmer *et al* [15]; uninfected (no parasites or pigment), acute (parasites in intervillous spaces), chronic (parasites in maternal erythrocytes and pigment in fibrin or cells within fibrin and/or chorionic villous syncytiotrophoblast or stroma), past (no parasites and pigment confined to fibrin or cells within fibrin).

Cytokines measurement

Sera samples obtained at enrollment were analyzed by standard sandwich enzyme-linked immunosorbent assay (ELISA) for interferon gamma (IFN-γ), interleukin-4 (IL-4) and interleukin-10 (IL-10) using pairs of cytokine-specific, monoclonal antibodies according to the manufacturer's instructions (Bioscincel, Germany). Each plate included a standard curve of recombinant human cytokine. All samples were run in duplicates and the mean value was used in all analyses.

Statistics

Data were entered in computer using SPSS for windows and double-checked before analysis. Data (cytokines) were not normally distributed; Mann-Whitney test U (2-group comparisons) or Kruskal-Wallis (> 2-group comparisons) tests were used to determine the significance of differences between the variables. Post hoc test for multiple means

comparisons was used for multivariate analysis. Correlations between continuous variables were assessed by the Spearman rank test. P < 0.05 was regarded as significant.

Ethics

The study received ethical clearance from the Research Board at the Faculty of Medicine, University of Khartoum.

Results

The triplet samples, maternal peripheral, placenta and cord sera were analyzed in 87 parturient women. While 53 women had past placental malaria infections, 34 showed no infections, according to placental histopathological examinations. 33, 54 were primiparae and multipare, respectively.

Cytokine concentrations differ between peripheral, placental and cord plasma

Table 1 and table 2 show the concentrations of IFN-γ, IL-4 and IL10 in the peripheral, placental and cord sera from all the recruited women. Cord sera contained significantly less concentrations of these cytokines than the peripheral and placental sera. The difference was not significant when the peripheral and placental sera concentrations were compared.

When comparisons were made according to the parity, similar pattern was observed. The levels of these cytokines were not different when the primiparae were compared to multiparae. The same findings were observed (no difference between the cytokines levels between primiparae and multipare) when data of the infected women were analyzed separately (data not shown).

Correlation between peripheral, placental and cord plasma concentrations of cytokines

Strong positive correlations were observed between peripheral and placental $r= 0.89$, $P < 0.000$, and the cord $r=0.82$, $P < 0.000$ and between the placental and the cord, $r =0.66$ $P< 0.000$ IFN-γ. Likewise strong positive correlations were observed between peripheral and placental $r=0.82$, $P < 0.000$ IL-4 and IL-10, $r= 0.15$, $P< 0.000$.

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There was no correlation between peripheral and cord $r= 0.2$, $P= 0.06$ or placental and cord, $r=0.13$, $P= 0.2$ IL-4. This was true with to regard peripheral and cord $r= 0.12$, $P=0.2$ or placental and cord, $r=0.1$, $P=0.3$ IL-10. The same findings were observed when the data of infected women were analyzed separately (data not shown).

Discussion

In the current study, IFN-γ, IL-4 and IL-10 concentrations (mainly peripheral and placental) were higher in uninfected women than in the infected women. There were no differences in the levels of these cytokines in primiparae than in multipare. Furthermore, cord sera had lower levels of these cytokines in comparison to sera from placenta and maternal peripheral blood. The high levels of these cytokines in sera of uninfected women, might suggest that these cytokines are involved in the control of parasitemia in peripheral blood and in placenta. The low amounts of these cytokines found in *P. falciparum*-infected women further supports this idea. The anti-inflammatory cytokine environment is thought to be maintained, in part, by the high progesterone levels in pregnancy, which induces both Th0 to Th2 conversion [16].

IFN-γ production by intervillous blood cells was associated with protection from malaria, and impaired IFN-γ production was mooted as a cause for the increased susceptibility to placental malaria [17]. In consistence with our previous findings, malaria-infected placentas had higher IFN-γ levels than did uninfected placentas [18]. In contrary, Moorman *et al.* found no detectable IFN-γ in placental biopsy specimens from primigravid specimens from Malawian women [19]. Differences in IFN-γ responses to malaria infection during pregnancy were reported between different African settings. In Neighboring Kenya IFN-γ levels were found in about 40% of placental plasma samples and were associated with malaria infection and poor fetal outcome [20].

The enhanced IL-4 and IL -10 expression, perhaps in concert with other anti-inflammatory immunomodulatory cytokines, curtails the potentially hazardous effects of Th1-related cytokine production on systemic immunity during pregnancy, thus ensuring

the retention of the fetal allograft [21]. Perhaps, IL-4 blocks NK activity of the decidua which may have potentially deleterious effect on the fetus like thrombosis, inflammation, and abortion [22].

Our finding of higher peripheral blood IL-10 levels in *P. falciparum*-infected mothers is in contrast to the previous recent observations [23]. IL-10 characterizes normal human pregnancy and is thought to prevent inflammatory responses that might damage the integrity of the materno-fetal placental barrier [20, 24]. During placental malaria, despite the placental shift toward Th1-type cytokines, IL-10 concentrations are elevated compared with healthy placentas [25]. IL-10 has a major role in controlling inflammatory responses and preventing materno-fetal placental barrier damages [9,19,25].

Likewise, high levels of IL-12 have been reported in uninfected women [26]. However, these studies should be compared cautiously, because of the difference in the endemicity. Furthermore, in the later study malaria infections were diagnosed by microscopy (current infections), while in our study the placental histopathology was the tool used to diagnose malaria placental infections and these were past infection.

The influence of endemicity on the results of our study is obvious as there were no significant differences in the levels of these cytokines between the primiparae and multiparae. Thus, in this area of eastern Sudan, the pathogenesis of malaria is the same irrespective to the parity. Previously we have observed that, pregnant women of eastern Sudan are susceptible to peripheral malaria as well as placental malaria irrespective to their age and parity [3,14].

Yet, gravidity-based differences in cytokine responses to malaria have been proposed to explain the difference in susceptibility to malaria between primigravid and multigravid women [17].

In contrast to the previous findings [26], our study showed that, cord sera had the lower cytokines levels. This might support the previous assumption, that because we investigated these cytokines in past malaria infections mainly. Yet, we investigated IFN-

γ, IL-4 and IL-10, while the former study investigated IL-12 and IL -15 and the difference in their passage through placental barrier and neonatal antigenicity may be varied in various cytokines.

Unlike Bouyou-Akotet *et al.,* reports [26], we found strong positive correlations between the peripheral and placenta sera concentrations of cytokines, suggesting that anti-malaria immune responses occurring in the placenta are influenced by the cytokines from mother's blood, and that the immune response during *P. falciparum* infection is not different in the peripheral and placental compartments.

Conclusions: the patterns of the immune responses that occur in placental, peripheral and cord blood were influenced by the malaria infections, irrespective to the parity. IFN-γ, IL-4 and IL10 are key mediators in the host response to *P. falciparum* infection during pregnancy in women living unstable malaria transmission. Immune response during *P. falciparum* infection is not different in the peripheral and placental compartments, further studies are required.

Authors` contributions

NB and KH carried out the study and participated in the statistical analysis and procedures, IA, MIE and EM coordinated and participated in the design of the study, statistical analysis and the drafting of the manuscript. AM and MS participated in the lab work. All the authors read and approved the final version.

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Table 1 The median (interquartile range) of plasma cytokine levels in infected (n = 53) and uninfected (n = 34) parturient Sudanese women

Cytokines, pg/ml	Mother	Placenta	Cord	P
IFN-γ				
Total	261.2(169.6-461.7)	249.8(169.6-388.6)	123.8(66.5-192.5)	0.02
infected	215.4(112.3-375.8)	226.8 (135.2-387.2)	123.8(80.8-224.0)	0.03
non-infected	358.6 (201.1-662.4)	278.4 (203.9-470.2)	89.4(32.1-169.6)	<0.000
P	0.01	0.04	0.03	
IL4				
Total	25.0(15.6-41.0)	26.3(15.6-39.6)	5.0(1.0-13.4)	<0.000
infected	22.3(10.3-30.3)	21.0(11.6-34.3)	5.7(2.6-13.0)	<0.000
non-infected	41.0(23.6-82.0)	33.0 (25.0-62.9)	5.0 (5.0-16.0)	<0.000
P	<0.000	0.002	0.4	
IL10				
Total	121.4(82.3-254.3)	148.7(86.2-276.3)	54.9(34.4-101.8)	<0.000
infected	109.7 (74.5-207.4)	121.4(78.4-211.3)	60.8 (36.4-106.2)	<0.000
non-infected	162.4 (97.0-469.1)	203.5 (110.6-367.6)	54.2 (27.6-86.2)	0.1
P	0.008	0.01	0.3	

Table 2 The median (interquartile range) of plasma cytokine levels in primiparae (n = 33) and multipare (n = 54) parturient Sudanese women

Cytokines, pg/ml	Mother	Placenta	Cord	*P*
IFN-γ				
Primipare	238.3(175.3-438.7)	272.7 (140.9-387.2)	152.4(89.4-218.3)	0.04
multipare	278.4 (146.7-467.4)	249.8(181.0-415.8)	100.9(49.3-165.2)	0.03
P	0.6	0.2	0.6	
IL4				
Primipare	23.0 (16.3-35.6)	23.6(12.3-37.6)	5.0 (1.0-9.6)	<0.000
multipare	26.3(13.6-48.0)	27.6 (17.3-43.0	6.3 (1.0-17.0)	<0.000
P	0.6	0.2	0.5	
IL10				
Primipare	125.3 (84.2-271.1)	172.2(86.2-334.4)	54.9(45.2-113.6)	<0.000
multipare	119.4 (82.3-240.7)	137.0(85.2-234.7)	54.9(27.6-96.0)	0.0002
P	0.6	0.6	0.1	

Cortisol, prolactin, cytokines and the susceptibility of pregnant Sudanese women to Plasmodium falciparum malaria

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Abstract

Understanding the hormonal and cytokine interactions that underlie susceptibility to the disease should be helpful in elucidating the pathogenesis of malaria during pregnancy. The current study was conducted in the Wad Medani hospital, in an area of central Sudan that is characterised by unstable malarial transmission. Its aims were to investigate the roles and interactions of cortisol, prolactin, interferon-γ (IFN- γ), interleukin-4 (IL-4) and interleukin-10 (IL-10) in pregnant women with *Plasmodium falciparum* malaria. The 82 pregnant subjects who were enrolled either had uncomplicated, *P. falciparum* malaria (the 47 cases) or were apparently uninfected and healthy women (the 37 controls) who were similar to the cases in terms of their mean age, weight, gravidity, gestational age and haemoglobin concentration. Compared with the controls, the cases were found to have significantly higher serum concentrations of total cortisol and IL-10 and significantly lower levels of prolactin and IFN-γ (but similar concentrations of IL-4). The hormone and cytokine concentrations measured in the infected primigravidae were similar to those recorded in the infected multigravidae. Among the cases, there was a significant positive correlation between serum cortisol and IL-10 ($r=0.188$; $P=0.025$) and significant negative correlations between prolactin and both IL-4 ($r=20.175$; $P=0.038$) and IL-10 ($r=20.186$; $P=0.027$) but no significant correlation between prolactin and cortisol.

Introduction

During pregnancy, immune responses appear to be influenced by *P. falciparum* infections, irrespective of parity. Cortisol, prolactin and some cytokines appear to be key mediators in the host response to *P. falciparum* infection, although further research on this subject is clearly needed. It has been estimated that 90% of the global malaria burden occurs in sub-Saharan Africa, where, during pregnancy, 40% of women are exposed to malarial infection (Steketee *et al.*, 2001). Malaria during pregnancy poses a substantial risk not only to the mother but also to her foetus and the neonate (Cot and Deloron, 2003). Malaria is the major health problem in Sudan, especially among pregnant women, who, irrespective of their age and parity, are more susceptible to *Plasmodium falciparum* malaria than their non-pregnant counterparts (Adam *et al.*, 2005).

Despite its obvious importance, the pathogenesis of human malaria during pregnancy is not completely understood. There are indications that changes in the serum concentrations of cortisol and prolactin may be associated with the loss of antimalarial immunity observed during pregnancy (Vleugels *et al.*, 1989; Bouyou- Akotet *et al.*, 2005). Cortisol suppresses the immune system and directly inhibits the activity of natural killer (NK) cells whereas prolactin is immunostimulatory and tends to increase the activity of NK cells (Gatti *et al.*, 1987; Jara *et al.*, 1991; Bouyou-Akotet *et al.*, 2004). Several cytokines have been reported to influence endocrine functions and, in malaria, cytokine concentrations are generally elevated (Kern *et al.*, 1989; Jaatella *et al.*, 1991; Perlstein *et al.*, 1993; Späth-Schwalbe *et al.*, 1994).

During pregnancy, the immune system may be biased towards type-2, humoral defence mechanisms rather than towards the type-1, cellular responses — a characteristic that may be fundamental for foetal wellbeing (Wegmann *et al.*, 1993). The systemic suppression of pro-inflammatory responses from T-helper-1 (Th1) cells [with increased circulating levels of interferon-c (IFN-γ) and tumour necrosis factor, and increased local expression of anti-inflammatory cytokines such as interleukin-4 (IL-4), IL-6 and IL-10] has been reported (Raghupathy, 1997). In investigating the pathogenesis of malaria during pregnancy, a better understanding of the hormonal and cytokine interactions that

underlie both infection control and disease should be helpful. The aims of the current study, conducted in a part of Sudan that experiences unstable malarial transmission (Malik *et al*., 2004), were to investigate the cortisol, prolactin, IFN-γ, IL-4 and IL-10 levels of *P. falciparum*-infected and uninfected pregnant women, and so add to the results of recent, local research on the pathogenesis of malaria during pregnancy (Adam *et al*., 2007a, b; Bayoumi *et al*., 2008).

SUBJECTS AND METHODS

The study was conducted, between the October and December of 2007, at the antenatal clinic of Wad Medani Teaching Hospital, in central Sudan. Pregnant women with singleton babies who presented with uncomplicated, *P. falciparum* malaria ('cases') were invited to participate in the study. Other pregnant volunteers who appeared to be healthy and uninfected with malarial parasites were used as controls, with the cases and controls roughly matched for age, parity, weight, haemoglobin and gestational age. Any woman who had antepartum haemorrhage, hypertensive disorder of pregnancy (with a diastolic blood pressure .90 mmHg) and/or diabetes mellitus was excluded.

 After taking witnessed, written, informed consent, relevant data on each subject's socio–demographic characteristics and parity were collected in an interview based on a structured questionnaire. Thin and thick smears were prepared from fingerprick blood samples and Giemsa-stained. The thick smears were used to evaluate malarial parasitaemias (asexual stages/ml), by counting the asexual stages against 200 leucocytes and assuming each subject had 8000 leucocytes/ml. Parasites were counted twice (once each by two microscopists) and the mean count was recorded.

Maternal haemoglobin concentrations were estimated in a Hemocue haemoglobinometer (HemoCue AB, A¨ ngelhom, Sweden). Venous blood (5 ml) was collected from each subject, into a plain tube, allowed to clot and then centrifuged so that the serum could be collected and kept at 220uC until assayed for cortisol, prolactin, IFN-γ, IL-4 and IL-10.

The cortisol and prolactin concentrations in each serum sample were evaluated using commercial radio- immunoassays (Izotop, Budapest) and a gamma counter (STRATEC

Biomedical Systems, Birkenfeld, Germany). Commercial sandwich ELISA (eBioscience, San Diego, CA), each based on a pair of cytokine-specific, monoclonal antibodies and each using the appropriate recombinant human cytokine as a positive control, were used to measure IFN-γ, IL-4 and IL-10 concentrations. All samples were run in duplicate, with the mean results used in the statistical analyses. Those performing the hormone and cytokine assays were unaware of the case/control status of the donor of each serum sample.

All the data were entered in a computer database, created using the SPSS for Windows software package (SPSS Inc., Chicago, IL), and double-checked before analysis. The cortisol data were found to be normally distributed and Student's t-tests were therefore used for comparing the mean values for each group of subjects. As the cytokine and prolactin concentrations were found to be not normally distributed, their median values were compared using Mann–Whitney U-tests. The levels of correlation between the continuous variables were assessed using Spearman rank tests. A *P*-value of <0.05 was considered indicative of a statistically significant difference.

The study received ethical clearance from the Research Board of the University of Khartoum's Faculty of Medicine.

RESULTS

During the study period, 45 pregnant women with uncomplicated, *P. falciparum* malaria (18 of them primigravidae) and 37 similar but apparently uninfected pregnant controls were enrolled (Table 1). The costs of the assay kits precluded the enrollment of one control for each case. Parasitaemias in the cases ranged from 1760 to 29,629 asexual stages/μl.

The mean (S.D.) serum concentration of cortisol in the infected cases was significantly higher than that in the uninfected controls [439.4 (172.0) *v*. 318.2 (210.3) ng/ml; *P*=0.005]. The cases also had significantly lower prolactin and IFN-γ levels and significantly higher IL-10 levels than the controls, although the cases and controls were similar in terms of their IL-4 concentrations (Table 2).

Among the cases, all the assay results for the primigravidae were similar to those for the multigravidae, not only in terms of the mean (S.D.) cortisol concentrations [383.8 (146.9) *v*. 458.0 (183.7) ng/ml; *P*>0.05] but also in terms of the median prolactin and cytokine concentrations (Table 3).

Also among the cases, there were significant positive correlations between the cortisol and IL-10 concentrations (r=0.188; *P*=0.025) and significant negative correlations between prolactin and both IL-4 (r=20.175; *P*=0.038) and IL-10 (r=20.186; *P*=0.027). There were, however, no significant correlations between cortisol and prolactin, IL-4 or IFN-γ, or between prolactin and IFN-γ.

There were also significant positive correlations, among the cases, between IL-10 and IL-4 concentrations (r=0.374; *P*<0.001) and between IFN-γ and IL-4 concentrations (r=0.687; *P*<0.001), although the IL-10 and IFN- γ concentrations of the cases did not appear to be correlated.

DISCUSSION

The current study was conducted to investigate the role of — and the interactions between — cortisol, prolactin, IFN-γ, IL-4 and IL-10 in the pathogenesis of malaria during pregnancy, in an area of Sudan characterised by unstable malarial transmission. The main findings of the study were the significant differences, in the levels of both hormones investigated and IFN-γ and IL-10 (but not IL-4), observed between the pregnant women with uncomplicated malaria and their uninfected counterparts. In terms of the hormones and cytokines investigated, the infected primigravidae were very similar to the infected multigravidae. Among the infected pregnant women, there were significant correlations between the IL-10 concentrations and both cortisol and prolactin concentrations.

The observation that pregnant women with *P. falciparum* malaria have higher serum levels of cortisol and lower levels of prolactin than their uninfected counterparts has been made several times (Vleugels *et al*., 1986, 1987, 1989; Bouyou-Akotet *et al*., 2005; present study). In malaria-endemic areas, it might be expected that multigravidae, who

have experienced previous foeto–maternal interactions and have, on average, presumably been more exposed to *P. falciparum* than the generally younger primigravidae in the same area, would portray different hormonal and cytokine pictures to those of the primigravidae. This difference would be a result of both immune cell priming, especially against malarial parasites, and the development of antibodies against the adhesion molecules that are responsible for the placental sequestration of malarial parasites (O'Neil-Dunne *et al*., 2001). So far, however, only Vleugels *et al*. (1986) have reported significantly higher cortisol levels in primigravidae with malaria than in multigravidae with the same disease. Such gravidity-related differences may be related to the level of malarial endemicity in the area. In central Sudan, and possibly in other areas where malarial transmission is unstable, the epidemiology and pathogenesis of malaria during pregnancy appears the same, irrespective of parity (Adam *et al*., 2007a, b; Bayoumi *et al*., 2008). The pregnant women in this region appear to be particularly susceptible to *P. falciparum* malaria, irrespective of their age and parity (Adam *et al*., 2005), and may even develop severe forms of the disease, such as cerebral malaria (Adam *et al*., 2004).

In the present study, the relatively high serum level of cortisol and relatively low serum level of prolactin seen in the infected women, and the correlation of the concentrations of both of these hormones with that of IL-10, indicate the effects of an integrated hypothalamus–pituitary–adrenal axis in these subjects. Activation of this axis in malaria may be the result of the release of cytokines and/or the stress generated by the disease itself. Cytokine concentrations are associated with the basal and peak levels of some hormones (Wilson *et al*., 2001). As discussed above, both cortisol and prolactin appear to modulate the immune system and influence the activity of NK cells (Gatti *et al*., 1987; Jara *et al*., 1991; Bouyou-Akotet *et al*., 2004). Several cytokines have been reported to influence endocrine functions and, in malaria, serum concentrations of some cytokines are generally raised (Kern *et al*., 1989; Jaatella *et al*., 1991; Perlstein *et al*., 1993; Späth-Schwalbe *et al*., 1994). In addition, IL-10 production is up-regulated by prolactin (Kim *et al.*, 2003), perhaps via the activation of lymphocytes and macrophages (Matalka, 2003).

During pregnancy, the enhanced expression of cytokines such as IL-10 curtails the potentially hazardous effects of Th1-related cytokine production on systemic immunity,

thus ensuring the retention of the foetal allograft (Matthiesen *et al*., 2003). Possibly, IL-10 blocks the activity of NK cells in the decidua, which could otherwise cause thrombosis, inflammation, and/or miscarriage (Clark *et al*., 1998). An anti-inflammatory cytokine environment is thought to be maintained during pregnancy, in part, by high progesterone levels, which induce Th0 to Th2 conversion (Szekeres-Bartho and Wegmann, 1996).

In the present study, the serum concentrations of IFN-γ were lower in the infected women than in the uninfected controls (although, in the infected women, the IFN-γ concentrations were not correlated with those of cortisol or prolactin). Previously, IFN-γ has been associated with protection from malaria, with impaired IFN-γ production mooted as a cause for increased susceptibility to placental malaria (Moore *et al*., 1999). Although Rogerson *et al*. (2003) found no detectable IFN-γ in placental biopsy specimens from Malawian primigravidae, IFN-γ was found in about 40% of placental plasma samples collected in neighboring Kenya, being associated with malarial infection and poor foetal outcome (Moorman *et al*., 1999). The contrasting results of these two studies may again reflect differences in malarial endemicity in the study areas. The malarial infections investigated in the present study were current/acute, not the chronic or past infections often revealed by placental histopathology.

In conclusion, it appears that, irrespective of parity, cortisol, prolactin and certain cytokines are key mediators in the host response to *P. falciparum* infection during pregnancy in women living in central Sudan, where malarial transmission is unstable. It remains possible, however, that the hormonal and cytokine perturbations seen in the infected pregnant women living in this region are simply the result of malarial infection, and not factors in the aetiology of the malaria. Further research is needed to elucidate the very complex nature of malaria during pregnancy.

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Table 1: The baseline characteristics of the 82 pregnant women with and without *Plasmodium falciparum* malaria who provided blood samples.

Variables	Women with malarial infection (the cases)	Women without malarial infection (the controls)	*P*
No. of subjects	45	37	
Mean value and SD for:			
Age, years	26.8(6.9)	26.1(6.7)	0.6
Gravidity	3.1(3.5)	2.5(2.4)	0.4
Gestational age, weeks	29.5(8.6)	27.7(9.7)	0.5
Weight, kg	65.4(6.6)	67. 6(11.2)	0.3
Haemoglobin, g/dl	10.2 (1.0)	10.8(0.9)	0.6

Table 2: The cytokine and prolactin concentrations detected in the sera of the 82 pregnant women with and without *Plasmodium falciparum* malaria who provided blood samples.

	Median concentration and (interquartile range)		
Variable	Women with malarial infection (the cases)	Women without malarial infection (the controls)	*P*
IFN-γ (pg/ml)	150.5(142.4-412.2)	311.5 (66.1-608.1)	0.016
IL-4 (pg/ml)	49.0 (19.1-74.5)	45.3(40.3-60.0)	0.856
IL-10 (pg/ml)	506.2 (252.0-1435.2)	39.8 (10.0-112.7)	<0.001
Prolactin (mU/litre)	5365.0(4362.0-7730.0)	7130.0 (5244.0-9322.5)	0.02

Table 3.3: The cytokine and prolactin concentrations detected in the sera of the 18 primigravidae and 27 multigravidae with Plasmodium falciparum malaria who provided blood samples.

	Median concentration and (interquartile range)		
Variable	Primigravidae	Multigravidae	*P*
IFN-γ (pg/ml)	73.0 (72.6–168.4)	78.3 (41.7–122.4)	0.3
IL-4 (pg/ml)	43.7 (33.5–75.1)	57.9 (15.0–76.6)	0.9
IL-10 (pg/ml)	129.4 (52.1–270.2)	72.5 (33.1–107.1)	0.1
Prolactin (mU/litre)	5175.0 (4817.6–8008.7)	5480.0 (4343.7–7848.7)	0.9

Peripheral, Placental and Cord Cytokines Profile in Spontaneous Labor and Elective Caesarean Section

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ABSTRACT

Background: Cesarean section delivery can lead to much maternal morbidity. Different cytokines have been reported to be influenced by the mode of delivery. Objective: To investigate the influence of mode of delivery on maternal, placental and cord sera of interferon gamma (IFN-γ), interleukin-4 (IL-4) and interleukin-10 (IL-10) levels. **Methods:** These three cytokines were measured using ELISA in peripheral, placental and cord sera of two groups of women (38 in each group), either delivering vaginally or by elective cesarean section. **Results:** Concentrations of IFN-γ, IL-4 and IL-10 in the peripheral and placental sera were higher in vaginal delivery, while cord cytokines were not significantly different in the two groups. Cord sera contained significantly less concentrations of these cytokines than the peripheral and placental sera. **Conclusion:** It appears that the levels of IFN-γ, IL-4 and IL-10 are influenced by the mode of delivery.

Keywords: Cytokines, Cesarean, Labor, Vaginal

INTRODUCTION

The rate of cesarean section is rising worldwide and Sudan is not an exception (1, 2). Cesarean section delivery can lead to higher maternal morbidity and is associated with increased risks of asthma and atopy in the delivered children (3, 4).

Cytokines play an important role during labor and they influence immunity of the fetus and neonate. The mode of delivery might influence the establishment of the infant's

microflora (1). Thus, during the transitional period from the normally sterile intrauterine environment to the extrauterine one- which is characterized by exposure to multiple antigenic stimuli- neonatal defense is going to be built accordingly (2). Recently, production of different cytokines and their balance have been reported to be influenced by the mode of delivery (7-9). Further understanding of the relationship between mode of delivery and immune system of the newborn is needed. The current study was conducted to investigate the influence of mode of delivery on maternal, placental and cord sera IFN-γ, IL-4 and IL-10 levels.

MATERIALS AND METHODS

The study was conducted at the labor ward of New Halfa hospital, eastern Sudan in the period of October 2006 through March 2007 to investigate whether maternal, placental and cord cytokine profiles depend on mode of delivery.

Healthy women -vaginal or elective cesarean delivery- and their singleton neonate were approached to participate in the study. A structured questionnaire was administered to gather socio-demographic characteristics.

The study received ethical clearance from the Research Board at the Faculty of Medicine, University of Khartoum.

Immediately after delivery, 5 mL of maternal, placental and cord blood were collected, using the biopsy-pool method for placental blood. The samples were centrifuged and the sera were kept at -20 until processed for cytokines in the laboratory.

Sera were analyzed by ELISA for IFN-γ, IL-4 and IL-10 levels according to the manufacturer's instructions (eBioscience, Inc. 6042 Cornerstone Court West San Diego, CA 92121, USA).

Statistical Analysis: Data were analyzed using SPSS for Windows. The socio-demographic characteristics were compared using Student's t-test. Cytokines data, which were not normally distributed, were compared by Mann-Whitney U and Kruskal-Wallis

tests. Correlations between continuous variables were assessed by the Spearman rank test. *P*-values less than 0.05 were regarded as significant.

RESULTS AND DISCUSSION

Seventy six (38 in each group) women and their neonates were enrolled. The two groups were well matched in the basic data; age, parity, gestational age, haemoglobin and birth weight (Table 1).

Concentrations of IFN-γ, IL-4 and IL-10 in the peripheral and placental sera were higher in vaginal delivery, while the levels of these cytokines were not significantly different in the cord sera. Significantly less concentration of these cytokines were noted in cord sera than in the peripheral and placental ones (Table 2).

Strong positive correlations were observed between each cytokine in peripheral and placental sera: IFN-γ (r= 0.89, P < 0.05); IL-4 (r=0.82, P< 0.05); and IL-10 (r= 0.15, P < 0.05). There was no correlation between peripheral and cord (r= 0.2, P = 0.06), or placental and cord sera concentrations (r= 0.12, P = 0.2) of IL-4. Similarly a lack of correlation between peripheral and cord sera IL-10, (r =0.13, P = 0.2), or between placental and cord sera concentrations of this cytokine(r= 0.1, P =0.3) was observed.

The current study was conducted to investigate the influence of the mode of delivery on cytokine levels. The peripheral and placental levels of IFN-γ, IL-4 and IL-10 were significantly higher in women who delivered vaginally, with strong positive correlations between peripheral and placental levels. The cord levels of these cytokines were not significantly different between the two groups, but cord levels were lower than the peripheral and placental levels. These results are in agreement with previous reports demonstrating that peripheral and placental and not the umbilical cord cytokine productions depend on the mode of delivery (7, 8). Cytokines play an important role in the defense against infections and the regulation of the immune response; therefore, their increase during labor is implicated in the protection of the mother and the neonate against perinatal infections.

In a previous report, no association was reported between cesarean section and the neonatal levels of IL-10 (9), a cytokine with inhibitory effects on the secretion of Th1 and Th2 cytokines (10). Yet an elevated level of IFN-γ at birth was found to associate with asthma and atopy in childhood (11).

Our study did not show any difference in the cord sera concentrations of these cytokines. Ly et al., reported that the cytokine levels were significantly higher in cesarean delivery (9). Interestingly, in the current study, there were positive correlations between the peripheral and placental cytokines. This indicates the interactions between the two sources of these cytokines mainly the maternal and the placental ones. In summary, it seems that levels of IFN-γ, IL-4 and IL-10 are influenced by mode of delivery.

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Table 1 The mean (SD) of the demographic characteristic of the two groups of women, those who delivered vaginally and those delivered by elective cesarean section.

Variable	Vaginal delivery	Cesarean section
Age, years	25.7(7.3)	26.3(6.1)
Parity	3.1(2.5)	2.4(2.1)
Weight, Kg	51.1(13.1)	59.9(12.5)
Birth weight, gm	2932.0(585.4)	2922.2(502.9)

Table 2 The median (interquartile range) of cytokine levels in vaginal and cesarean section delivery

Cytokines, pg/ml	Mother	Placenta	Cord	*P*
IFN-γ				
vaginal	352.8(169.6-732.2)	289.8(181.0-559.0)	123.8(77.9-238.3)	S
cesarean section	215.4(169.6-307.0)	244.0(186.8-344.3)	83.7(40.7-166.3)	S
P	S	S	NS	
IL4				
vaginal	28.3(17.6-65.6)	30.3(17.3-60.9)	5.0(1.0-13.0)	S
cesarean section	23.6(18.3-31.0)	24.3 (18.0-31.6)	7.6 (1.0-16.0)	S
P	S	S	NS	
IL10				
vaginal	168.3 (94.0-387.1)	180(103.7-277.7)	51.0 (31.5-90.1)	S
cesarean section	105.7(82.3-156.6)	109.7(85.2-217.1)	70.6 (33.4-106.7)	S
P	S	S	NS	

* N = not, S = significant

Cytokines Profiles in Sudanese Women with Preeclampsia

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Background: Cytokine imbalance in preeclampsia may be one of the etiological factors for preeclampsia. **Objectives:** The study was conducted to investigate interferon gamma (IFN-γ), interleukin-4 (IL-4) and interleukin-10 (IL-10) in preeclampsia. Enzyme-linked immunosorbent assay (ELISA) was used to measure the concentrations of these three pro-inflammatory cytokines in sera from 33 Sudanese women with preeclampsia (at presentation and 7 days later) and 32 women with normal pregnancy as a control group. **Results.** The levels of IFN-γ and IL-4 were slightly— not statistically significant— higher in the women with preeclampsia. IL-10 was significantly higher in the women with preeclampsia. Women with preeclampsia had significantly lower levels of IFN-γ and IL-4 and significantly higher levels of IL-10 7days later in comparison with the presenting levels. **Conclusion.** Thus, the significantly raised levels of IL-10 in women with preeclampsia suggest its role in pathogenesis of preeclampsia, and further research is needed.

Keywords: Cytokines, Preeclampsia, Pathogenesis, Sudan.

INTRODUCTION

Hypertensive disorders of pregnancy occur primarily in humans, and are estimated to cause 10%–15% of maternal deaths (1). Preeclampsia, defined by hypertension and

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proteinuria, is the well-described disorder (2). It is an important cause of maternal, perinatal morbidity and mortality worldwide (3).The pathogenesis of preeclampsia is obscure, although abnormal placentation is currently considered among the more plausible hypotheses. Recent findings and theories have variously imputed roles for systemic inflammation, abnormalities in cardiovascular adaptation to pregnancy.

The immune system has been implicated in the pathophysiology of preeclampsia with modifications in the cellular immunity and cytokines production (4), which are sought to have an important role in the maintenance of pregnancy (5).The current study was conducted to investigate the role of interferon gamma (IFN-γ), interleukin-4 (IL-4) and interleukin-10 (IL-10) in the pathogenesis of preeclampsia, to add to our ongoing research on preeclampsia and these cytokines (6).

MATERIALS AND METHODS

Patients

The study was conducted in Khartoum teaching hospital, Sudan during the period of March through July 2007. Patients with preeclampsia were approached to participate in the study. Volunteer women with normal pregnancy at the same gestational age were selected as a control group. Well structured questionnaires were used to gather sociodemographic characteristics. Preeclampsia was defined as persistently high blood pressure ≥140/90 mmHg on 2 or more occasions 6 hours apart and proteinuria ≥ +2 by dipstick or ≥300 mg/day in 24 hours' urine collection. Gestational age was calculated from the last menstrual period and confirmed by ultrasound in suspected cases. Blood pressure was measured in all patients and controls with mercury sphygmomanometer.

Sample Collection

Five ml of blood was collected from both groups by venipuncture in plain tubes, in women with preeclampsia, another 5 ml of blood were collected 7 days later. Samples were kept at room temperature for 30 minutes, centrifuged at 2000 rpm for 10 minutes

and serum was stored at -20 degrees until the assay. All of these women were pregnant and not in labour at presentations or 7 days later.

Cytokines Measurement

The levels of INF-γ, IL-4 and IL-10 were measured for the patient and control group by standard sandwich enzyme linked immunosorbent assay (ELISA) using pairs of cytokine-specific, monoclonal antibodies according to the manufacturer's instructions (eBioscience, Inc. 6042 Cornerstone Court West San Diego, CA 92121 USA). All samples were run in duplicates and the mean value was used in all analysis.

Statistics

Data were entered in computer using SPSS for windows and double-checked before analysis. Data was not normally distributed; the Mann-Whitney U-test was used to determine the significance of difference between the variables. Correlations between continuous variables were assessed by the Spearman rank test. $P < 0.05$ was regarded as significant.

Ethics

The study received ethical clearance from the Research Board at the Faculty of Medicine, University of Khartoum.

RESULTS

The two groups were well- matched in their age, gravidity, weight and gestational age Table 1 shows the medians and intercortiles for IFN-γ, IL-4 and IL-10 levels in sera of patients with preeclampsia and in the control group at presentation. The levels of IFN- γ and IL-4 were slightly higher—not statistically significant—in women with preeclampsia. The level of IL-10 was significantly higher ($P = 0.002$) in women with preeclampsia. In women with preeclampsia, the levels of IFN-γ and IL-4 were

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significantly lower; level of IL-10 was significantly higher seven days later in comparison with the presenting levels, Table 2.

Correlation Between the Three Cytokines at Presentation A significantly positive correlation was observed between IFN-γ and IL-4 (r = 0.495, P = 0.007) and IL-4 and IL-10(r = 0.445, P = 0.009). There was no correlation between IFN-γ and IL-10 (r = 0.094, P = 0.602).

DISCUSSION

The study was conducted to investigate the pattern of cytokine production in Sudanese patients with preeclampsia. The main finding of this study is the slightly raised level of IFN-γ, IL4 and significantly raised level of IL10 in the preeclamptic group. Recently, Mansouri and colleagues reported significantly higher levels of IFN-γ, IL4 without significant difference in the level of IL10 in Iranian women with preeclampsia (7). However, on the contrary IL10 production has been reported to be significantly lower in women with preeclampsia in comparison with the control group (8). On the other hand, no significant difference was reported in the level of IFN-γ in women with preeclampsia (9). Likewise, IL4 is raised slightly and a positive correlation was observed in the current study. Perhaps the increase levels and the correlation between IFN-γ and IL4 indicate a compensatory mechanism. The enhanced cytokines expression, perhaps in concert with other anti-inflammatory immunomodulatory cytokines, curtails the potentially hazardous effects of Th1-related cytokine production on systemic immunity during pregnancy, thus ensuring the retention of the fetal allograft (10). Perhaps, these cytokines blocks NK activity of the decidua which may have potentially deleterious effect on the fetus like thrombosis, inflammation, and miscarriage (11). Interestingly, in our study IL10 was significantly higher in women with preeclampsia. This is in line with the previous reports (12). Yet, Borekci *et al.,* reported a significantly lower level of IL10 in women with preeclampsia and Mansouri *et al*., 2007, reported no difference in IL10 level in women with preeclampsia too (7, 13). Furthermore, in this study the levels of IFN-γ and IL4 cytokines were significantly lower 7 days later, but IL10 was higher. These patients

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received different antihypertensive drugs (mainly methyldopa, hydralazine and nifedipine). Drugs might have altered Th1/Th2 cytokine balance in women with preeclampsia (14), or the levels of these cytokines levels have changed according to the blood pressure itself rather than the drugs (12). This area needs to be investigated in the future. Preeclampsia is associated with both local and systemic changes in type1/ type2 cytokine balance compared to normal pregnancy. Decidual and peripheral blood mononuclear cells from patients with pre-eclampsia are generally primed to synthesize high levels of the Th1 cytokines, (15). These variations may be due to the unknown aetiology and pathophysiology of preeclampsia, and this raises a big question to be answered by further research in this field, whether these cytokines play an etiological role or their pattern of production is sequelae to the pathology. Interesting the levels of these cytokines were low in comparison with their levels in an other important maternal morbidity—malaria—that we are investigating too (16). Recently, some evident emerging out concern the interactions between malaria and hypertension (17), but still this area needs to be investigated in the future.

A major problem with preeclampsia is the lack of understanding of the aetiology and pathophysiology of the condition. The Th1/Th2 cytokine imbalance in preeclampsia may be considered as sequelae to the hypoxic nature of the disease or an aetiological factor and further research is needed to understand the very complex nature of preeclampsia.

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Table1: showing the mean (SD) of characteristics of the women with preeclampsia and controls

Variables	Women with preeclampsia (n=33)	Control (n=32)	*P*
Age	29.5(5.7)	29.5(5.90	0.9
Gravidity	2.1(2.1)	1.8(1.9)	0.3
Gestational age, weeks	35.2(2.8)	35.6(2.9)	0.5
Weight, kg	59.7(7.4)	57.0(6.8)	0.6

Table (2): The median ((interquartile range) of cytokines in women with preeclampsia and control group

Cytokine pg/ml	Women with preeclampsia (n=33)	Control (n=32)	P
IFN-γ	210 (142.40-287.06)	125.38 (85.67-184.95)	0.437
IL-4	10.3 (3.33-18.35)	9.69 (3.69-14.63)	0.780
IL-10	8.6 (2.39-16.66)	6.99 (3.39-16.84)	0.002

Table (3) the median (interquartile range) of cytokines in patients with preeclampsia at presentation and day seven.

Cytokine, pg/ml	At presentation	Day seven	*P*
IFN-γ	210.48 (142.40-287.06)	136.73 (81.42-192.04)	0.035
IL-4	10.36 (3.33-18.35)	9.99 (2.15-15.36)	0.000
IL-10	8.61 (2.93-16.66)	10.63 (6.18-25.41)	0.000