The role of histology, immunohistochemistry and molecular biology in the diagnosis and study of malaria and low birth weight in an area of unstable malaria transmission in central Sudan

By

Amal Hussein Mohammed Ali

M.S.c in Medical Laboratory Sciences (Histopathology and Cytology)

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Supervisor

Professor Ishag Adam, MD, PhD

Faculty of Medicine

University of Khartoum, Sudan

Co-supervisor

Dr. Magdi M. Salih, M.S.c, PhD

Histopathology and Cytology Department

Faculty of Medical Laboratory Sciences

University of Khartoum, Sudan

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قال تعالى: (إِنَّ الْلَّهَ لا يَضُعِّفُنَّ بِمَا بَعْضَهُ مَنِ احْتَضَنَّهُ). قالوا: (إِنَّ ذَٰلِكَ لَصَالِحٌ). قال: (يَتَّبِعُهُ بِمَا يَتَّبِعُهُ). وَيَسْتَفْدِعُهُ بِمَا يَسْتَفْدِعُهُ. وَيَعْلَمُهُ بِمَا يَعْلَمُهُ. وَيَسْتَفْنَى بِمَا يَسْتَفْنَى. 

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إهـداء

إلى من كانا لي بحبيهما وعطائهما ودعائهما منارة أضاءت دربي ... والدي العزيز ... والدتي الغالية ... حفظكم الله لي من كل شر ...
إلى من كان سندًا قوياً لي في حياتي العلمية ... والعملية ... زوجي ... جزاك الله خيراً عني ...
إلى الرائعة التي تعطر حياتي موجودًا قريبي ... أختي الحبيبة إِشراقه ... جزاك الله كل خير عني ...
إلى أحبتي ... وأخوتي ... أماني ... محمد ... أسامة ...
إلى ربيع حياتي ... رحاء ... اشرف ... ربي ...
إلى كل من وقف بجانبي ... بتشجيع ... بنصيحة ... بمعلومة
أحمد الله كثيرا إنكم كنتم وحازتم بجانبي ... حفظكم الله وجزاكم الله كل خير على وان أنسى أن أهدي عملي البسيط هذا لكل أم عانت من إصابتها بالملاريا وهي تضع مولودها و لكل رضيع أصيب بالملاريا في شهرته الأولى.
لكم جميعا أهدي هذا المجهود المتواصل ... دعاء الله أن يكون أضافه في الجهاد المبذول للقضاء على مرض الملاريا في بلادي ...

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<td>PAM</td>
<td>Pregnancy-associated malaria</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxlin and eosin</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>MSP1</td>
<td>Merozoite surface protein 1</td>
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<td>E V T</td>
<td>Extra villous trophoblast</td>
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<td>PM</td>
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<td>L B W</td>
<td>Low birth weight</td>
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<td>M IF</td>
<td>Macrophage migration inhibitory factor</td>
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<td>T N F</td>
<td>Tumor necrosis factor</td>
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<td>IFN-γ</td>
<td>Interferon gamma</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>CSPGs</td>
<td>Chondroitin sulfate proteoglycans</td>
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<td>IRBC</td>
<td>Infected red blood cells</td>
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<td>H A</td>
<td>Hyaluronic acid</td>
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<td>C S A</td>
<td>Chondroitin sulfate A</td>
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<td>C N S</td>
<td>Central nerves system</td>
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<td>F T F</td>
<td>Fibrin-type fibrinoid</td>
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<td>P T D</td>
<td>Pre term delivery</td>
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<td>D P X</td>
<td>Distrene plasticizer xylene.</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<td>I U G R</td>
<td>Intra uterine growth retardation</td>
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Abstract

Background: *Plasmodium falciparum* malaria infections during pregnancy are associated with poor pregnancy outcomes such as maternal anemia and low birth weight (LBW). There are few published data on submicroscopic malaria and LBW.

Objectives: The study was conducted at Medani Teaching Hospital in Central Sudan- an area which is characterized by unstable malaria transmission- to investigate the association between placental malaria, submicroscopic malaria and LBW.

Methods: A case control study was conducted (87 women in each arm); cases were women with single LBW (< 2500g) and controls were women with single babies weighting 2500-4200 g. Socio-demographic characteristics were gathered. Histology and polymerase chain reaction (PCR) were used to detect placental malaria in the maternal blood, placenta and to look for cord parasites.

Result: Twenty-seven (31.0%) versus 22 (25.3%), P = 0.500 of the cases and controls, respectively, had placental malaria infections as determined by histological examination. In comparison to the controls, the submicroscopic malaria infection prevalence rate was significantly higher in the cases; 24 (27.6%) vs. six (7.0%), P < 0.001. Multivariate analysis showed that while malaria infection of the placenta (based on histology) was not associated with LBW, submicroscopic *P. falciparum* infection (OR=6.89, 95% CI=2.2–20.8; P = 0.001), or a combination of histologically determined and submicroscopic infections (OR=2.45, 95% CI=1.2–4.9; P = 0.012), were significantly associated with LBW.

Conclusion: In Central Sudan, pregnant women were at a higher risk of having an LBW delivery if they had submicroscopic infections rather than a histological diagnosis of placental malaria.
مستخلص الدراسة

المقدمة: أن الإصابة بطفيل الملاريا المنجلية في فترة الحمل يؤدي إلى الولادة الفقيرة، بما في ذلك انخفاض الوزن عند الولادة. وما يلاحظ إن المعلومات المنشورة بخصوص الملاريا المنجلية وعلاقاتها بالانخفاض وزن الجنين لشخصية.

الأهداف: الدراسة الحالية أجريت في مستشفى مدني تعليمي في غرب السودان، وهي منطقة تتسم بعدم ثبات انتقال الملاريا فيها. تحاول الدراسة التحقق من العلاقة ما بين المشيمة المصابة بالملاريا غير المنجلية بالمجهر المرضي والولادة.The procedure: أجريت دراسة مراقبة حالات حيث تم دخول المراقب في 87 حالة في كل جانب من جانب الدراسة حيث شكلت امراضة الأطفال حديثي الولادة الذين يزنون أقل من 2500 غرام التراوح الأول بينما شكلت امراضة الأطفال حديثي الولادة الذين يزنون وزنا طبيعيًا عند الولادة تراوح بين 2500-4000 غرام. وقد جمعت الخصائص الاجتماعية والديموغرافية، وقد تم استخدام طرق التشخيص النسجي وتحليل النشاط الجنيني لفحص دم الأمهات والمشيمة ونسبة من الحالات السري.

النتيجة: نسبة وضوح (31.0%) مقابل 22 (25.3%) (P = 0.500) من الحالات والضوابط، على التوالي، وكانت الإصابة بالملاريا المشيمة وفقاً لما يحضه الفحص السنجوي. ومثابرة الحالات بالضوابط؛ 24 (27.6%) مقابل ستة (7.0%) (P = 0.010). وأظهر التحليل متعدد المتغيرات أنه في حين أن عدداء الملاريا من المشيمة (على أساس الفحص النسجي) لم يترافق مع نقص الوزن عند الولادة، فلدقت تراقت الإصابة بالملاريا دون المجهري بتفسان وزن الأطفال حديثي الولادة: (P = 0.001، OR = 6.89، 95% CI = 2.20-21.80) (P = 0.012، OR = 9.42، 95% CI = 4.20-21.80) في حالة الفحص دون المجهري المرتبط بالفحص النسجي.

الخلاصات: أثبتت الدراسة أن المرض يتعرض النساء الحوامل في غرب السودان لخطر انتقال أمراض ناقل إنساً ونسبة من حالات الصيدلة.

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Chapter One

Introduction and Literature Review

1.1 Introduction

In spite of the intense efforts to control malaria, malaria remains a major challenge to health authorities and impedes economic and social growth in the developing countries (1). Several factors contributed to further complicate problem of malaria, these include; the emergence of drug-resistant strains of *Plasmodia*, evolution of insecticide-resistant species of mosquitoes, global climatic changes, rapid increase of the world population, poor health facilities, civil disturbances and armed conflicts in various parts of the developing world (2). The natural history of infection with *P. falciparum*, which causes the most severe infections and nearly all malaria-related deaths, has been well characterized in areas of high endemicity in Africa (3). World Health Organization (WHO) established criteria for severe malaria in order to assist future clinical and epidemiological studies. These criteria include cerebral malaria, pulmonary edema, acute renal failure, severe anemia, and bleeding. Acidosis and hypoglycemia are the most common metabolic complications. Any of these complications can develop rapidly and progress to death within hours or days. Severe malaria accounts for
approximately 5% of imported malaria cases. The case fatality rate in returning travelers with \textit{P. falciparum} malaria varies from 0.6% to 3.8%, and for severe malaria it may exceed 20%, even when managed in intensive care units. In various studies risk factors for severe malaria and death include age greater than 65 years, female sex especially when associated with pregnancy (4). Malaria is most frequent in first pregnancy, peaking between 13 and 16 weeks, and declining toward term. Age may be an independent risk factor, as younger pregnant women have been found to be more susceptible to malaria in some settings. Adolescent and young adult women are more commonly parasitemic than older adults, and this may reflect the development of malarial immunity (5). Children under 5 years have a primary malaria attack during their first year of life, while most toddlers and juveniles have already developed resistance against severe disease but still experience a few clinical episodes. African adolescents and adults, in contrast, are often clinically immune; they remain free of malaria symptoms, despite continuous exposure to the parasite, but maintain low-grade infections throughout the transmission season. The virulence of \textit{P. falciparum} is because of the ability of infected red blood cells to clump in the capillaries of vital organs, in addition to the adhesion of parasites to the host endothelial cells and blood cells, which leads to blockage of the micro vascular circulation. Another virulence factor of this parasite is its ability to
generate variability within genetic families. This diversity relies on the extensive allelic polymorphism, the antigenic variation and the sexual reproduction that ensures genetic mixing. The genetic diversity of *P. falciparum* is also responsible for the parasite’s resistance to antimalarial drugs. The parasite polymorphism markers most commonly used in molecular epidemiology are the merozoite surface proteins namely msp-1 and msp-2, and other antigens associated with the surface of the merozoite such as msp-3, GLURP, SERP and S-antigen (6). When *P. Falciparum* infect red blood cells, it produces molecules that the blood cells then exhibit on their surface. These molecules are what alert the immune system into launching an attack, the first interaction between malaria merozoite and red blood cell it will invade it mediated by this molecule, the *P. falciparum* merozoite surface protein 1(MSP1) and merozoite surface protein 2(MSP2) which is thought to have role in biology of the host parasite interphase and related protein coded by gene families in each of the different *Plasmodium* species (7). The *P. falciparum* genome is 23 megabases long, consist of 14 chromosome and encodes for approximately 5300 genes a large number of genes exhibit extensive polymorphism, the loci encoding proteins displayed on the surface of the sporozoite, e.g. (CSP) and the merozoite, e.g. (MSP1,MSP2,AMA1) (8). Infections of a malaria parasite could include a single genotype of cells (single-clone infections) or two to several
genotypes (multiclone infections). Clonal diversity of infection plays an important role in the biology of the parasite, including its life history, virulence, and transmission (9). Genotyping of *P. falciparum* infections with polymerase chain reaction (PCR)-based methods has therefore been introduced in epidemiological studies. Polymorphic regions of the msp1, msp2 are the most frequently used markers for genotyping, but methods may differ. Genotyping by PCR is a powerful tool for studies on genetic diversity of *P. falciparum* (10). To investigate the impact of transmission on the development of immunity to malaria and on parasite diversity in holo- and mesoendemic transmission conditions, respectively. Konaté and other authors (11) analyzed *P. falciparum* msp1 block 2 and msp2 genotypes of isolates collected from uncomplicated and severely infected people. Allele frequencies differed in both group, indicating considerable micro-geographical heterogeneity of parasite populations. The complexity of the infections, estimated using individual or combined msp1 and msp2 genotyping (11).
1.2 Epidemiology of Malaria

Human malaria is caused by four species of parasitic protozoa of the genus *Plasmodium*: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. Malaria is the most important tropical disease and causes death of more people than any other transmissible disease. Approximately 36% of the world population lives in risk areas. Worldwide estimates of patient numbers is around 515 million annually, and 1.5 to 2.7 million people die due to complications of malaria. *P. falciparum* is responsible for most of the infections and almost all deaths, occurring in many countries but mainly in the African continent. The World Health Organization (WHO) listed 101 countries as endemic for malaria. The majority (70%) of all malaria cases are in Sub-Saharan Africa (12).
1.3 List of Malaria Endemic Countries

The World Malaria Report 2011 summarizes information received from 106 malaria-endemic countries and a range of other sources. It analyses prevention and control measures according to a comprehensive set of indicators, and highlights continued progress towards global malaria targets. This year's report builds primarily on data received from Source: World Malaria Report -Country profiles 2010 (13).
1.3.1 Malaria Endemic Countries

Figure 1: worldwide distribution of malaria from mid-19th century to 2010, Source WHO
1. 4 Epidemiology of Malaria in Sudan

In Sudan malaria is considered as one of the main health problems, with high morbidity and mortality particularly among children and pregnant women. It constitutes approximately 26% of all out-patients and about 18% of in-patients, and about 20% of deaths in clinical and other governmental institutions (14). 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 (15). In eastern Sudan, transmission and intensity of malaria is perennial and moderate rather than low (16). There was a significant positive correlation between malaria cases and rainfall in the area, and malaria epidemics were found to be associated with heavy rains (17). The predominant parasite species is *P. falciparum*, whereas *P. ovale* is sporadically distributed. *P. malaria* is particularly considered to be available only in Southern Sudan, (18). In New Halfa, Eastern Sudan, cerebral malaria is more frequent during adolescence and early adulthood, and it is the major cause of malaria mortality (19). Uniquely in this area, cerebral malaria may be associated with latent parasitaemia in partially immune adults (20). This study will be conducted in central Sudan which
consider as the higher of malaria endemicity stratum, where transmission
intensity is high, and rainy season. The main malaria parasite in the
region is \textit{P. falciparum} (95\%), \textit{P. vivax} (3\%), and \textit{P. Ovale} (2 \%) (21)
and the vector is \textit{Anopheles arabinsis}, \textit{P. falciparum} survives the long dry
and transmission–free season as asymptotic sub-patent infections (22).
1.4.1. Map of Malaria in Sudan

The spatial limits of *Plasmodium falciparum* malaria transmission map in 2010 in Sudan (23)

![Malaria distribution in Sudan](image)

**Figure 2: Malaria distribution in Sudan, Source WHO**
1.5 Histology of placenta

Placenta is formed from elements of the membranes which surround the developing fetus as well as the uterine endometrial. It provides the physiological exchange between the fetal and maternal circulation. Early placenta develop when the stromal cell of the endometrial stratum functionalis at the implantation site to large polyhedral decidual cells this change extended to whole endometrial lining of the uterus. The decidua beneath the developing embryo is known as deciduas basalis and with the trophoblast will form the future placenta. The complex villous structure of mature placenta contains mesenchymal core contain capillaries served by afferent and efferent fetal blood vessels. The placenta develops with fetus to full term and contains huge number of villi with increased in surface area which filled with maternal blood. The elements membranes of placenta surround the developing fetus as well as the uterine endometrial and provides the mean for physiological exchange between the fetal and maternal circulation (24).

1.6 Physiology of the Placenta

Development of the placenta is a highly regulated process that is essential for normal fetal growth and development, and for maintenance of a healthy pregnancy. The placenta fulfills several critical roles as the interface between mother and fetus: preventing rejection of the fetal
allograft; transporting and metabolizing nutrients, and providing peptide and steroid hormones. Development of the placenta and fetus is a continuous process that begins at the time of fertilization. Four days after fertilization, the morula (a solid mass of blastomere cells) enters the uterus. As uterine fluid penetrates its outer layer (the zona pellucida), a blastocyst with a cavity is formed. Further of fluid results in separation of the expanded blastocyst into two parts and the inner cell mass-group blastomeres which form the embryo. Trophoblast- is a thin outer cell layer of blastomeres which form the placenta and fetal membranes. The blastocyst is bathed in uterine secretions that provide the embryo with oxygen and metabolic substrates. However, this soon becomes inadequate for further development and the embryo must then implant in the uterine wall, the progenitor villous trophoblast cell is the stem cell of the placenta. It proliferates throughout gestation, differentiating along two pathways to form either inner single layer of mononuclear cells cytotrophoblast (stem cell) or an outer layer of invasive extra villous trophoblast (EVT) or syncytiotrophoblast. The EVT is responsible for invasion, thereby anchoring the placenta to the decidua and myometrium. The syncytiotrophoblast is a specialized epithelium covering the villous tree and has several functions, such as transport of gases, nutrients, and waste products and synthesis of peptide and steroid hormones that regulate maternal systems (25).
1.7 Epidemiology of Malaria during Pregnancy

Pregnancy-associated malaria remains a major public health problem in endemic regions because of its detrimental effect on pregnancy outcome. It is important to assess the different burden of *P. falciparum* on pregnant women to determine accurately the morbidity caused by malaria in this population at risk. Malarial infection in non-immune women is a risk factor for pregnancy loss. It is estimated that more than 50 million pregnancies occur every year in malaria endemic areas, and approximately half of these occur in Sub-Saharan Africa, where *P. falciparum* transmission is most intense. Pregnancy-associated malaria is one of the major public health problems in Africa with a high burden of maternal and fetal morbidity leading to 100,000 infant deaths per year (26). Malaria accounts for over 10,000 maternal and 200,000 neonatal deaths per year, pregnant women infected with malaria usually have more severe symptoms and poor outcomes, with higher rates of miscarriage, premature delivery, low-birth-weight neonates, and neonatal death (27). Clinical immunity is usually lost during pregnancy, especially among primigravidae women, or after migration to areas where the disease is not endemic (28). Placental malaria lesion thought to be of immunologic origin that has been associated with poor fetal outcome. It is characterized by a prominent inflammatory infiltrate in the intervillous space, composed mainly of monocytes and macrophages that can stimulate a
maternal disorder involving the placenta; also there is an increased fibrin deposition and prominent syncytial knots (29).

There should be some evidence and a leading study that focused on microscopic and submicroscopic \emph{P. falciparum} infection and its implication on the virulence and severity character of parasites sequestered in placenta and present in peripheral blood and cord blood. Expression of genotype would provide a more definitive answer to question of whether detection of individual parasite clones in only peripheral or placental blood or cord blood reflects their influences on the birth weight of the newborns. Thus it is of value to investigate microscopic detection of \emph{Plasmodium} on Giemsa-stained blood smears and PCR- diagnostic methods (which are considered sensitive and specific method for analyzing clonally diversity of \emph{P. falciparum}), and histological technique to detect the presence and feature of parasite in the placenta.

1.8 Pathological Changes

Cytoadherence to human cell surfaces is an important component of \emph{P. falciparum} pathogenesis. When \emph{P. falciparum} parasites mature from rings to trophozoites -within red blood cells- it can induce the formation of sticky knobs on the surface of erythrocytes. The knobs bind to receptors on a variety of cell types in capillaries and venules, including endothelial cells. Endothelial binding leads to sequestration of infected
red cells within these small vessels. This leads to partial blood flow obstruction, endothelial barrier breakdown, and inflammation. Sequestration can be demonstrated in any organ of a patient infected with *P. falciparum*. The most catastrophic clinical manifestation of sequestration is cerebral malaria. A glomerular pathology and fluid loss due to alterations in the renal microcirculation also probably contribute to renal failure. Low nitric oxide, low arginine (the pre-cursor of nitric oxide), and elevated arginase activity in peripheral blood. With nitric oxide depletion due to intravascular hemolysis in the setting of severe malaria subsequently develop pulmonary hypertension and myocardial wall stress (30). There may be a reticulocytosis, leucopenia with relative monocytosis is common in black water fever, an acute intravascular hemolysis during falciparum malaria in non-immune the effect are those of massive hemolysis, fever, also malaria causes hyperplasia of the lymph reticular system in acute attack lymph node, spleen, liver, enlarge and become dark brown-grey the sinusoids are congested with parasite red blood cells and haemozoin pigment is plentiful in splenic macrophages(31). Placenta pathology was associated with decreased fetal viability, intra-uterine growth retardation, gross post-natal growth impairment and increased disease severity in pregnant women and reduction of maternal blood flow in the placenta is a key pathogenic factor in malaria during pregnancy (32).
1.8.1 Immune-Pathological Change

People subject to repeated infections in malaria endemic areas rarely develop complete or sterile immunity to malaria. They frequently carry small numbers of parasites in the blood, with little symptoms of the disease, illustrating a phenomenon termed semi-immunity. Malaria parasites are susceptible to several immunological effectors mechanisms. The presence of extensive repetitive regions is a feature of many \textit{P. falciparum} proteins. Available evidence suggests that the structural characteristics of the repeats and their location on the surface of parasite proteins promote immunogenicity. The repeated alteration may help the parasite evade host immunity by: exhibiting sequence polymorphism, preventing the normal affinity and isotype maturation of an immune response. Sequence diversity in non-repetitive regions and antigenic variation in parasite molecules located on the surface of infected red blood cells also play a role in immune evasion. Some sequence in other instances can cause autoimmune responses (33).

Cerebral malaria, a major cause of death during malaria infection, is characterized by the sequestration of infected red blood cells in brain micro vessels. The parasite infected erythrocyte can mimic the leukocytes interaction with endothelial cells. This process is associated with a transfer of malaria antigens to the endothelial cells which can inappropriately activate the immune system and opening of the
intercellular junctions, which can trigger blood-brain-barrier leakage during cerebral malaria. This transfer of infected red blood cells antigens can thus transform endothelial cells into a target for the immune response and contribute to cerebral malaria pathogenesis (34). In placentae infected heavily with *P. falciparum* revealed large intervillous accumulations of erythrocytes containing parasites together with monocytes which had ingested pigment. These appearances were associated with focal syncytial necrosis, loss of syncytial microvilli and proliferation of cytotrophoblastic cells. In addition, marked irregular thickening of trophoblastic basement membranes was observed (35). Inflammatory cytokines are beneficial in controlling and eliminating parasites this cytokine production from the cell and tissue responsible of placenta inflammation include α and β chemokines which produced by macrophage (36). Macrophage migration inhibitory factor (MIF) produced by T lymphocyte, macrophages, lymph (37). Amorsolo and others (38) observed that both fetal and maternal cells secrete inflammatory and immune-regulatory cytokines they found that there is increase in (IFN)–γ, tumor necrosis factor (TNF)–α, and Interleukin (IL)-10 in responses to malaria (38). Elizabeth and others (39) observed that interactions of fibrinogen with macrophages promote adhesion and stimulate macrophage production of chemokines including MIP-1α, MIP-1β, MIP-2, and MCP-1 (39). Other studies suggest that significantly
increased expression of interleukin (IL)-1β, IL-8, produced by maternally derived hemozoin-laden placental macrophages (40). Monocytes showed significant increases in human leukocyte antigen D-related, CD54, CD80, and CD86 surface markers in intervillous blood (41). A marked increased in the number of monocytes, macrophages, cytotoxic T cells and B lymphocyte cells in the intervillous space of infected placenta was associated with increase of cytokines of CD3+, CD8+, CD15+, CD20+,CD56+,CD68+, and TIA-1+ cells in the intervillous areas (maternal) and indicate the severity of the infection (42).

1.9 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 (43). However, in spite of this depression, the maternal immune system continues to respond to the parasite and antibodies preventing P. falciparum attachment to the placenta can be produced and associated with better outcomes of the fetus (44). It has been shown that there were significant variations in risk and severity of infection between primigravidae and multigravidae with risk and severity decreasing in proportion to the number of pregnancies (45). This suggests that immune build-up is
achieved after several pregnancies and infections. Cell mediated immunity is particularly suppressed during pregnancy, and the mother is increasingly reliant on humoral immunity. This immune-suppression was believed to account for pregnant women increased risk of infection, including malaria (46). 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. 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 (47). This was supported by the finding that cellular immune responses to *P. falciparum* are depressed in pregnant women in comparison with non-pregnant control ones (48).

1.10 Mechanisms of *P. falciparum* Adherence to Red Blood Cells

Adherence of *P. falciparum*-infected erythrocytes to the microvascular endothelium of specific organs and consequent sequestration is believed to be responsible for the development of malaria pathology. A number of studies have shown that cell adhesion molecules expressed on the surface of endothelial cells mediate the adherence. Recent studies
indicate that a subpopulation of red blood cells adhere to chondroitin 4-sulfate. This adhesion can be effectively inhibited by C4S oligosaccharides. In pregnant women, the placenta specifically selects C4S-adherent PRBCs, and thus these phenotypes multiply and sequester in the intervillous spaces (49). In pregnant women infected with \textit{P. falciparum}, the infected red blood cells selectively accumulate in the intervillous spaces of placenta leading to poor fetal outcome and severe health complications in the mother. Moreover, there is evidence that chondroitin sulfate A and hyaluronic acid which is also present on the placental lining, known to mediate \textit{P. falciparum} adhesion to human placenta (50).

\textbf{1.11 Genetic Diversity in msp-1 and msp-2 Genes}

Genetic diversity presented by \textit{P. falciparum} field isolates, the occurrence of variant forms of the parasite in different geographic areas, and occultation of multiple genotypes during a single mosquito, constitute one of the main obstacles to the design of a malaria vaccine. Merozoite surface protein (MSP)-1 and merozoite surface protein-2 (MSP-2) are 2 proteins causing immune responses in humans and are important candidates for development of blood stage malaria vaccines. The block 2 MSP-1 is particularly polymorphic and 3 distinct allelic families have been described as Mad 20, K1 and Ro33. The polymorphic central
domain of the gene encoding MSP-2 belongs to 2 distinct families; Ic and Fc27. Allelic forms of these antigen genes have been reported from different parts of the world (51). The msp-1 gene is divided into 17 blocks, based on analysis of sequence diversity: seven highly variable blocks are interspersed with five conserved and five semi-conserved regions. Block 2 of the msp-1 gene appears to be subjected to rapid intragenic recombination. The msp-2 gene, also known as polymorphic merozoite surface antigen (msa-2) gene, codes for a merozoite surface polymorphic glycoprotein that has been widely studied as one of the major vaccine candidate. The sequencing of DNA has shown that a single copy of msp-2 gene has conserved N- and C-terminal domains (blocks 1 and 5), two non-repetitive variable regions (blocks 2 and 4), and a polymorphic central region (block 3) containing variable numbers of tandem repeats, which also vary in sequence -6,7,17,18 and length. Genes in which polymorphism has arisen through intragenic recombination in repetitive segments are characterized by repeat motifs with length variability differing between strains (52).
1.12 Effects of Malaria During Pregnancy: Anemia, Maternal Mortality and Still Birth

Malaria is known to have a negative impact on pregnant women and their fetuses. Maternal infection significantly increased the risk of infection in newborns. Haemoglobin concentration and birth weight was lower in infected mothers, although not significant. HIV infection was recorded in 6.0% of mothers and increased by 5-folds the risk of malaria parasite infection. Attendance at antenatal clinic and level of education significantly influenced the use of sulfadoxine-pyrimethamine and mosquito net resulted in improved pregnancy outcome especially in primiparous, though the difference was not significant. Malaria infection in pregnancy is common and increases the risk of neonatal malaria infection. Preventive strategies are poorly implemented and their utilization has overall reasonable effect on malaria infection and pregnancy outcome (53). The effect of malaria infection on the progress and outcome of pregnancy was carried out where pregnant women were highly susceptible to the infection compared to the general population. *P. falciparum* infection was predominant. The infection rate was also found to be higher, in second trimester compared to first and third semesters. Primigravidae seemed to be at a greater risk as the mean parasitaemia level was higher and the outcome poor as compared to multigravidae.
Infection during pregnancy caused severe maternal complications like abortion, premature labour, and still-births, which were higher in *P. falciparum* infection. Microcytic anaemia combined with dimorphic anaemia was predominant in the infected group. Cord blood in 4 cases and on baby's blood was found positive for malaria parasite, showing transplacental passage of malaria parasites, which is rare. The infection was found to have a definite bearing on the low birth weight of babies. Chemoprophylaxis could obviate much of the complications (54).

1.12.1 Malaria in Pregnancy and Low Birth Weight

Malaria-related low birth weight is a major public health problem in tropical countries and is thought to kill tens of thousands or hundreds of thousands of infants each year. Previous studies showed that a baby is twice as likely to be born with a low birth weight if the mother has malaria infected placenta at delivery. During pregnancy malaria parasites in the placenta can interfere with the transfer of oxygen and nutrients from the mother to the unborn baby, therefore, increases the risk of spontaneous abortion, stillbirth, preterm birth, and low birth weight (55). Additional studies proved that despite the prevalence of placental infections for women of all gravidities, ranging from 5 to 52%, the risk of low birth weight associated with infection was relatively consistent, with babies born to mothers with an infected placenta being twice more likely
to be of low birth weight than those born to mothers with an uninfected placenta (56). Shulman and Dorman in 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 birth weight baby (57). In Africa about 5-14% of all low birth weight 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 (58). In Tanzania nearly 1 in 5 of born children had a low birth weight, and >20% of these children were born prematurely (59). In Uganda it has been reported that malaria is an important cause of stillbirth and low birth weight (60). Taha et al., in 1993 conducted study in Central Sudan to assess the contribution of mesoendemic malaria to low birth weight; they found that risk of low birth weight associated with malaria was higher among primiparous women than among multiparous women. The mean birth weight of infants whose mothers had malaria during pregnancy was significantly lower than the mean birth weight of infants whose mothers did not (61). While in eastern Sudan low birth weight associated with malaria infection has been reported (62). But on other hand Adam et al. in 2009 (63) disagree the previous idea when they found that there is no significant association between placental malaria and low birth weight. Thus, placental malaria infections affect
pregnant women in this area of eastern Sudan regardless of their age or
parity and this result reject the results of other studies by Ian et al,
1983(64) in the Gambia, West Africa which found that no distinct
gradient linking birth weight with ascending density of placental
parasitaemia was observed. Singleton birth weights of 2.5 kg or less
occurred more frequently in association with malarious than non-
malarious placentae and the association was more marked among first
born than later birth rank infants. Differences between the weights of
malarious and non-malarious placentae were small and not significant
(64). Later on Elbashir et al., in 2011(65) which reaches to same result
that the birth weight was not associated with histopathological placental
malaria infections, and the explanation for this remains unclear. They
found that the women had very low prevalence of peripheral, placental
and cord microscopically detected parasitaemia, hence the role of
submicroscopic parasitaemia on low birth weight cannot be excluded
(65).

In Gabon, it has been found that women with submicroscopic \textit{P.}
\textit{falciparum} infections had a 13-fold higher risk of LBW delivery
compared with non-infected pregnant women (66). Remarkably, malaria
infections in pregnant Kenyan women as estimated by real-time
quantitative PCR were strongly associated with LBW delivery, but malaria detected by nested PCR showed a weaker association (67).

Other studies confirmed that in Malawi there was statistically significant association between submicroscopic \textit{P. falciparum} infection and LBW (68-70).
1.12.2 Submicroscopic Placental Malaria

Pregnant women and the developing fetus are commonly suffered from the adverse effects of *P. falciparum* infections. Epidemiological studies have documented that pregnant women are more susceptible to malaria than non-pregnant women, and that young primigravidae are more likely to be slide-positive, have higher parasitemias, and develop anemia than multigravidae the previous studies also show that the accumulation of *P. falciparum*-infected erythrocytes in the intervillous space of the placenta increases a woman's risk of premature deliveries and low birth weight (LBW) babies (71). Virulence of *P. falciparum* parasite might be explained by its ability to generate variability within genetic families. This diversity relies on the extensive allelic polymorphism. The genetic diversity of *P. falciparum* is also responsible for the parasite’s resistance to antimalarial drugs. The parasite polymorphism markers msp-1 and msp-2 used in molecular epidemiology to detect relation with the complication of the disease (72). Thus substantial information has been obtained about malaria in pregnant women under different environmental conditions. In my country Sudan malaria-associated low birth weight is a major public health problem. Understanding the relative contributions of maternal factors that cause low birth weight during malaria could focus the effort to develop interventions. Accordingly, this study will be achieved to provide critically needed data in this field. Its aim is to
investigate malaria during pregnancy and its effect on low birth weight during malaria infection. Pregnant women residing in Yaoundé, Cameroon have *P. falciparum* detected by microscopy and PCR – it has been found that mixed species infections were significantly higher in women ≤ 20 years old and primigravidae. Neither submicroscopic infections nor number of parasite genotypes decreased significantly with age or gravidity. Thus, pregnancy-associated immunity helps reduce malaria to submicroscopic levels, but does not reduce the number of circulating parasite genotypes (73). Peripheral malaria parasitaemia detected by microscopy in pregnant women does not always provide an accurate estimation of the prevalence of placental malaria parasitaemia. Approximately one in every three of the pregnant Sudanese women, who had been found smear-negative for malaria, had PCR-based evidence of submicroscopic infection (74). It has been found that women with submicroscopic levels of *P. falciparum* infection (detected by using the real-time PCR) at delivery had a 13-fold increased risk of delivering a child with LBW compared with non-infected pregnant women, While results show that women with a microscopically detectable *P. falciparum* infection have a 2-fold increased risk for LBW compared with women with a sub-microscopic *P. falciparum* infection and 29-fold higher risk compared with non-infected women (75). Other study found that *P. falciparum* infections of the placenta might occur in the absence of
parasites in peripheral blood women with placental malaria and women with malaria in peripheral blood samples by microscopy, had lower-birthweight babies. This mean that positive results by PCR in the absence of microscopic parasitaemia were not associated with LBW (76). Mockenhaupt et al., agreed with other authors that microscopy of peripheral thick blood films fails to detect more than half of the P. falciparum infections diagnosed by microscopy of placental blood film. This may be because peripheral parasitaemia is below the threshold of microscopy or parasites may be sequestered in placental tissue and evade circulation (77). Other studies reflect that the mean birth weights of babies did not differ with malaria infection status, while mean haemoglobin concentrations were significantly lower in women with microscopically detected infection compared to uninfected women (78). Demba Sarr et al. published in 2010 (79) different results describing that during placental malaria parasites accumulate in the placenta with an increased risk of LBW. This accumulation can be related to low blood flow through the placenta and to specific adhesion of infected red blood cells on syncytiotrophoblast (79). Indu Malhotrain found the same result in 2005(80) that malaria infection in pregnant women can result in intrauterine growth restriction, which results in the birth of under weight babies, thereby increasing their risk for infant death. The mechanisms causing intrauterine growth restriction remain incompletely understood
but correlate with the presence of placental malaria and the intensity of malaria infection in pregnant women. Therefore, the ability to detect clinically significant malaria infection would lead to more effective testing and management of women in pregnancy. Using peripheral blood samples, -PCR detected clinically significant malaria (80).

Submicroscopic infections by *P. falciparum* during pregnancy are very common and they might be important in developing immunity against gestational malaria besides causing adverse effects to the mother such as anemia and the fetus such as low birth weight. Moreover, in similar study to the current study which using different methods to diagnose *Plasmodium* in maternal peripheral blood, placenta, and umbilical cord blood, they used light microscopy, nested PCR reaction, and histopathology. In maternal blood, they found that frequency of umbilical cord blood infections detected by PCR was higher than by light microscopy high frequency of concomitant infection of mother and placenta and when the maternal blood was heavily infected which lead to high infected birth weight, they found that surveillance of maternal blood may be a good reflection of placental infection, On the other hand, concomitant infection of mother and cord blood, or placenta and cord blood was relatively low, suggesting a low neonatal risk (81).

Uneke, reported in 2008 (82) that pregnant women often have a high frequency and density of *P. falciparum* parasitaemia, with high rates of
maternal morbidity including fever and severe anemia, with abortion and stillbirth, and with high rates of placental malaria and, consequently, LBW in newborns caused by both prematurity and intrauterine growth retardation (82).

The specific expression variants of the *P. falciparum* genotyping in four women including prevalence of slide and submicroscopic infections, and precise detection of clonal diversity of parasite by using PCR will help to answer some of the previous questions about *P. falciparum* infections in pregnant women and their relation with LBW.

### 1.12.3. Malaria during Pregnancy in Sudan

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 *P. falciparum* infection every year, and one in four women have evidence of placental infection at the time of delivery (83). In New Halfa, eastern Sudan, an area of unstable malaria transmission, Adam *et al.* reported that the prevalence of *P. falciparum* malaria is considerable in pregnant women and severe cases occur (84). Pregnant women with blood group O were at higher risk of past-chronic placental malaria infection in the area (85) and maternal death due to severe pulmonary oedema caused by *P. falciparum* malaria was recorded (86).
*P. falciparum* malaria, different severity manifestations were observed, and there were higher perinatal mortalities recorded in the area (87). Further study in the same area illustrated that there was a high prevalence of anemia and folate deficiency and that ferritin, serum folate and vitamin B12 levels were not significantly associated with anemia (88). Other study in Wad Medani in central Sudan investigated the relation between iron level, iron supplementation, and susceptibility to infection with malaria; they found that anaemia was less frequent in women with placenta of *P. falciparum* infection (89). In Gadarif area of eastern Sudan, placental malaria infections have been found to affect pregnant women irrespective of their age or parity (90). In Wad Medani, central Sudan, more than 50% of the women were porous and different forms of clinical presentations of severe malaria were observed, including cerebral malaria and hyperpyrexia (91).

Adam and his group confirmed in 2011 that placental malaria lesion thought to be of immunologic origin that has been associated with poor fetal outcome. It is characterized by a prominent inflammatory infiltrate in the intervillous space, composed mainly of monocytes and macrophages that can stimulate a maternal disorder involving the placenta, also there is increased fibrin deposition and prominent syncytialknots, the quantity of macrophage and there cytokine secretion
increases in pregnant woman infected with malaria, this may lead to anemia and low birth weight (92) The presentation of pregnancy malaria varies according to the pre-existing immunity of the mother.

Among women without prior exposure to malaria, or those living in areas of low transmission and have little immunity they induce severe syndromes, such as cerebral malaria and pulmonary edema. In addition, a pregnant woman who develops a severe malaria syndrome is more likely to die than her non-pregnant counterparts. Women who live in areas of stable malaria transmission enjoy considerable systemic immunity. These women experience few symptoms during episodes of pregnancy malaria, although they commonly develop severe anemia as a consequence of the infection. Intermittent treatment with anti-malarial drugs reduced severe anemia in first-time mothers by 39% (93).
1.13 Objectives

1.13.1 General Objective

The aim of this study is to investigate the epidemiology of submicroscopical malaria, and its effect on low birth weight.

1.13.2 The Specific Objectives

1. To determine the prevalence of submicroscopic parasitaemia at the maternal, placental and cord levels.

2. To investigate predictors of submicroscopic parasitaemia such as age, parity, residence and gender.

3. To investigate the association of low birth weight and submicroscopic parasitaemia.
Chapter Two

Patients and Methods

2.1 Study Design

This was a case-control study.

2.2 Study Site and Duration

This study was conducted at the labor ward of Medani Teaching Hospital, during the period of October 2011 to September 2012. Central Sudan is an area that is characterized by unstable malaria transmission and *P. falciparum* malaria is the predominant parasite species in the area (94).

2.3 Study Population

Cases were women who delivered low birth weight. Low birth weight was defined as a newborn weight <2500 gm (95). Controls were the subsequent women who delivered singleton baby of normal birth weight (2500-4200 gm). Those with diabetes mellitus, hypertensive disorder of pregnancy, antepartum hemorrhage and those who delivered birth weight more than 4200 gm were excluded from cases and controls. Then after signing an informed consent both case and control were approached to participate in the study.
Structured questionnaires were administered to collect information about socio-demographic characteristics and parity. Then women were enquired for using bed nets and history of malaria.

After birth babies were weighted and their gender was recorded. Then peripheral, and placental and cord blood samples were taken. Blood smears were prepared and stained with Giemsa for detection of malaria parasite; in addition full thickness placental blocks of around 2-3 cm was taken from the placentae, kept in neutral buffer formalin for histopathology examinations.

2. 4. Malaria Diagnosis

Maternal, placental and cord thick and thin blood films were prepared, stained with Giemsa and examined by light microscopy under an oil-immersion objective, at × 1000 magnification. Giemsa stain for parasite demonstration; Giemsa stain its type of Romanowsky- stained was used to stain many organism with their cellular environment, the constituent of Giemsa stain were: Giemsa powder, glycerol, methanol is used with acetate buffered distilled water, Giemsa stain give the parasite dark blue color and back ground pink to pale blue and nuclei is blue (96).
2.5. Basic Histological Method-Tissue Preparation

In histopathology tissues were fixed before they were examined microscopically, it follows that fixation in preparation of the section through to make diagnosis. Fixation is complex of chemical events, the aim of it is to prevent autolysis and bacteria attack, tissue which has been fixed should not change its shape, volume or loss rearrangement, neutral buffered formaldehyde (PH:7) was used as fixative, sometime artifact formalin pigment is making due to tissue fixed in acidic formalin it appears as brown black deposit in blood rich tissue, the removing of this pigment by treating unstained section with saturated alcoholic picric acid which have deleterious effects on subsequent staining techniques for that the use of buffered neutral formalin will minimize this problem. Another pigment similar to formalin pigment is malaria pigment but it have small different manner, This pigment is important in the current research as it indicates the presence of parasite inside infected red blood cell and also seen in phagocyte cell which have ingested infected red blood cell and can be removed from tissue section with saturated alcoholic picric acid for 12-14 hour (97).

After fixation, the tissue were treated with tissue processor to embed in solid medium to make supporting to the tissue and enable thin section to be cut by microtome. The stages involved were: dehydration by alcohol
–clearing with xylene –impregnation with paraffin wax and finally embedding the tissue in the paraffin wax blocks.

Microtome is a mean by which tissues can be sectioned and attached to surface for examination by microscopy. There are four type of microtome Cambridge Rocking microtome, Rotary microtome, Base sledge microtome, Rotary rocking microtome, sliding microtome, Ultra microtome. The microtome has different types of knife Wedge, Plano concave, Biconcave, Tool edge (98).

2.6 Criteria of Stain

Haematoxylin and eosin stain is most widely used histological stain. Haematoxylin used for the cell nuclei and intranuclear details which stained, blue – black color, and eosin stain cell cytoplasm and most connective tissue fiber pink ,orange or red color. Haematoxylin is extracted from logwood, Haematoxylin itself is not a stain, it is oxidation product, haematin which is natural dye. Haematoxylin has different types, Mayers 1903, Harr 1900, Coles 1943, Carazzis 1911, Gills 1974, iron Haematoxylin which also have different type Weigerts, Heidenhains, Loyez, Verhoeffs (99).

The presence of placental malaria infection was based on the pathological classification of malaria 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 chronic villous syncytiotrophoblast or stroma), past (no parasites and pigment confined to fibrin or cells within fibrin) (100).

2.7. Extraction of Parasite DNA and PCR

Three drops of blood were collected on a piece of filter paper (whatman 3) from peripheral finger, placental, and cord blood. The spots were allowed to dry, and then each piece of filter paper was stored separately in a plastic bag, until malaria parasites in the blood spots were investigated.

Genomic DNA was extracted from blood spots and in an assay based on nested polymerase chain reaction PCR for DNA from *P. falciparum* (101).

Positive samples PCR for *P. falciparum* are subsequently genotyped for *msp1* and *msp2* by nested PCR using internal primers specific for 3 known allelic families of Block 2 region of gene coding for merozoite surface protein 1 (MSP1), and for 2 allelic families of central polymorphic repeat region of MSP 2 (102).
2.7.1 DNA Extraction

DNA was extracted from filter paper blood samples using the Chelex method (103). The blood spots on filter papers were excised using sterile a scalpel on a glass plate and transferred into a sterile 1.5 ml eppendorf tube using a sterile forceps. The scalpel, glass and forceps were cleaned between each sample using 5M HCL, to reduce the possibilities of contamination, neutralized with 5M NaOH, and wiped up with 70% ethanol. One ml 0.5% Saponin in 1x Phosphate buffer saline (PBS) [0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, ph 7.4 at 25º C.] (SIGMA) was added to each sample with occasional inverting the tubes for a few minutes and then incubated at 4ºC overnight to wash residues of red blood cells and red blood inhibitors. The brown Saponin red blood cells mixtures was removed using a sterile tips and replaced by one ml PBS (Sterile and autoclaved), tubes were mixed gently and placed at 4º C for 15 -30 minutes to ensure complete washing of the Saponin, remaining cells debris, and proteins. The PBS was removed with sterile tips leaving the filter paper. In a sterile 1.5 appendorf tube, 50µl of stock 20% autoclaved Chelex -100 (BIO RAD) was added to 150 µl a sterile nuclease-free water and then heated to 100ºC in a heated block, the filter papers were taken with sterile clean forceps and immersed in a hot Chelex solution, vortexed vigorously for 30 seconds and then replaced in the heated block again for a further10
minutes, vortxed again once during the incubation and once afterwards. The tubes were spined at 10,000 r.p.m for 10 mins and the supernatant were transferred carefully to fresh sterile 1.5 ml appendorf tubes. The samples were centrifuged for a further 5 minutes at 10,000 r.p.m and supernatant containing the DNA were aliquoted into new 0.5 ml (polypropylene) PCR tubes and stored one at –20 ºC.

2.7.2  Polymerase Chain Reaction

Extracted DNA was brought from -20ºC, thawed and centrifuged again to pellet down any remaining Chelex and kept on ice cryo-rack for processing, at the same time stock primers, dNTPs, and buffer were brought to room temperature (RT) and kept on ice cryo-rack for thawing and sterile PCR water was brought out from refrigerator and aliquoted on 1.5 ml tube and kept as above.

Sequences of the primers MSP1 and MSP2 were used in the study:
<table>
<thead>
<tr>
<th>Primers name</th>
<th>Primer sequences</th>
<th>Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSP1-01</td>
<td>CACATGAAAGTTATCAAGAACTTGTC</td>
<td>Forward</td>
</tr>
<tr>
<td>MSP1-02</td>
<td>GTACGTCTAATTTCATTTCACG</td>
<td>Reverse</td>
</tr>
<tr>
<td>K1</td>
<td>GCAGTATTGACAGGGTTATGG</td>
<td>Forward</td>
</tr>
<tr>
<td>K2</td>
<td>GATTGAAAGGTTATTTGAC</td>
<td>Reverse</td>
</tr>
<tr>
<td>MAD-20</td>
<td>AAATGAAGAAGAAATTTACTACAAAAAGGTGC</td>
<td>Forward</td>
</tr>
<tr>
<td>MAD-20</td>
<td>ATCTGAAGGATTGTCATCGTCTTTGAATTACC</td>
<td>Reverse</td>
</tr>
<tr>
<td>RO33</td>
<td>TAAAGGATGGAGCAAATACTCAAGTTGTTG</td>
<td>Forward</td>
</tr>
<tr>
<td>RO33</td>
<td>CATCTGAAGGATTTGCAACCTGGGACAGATC</td>
<td>Reverse</td>
</tr>
<tr>
<td>MSP-2-01</td>
<td>GAAGGTAAATTAAACATTGTC</td>
<td>Forward</td>
</tr>
<tr>
<td>MSP-2-02</td>
<td>GAGGGATTTGCTGCTCCACAG</td>
<td>Reverse</td>
</tr>
<tr>
<td>Fc27</td>
<td>AATACTAGAGTGTTAGTGCTCARATGCCTCA</td>
<td>Forward</td>
</tr>
<tr>
<td>Fc27</td>
<td>TTTTTATTTTGCGATCGTCCAGAAGTTGAAC</td>
<td>Reverse</td>
</tr>
<tr>
<td>IC/3D7</td>
<td>AGAAGTATGGCCAGAAGTAAKCTYCTACT</td>
<td>Forward</td>
</tr>
<tr>
<td>IC/3D7</td>
<td>GATTGAAATTCCGGGGGATTCCAGTTGTTG</td>
<td>Reverse</td>
</tr>
</tbody>
</table>
2.7.3 Primer Preparation

Each of the upstream and downstream primers were prepared as follows: 10µl of each stock primer (100 µM) were added to 90µl PCR water and aliquoted in 0.5 ml PCR polypropylene tube to yield a concentration of 10µM, and the solution was mixed carefully using sterile tips to ensure the homogeneity.

2.7.3.1 Dexonuclotides Preparation

All four dexonuclotides with 100 mM concentration were prepared by adding 10µl of each nucleotides (total volume 40), in 60 µl of sterile PCR water to a final concentration 10 mM in a PCR tube, vortexed to collect any dNTPs from the tube surface in the button of the tube.

2.7.3.2 Master Mix Preparation

Samples and reagents were brought out from the freezer and kept on ice in a frozen cryo-rack during assembly procedure. A4 worksheet with PCR samples data were recorded for each samples to be tested. Master mix (MM) was prepared using specific primers for each MSP1 and MSP2 alleles. The amount of each reagents were calculated to go into the MM in 1.5 µl sterile tube, according to the number of samples to be processed with an extra one more samples than actually being tested to compensate for retention of solution in pipette tips and tube. PCR reagents, except for
samples DNA, were added in the order listed on the worksheet, adding water first and Taq polymerase last. The specified volume of MM was added into each tube, all reagents were kept in a frozen-cryo-rack during mixing and returned to the freezer immediately after use, caps were closed tightly and the PCR tubes were moved to samples loading area.

In the samples preparation area specified volume of sample was loaded into an appropriately labeled PCR tube. To avoid contamination, the tips were always changed and the avoidance of touching the side tube and capped was recommended.

2.8 PCR Amplification of MSP1

Genomic DNA was amplified using primers and conditions described by Sonounou in 2004(104). In the first round the outer primers MO-1 and M0-2 (Table), and in the second round the nested PCR for MSP-1 allele families for K1, MAD20 and RO33 were used.

The amplification were carried out using 10 x PCR buffer (10mM tris-HCl, Ph 8.3, 50 mM KCL), 1.5 mM MgCl2, 200 µM of each dNTPs (Sigma) 200 nM of the oligonucleotides, 1 U of Taq DNA polymerase (5 U) (Vivantis), and 2 µl DNA template. In a final volume 20 µL. Reactions were performed in a T3 thermocycler (Whtman Biometera) with the following thermal profile: primary
denaturing at 94 °C for 3 min, denaturing at 94 °C for 25 sec, annealing temperature at 50 °C for 35 Sec, extension at 68 °C for 2 minutes 30 sec, final extension at 72 °C for 3 min for 30 cycles for the outer PCR and the nested PCR. 2 µl of the outer PCR were added into 18 µl master mix to a final volume of 20.
Table 1: Master mix for the outer and nested MSP1 and MSP2

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration</th>
<th>Final Concentration</th>
<th>X1</th>
</tr>
</thead>
<tbody>
<tr>
<td>H20</td>
<td></td>
<td></td>
<td>14.6</td>
</tr>
<tr>
<td>10x buffer</td>
<td>10x</td>
<td>1x</td>
<td>2</td>
</tr>
<tr>
<td>dNTPs</td>
<td>10 mM</td>
<td>200 µM</td>
<td>0.4</td>
</tr>
<tr>
<td>Primer-1</td>
<td>10µM</td>
<td>200 nmol</td>
<td>0.4</td>
</tr>
<tr>
<td>Primer-2</td>
<td>10µM</td>
<td>200 nmol</td>
<td>0.4</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>5 Units</td>
<td>1 unit</td>
<td>0.2</td>
</tr>
</tbody>
</table>
2.9 PCR Amplification of MSP2

In the MSP-2 genomic DNA was amplified using primers and conditions described by Snounou et al in 2004 (104). In the first round the outer primers MO2-1 and MO2-2 (Table 1), and in the second round the nested PCR for MSP-2 allele families for Fc27 and IC, The amplifications were carried out using 10 x PCR buffer (10 mM tris-HCl, Ph 8.3, 50 mM KCl), 1.5 mM MgCl2, 200 µM of each dNTPs (Sigma) 200 nM of the oligonucleotides, 1 U of Taq DNA polymerase (5 U) (Vivantis), and 2 µl DNA template In a final volume 20 µL. Reactions were performed in a T3 thermocycler (Whtman Biometera) with the following thermal profile: primary denaturing at 94 °C for 3 mines, denaturing at 94 °C for 25 sec, annealing temperature at 42 °C for 60Sec, extension at 65 °C for 2 min, final extension at 72 °C for 3 min for 30 cycles for the outer PCR. The temperature profile of the nested PCR were primay denaturation 94 °C for 3 mines, denaturation 94 °C, 25 sec, annealing 50 °C, 60 sec, extension, 700C, 2min and final extension 72 for 3 minutes, 2 µl of the outer PCR were added into 18 µl master mix to a final volume of 20.
2.10 Gel Electrophoresis

A 1.5 % agarose gel was prepared in 1 x TBE buffer with 0.5 µg/ml Ethidium bromide, 15 µl of the nested PCR products were loaded into the gel after mixing with 3 µl loaded dye (Bromophenol blue). Molecular weight marker of 100bp (Vivantis) was included into each lane of the gel. The gel was run at <5V/cm (distance between electrodes) until the bromophenol blue dye front has migrated to within 1 cm of the end of the gel. This is to ensure maximum separation of the bands.

2.11 DNA Visualization

The Nested PCR of MSP1 and MSP2 were visualized on UV transilluminator (UVP bio imaging system, Cambridge) and photograph gel.

Reactions which do not produce a band (-) or produce only a weak band (+) were repeated using double the amount of outer PCR as template.
Micrograph 1: one of the DNA extraction step by the Author
Micrograph 2: Gel photograph showing PCR amplified product of *P. falciparum*
Micrograph 3: Parasite clones plates using PCR
2.12. Definitions

Primigravidae were those who were at their first pregnancy; multigravidae had multiple pregnancies. Malarial infection status was defined as any positive by microscopic, submicroscopic/placental histology. Microscopic \textit{P. falciparum} infections at delivery in peripheral blood, placental/cord blood smear. Sub-microscopic infection was defined as negative thick blood smear but positive for \textit{P. falciparum} species with PCR in peripheral blood, placenta tissue/cord. The negative group was defined by the absence of \textit{P. falciparum} as assessed by thick blood smear, placental histology and PCR in peripheral blood, placenta tissue and cord. LBW delivery is the birth weight < 2500 gm.

2.13 Ethics

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

2.14 Statistics

Data were entered in computer using SPSS for windows version 16.0. Means and proportions were compared by Students’\textit{t}-test, Chi square and Fisher’s exact tests as appropriate. Univariate and multivariate analyses were performed where LBW as a dependent variable and maternal socio-demographic characteristics (age, parity, education, residence, antenatal care) and malaria infection [diagnosed by histology, submicroscopic
(maternal, placental and cord / both)] as possible influencing factors. $P \leq 0.05$ was regarded as significant.
Chapter Three

Results

Out of 820 deliveries, 98 (12.0%) had low birth weight delivery. Eighty seven of these women with LBW delivery fulfilled the inclusion criteria and had complete data including placental histology and PCR and therefore were included in the final analyses. These data were compared with equal number of controls with complete data. There is no significant difference between the two groups in the level of education, residence, antenatal care attendance and the other socio-demographic characteristics, (Table 2). While mean (SD) of the maternal age was significantly lower [25.5 (5.7) vs. 27.6(6.4) years; \( P = 0.022 \)], there was no significant difference in body mass index [23.8 (3.3) vs. 24.3 (2.2) kg/m2] in the group of low birth weight delivery vs. controls women. The coverage of bednets was low in both groups without significant difference (Table 2).

3.1 Malaria Infections

There was no positive blood film for malaria in maternal, placental and cord samples in the cases or controls. Twenty seven (31.0%) vs. 22 (25.3); \( P = 0.500 \) of the cases vs. controls had placental malaria infections on histology examinations. Three (3.4%), 1 (1.1%) and 23 (26.4%) vs. 2(2.3%), 2 (2.3%) and 18 (20.7%) of the placentae showed acute, chronic
and past infection in histopathology examination in the two groups respectively, while 60 (69.04%) vs. 65 (74.7%) of them showed no infection, $P = 0.04$, (figure 1).

In comparison to the controls, prevalence of submicroscopic malaria infection was highly significant in the cases, 24(27.6%) vs. 6(7.0%); $P < 0.001$. None (0%), 19 (21.8%) and 6 (7.0%) vs. 4 (4.6%), 2 (2.3%) and 0 (0%) were maternal, placental and cord submicroscopic malaria infection in the two groups of the study, respectively. One case had both placental and cord submicroscopic malaria infections.

Significantly higher number in the cases than in the controls had malaria infections (placental malaria infections on histology/ submicroscopic malaria infection), 46 (53.0%) vs. 26 (30.0%); $P = 0.002$. Out of these malaria infections, 6 (5 and 1 in the cases and controls, respectively) had both placental malaria infections on histology and submicroscopic malaria infections, (figure 7).

### 3.2 Effects of Malaria Infections on Birth Weight

While there was no significant difference in the mean (SD) of the birth weight between those who had placental malaria infections on histology (in both groups, $N= 49$) and those who had not [2843.0 (601.0) vs. 2834.7 (533.0) g], the mean (SD) of the birth weight was significantly lower in those who had submicroscopic malaria infection (in both groups, $N= 30$)
than in those who had no submicroscopic malaria infections, [2551.7 (497.0) vs. 2896.9 (545.0) g], (figure 10).

3.3 Malaria Infections as Risk Factors for Low Birth Weight

In a multivariate analysis, while presence of placental malaria infections on histology was not associated with LBW, submicroscopic *P. falciparum* infections (OR =6.89, 95% CI=2.2–20.8; *P* = 0.001) and all malaria infections [histology/ submicroscopic] were significantly associated with LBW (OR =2.45, 95% CI=1.2–4.9; *P* = 0.012), table 5.
Table 2: Socio-demographic characteristics of the cases and controls

<table>
<thead>
<tr>
<th>Number (%) of</th>
<th>low birth weight (N=87)</th>
<th>Controls (N=87)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primigravidae</td>
<td>31(34.3)</td>
<td>22(18.9)</td>
<td>0.186</td>
</tr>
<tr>
<td>Antenatal care ≤ three time</td>
<td>62(14.7)</td>
<td>68(23.1)</td>
<td>0.446</td>
</tr>
<tr>
<td>Educational level &lt; secondary</td>
<td>36(25.2)</td>
<td>38(16.8)</td>
<td>0.151</td>
</tr>
<tr>
<td>Rural residency</td>
<td>61(70.1)</td>
<td>57(65.5)</td>
<td>0.627</td>
</tr>
<tr>
<td>Bednets coverage</td>
<td>15(14.7)</td>
<td>12(16.1)</td>
<td>0.675</td>
</tr>
<tr>
<td>Male gender</td>
<td>47(54.0)</td>
<td>45(50.6)</td>
<td>0.760</td>
</tr>
<tr>
<td>Malaria infection</td>
<td>46(53.0)</td>
<td>26(30.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>Placental malaria (histology)</td>
<td>27(31.0)</td>
<td>22(25.3)</td>
<td>0.500</td>
</tr>
<tr>
<td>Submicroscopic malaria</td>
<td>24(27.6)</td>
<td>6(7.0)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table 3: Placental malaria infection as detected by Giemsa in the cases and controls

<table>
<thead>
<tr>
<th>Types of malaria infection</th>
<th>low birth weight (N=87)</th>
<th>Controls (N=87)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total malaria infection</td>
<td>46(53.0)</td>
<td>26(30.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>Placental malaria (histology)</td>
<td>27(31.0)</td>
<td>22(25.3)</td>
<td>0.500</td>
</tr>
<tr>
<td>Acute</td>
<td>3 (3.4%)</td>
<td>2(2.3%)</td>
<td>0.624</td>
</tr>
<tr>
<td>Chronic</td>
<td>1 (1.1%)</td>
<td>2(2.3%)</td>
<td>0.473</td>
</tr>
<tr>
<td>Past</td>
<td>23 (26.4%)</td>
<td>18 (20.7%)</td>
<td>0.852</td>
</tr>
<tr>
<td>No infection</td>
<td>60 (69.04%)</td>
<td>65 (74.7%)</td>
<td>0.456</td>
</tr>
</tbody>
</table>
Table 4: Submicroscopic malaria infection detected by (PCR) in the cases and controls

<table>
<thead>
<tr>
<th>Types and number (%) of malaria infection</th>
<th>low birth weight (N=87)</th>
<th>Controls (N=87)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total submicroscopic malaria infection</td>
<td>24(27.6)</td>
<td>6(7.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Maternal</td>
<td>0(0)</td>
<td>4 (4.6)</td>
<td>0.06</td>
</tr>
<tr>
<td>Placental</td>
<td>19 (21.8)</td>
<td>2 (2.3)</td>
<td>0.001</td>
</tr>
<tr>
<td>Cord</td>
<td>6 (7.0)</td>
<td>0(0)</td>
<td>0.001</td>
</tr>
<tr>
<td>Placental and cord</td>
<td>1</td>
<td>0(0)</td>
<td>0.83</td>
</tr>
<tr>
<td>The variable (factors)</td>
<td>Univariate analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------------</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
<td>P</td>
</tr>
<tr>
<td>Age</td>
<td>0.94</td>
<td>0.8–0.9</td>
<td>0.024</td>
</tr>
<tr>
<td>Primigravidae</td>
<td>1.64</td>
<td>0.8–3.1</td>
<td>0.139</td>
</tr>
<tr>
<td>Residence</td>
<td>0.81</td>
<td>0.4–1.5</td>
<td>0.517</td>
</tr>
<tr>
<td>Education level &lt; secondary</td>
<td>1.79</td>
<td>0.7–4.1</td>
<td>0.169</td>
</tr>
<tr>
<td>Lack of antenatal care</td>
<td>1.47</td>
<td>0.7–2.7</td>
<td>0.217</td>
</tr>
<tr>
<td>Body mass index</td>
<td>1.06</td>
<td>0.9–1.1</td>
<td>0.240</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>1.28</td>
<td>0.9–1.7</td>
<td>0.084</td>
</tr>
<tr>
<td>Gender</td>
<td>0.88</td>
<td>0.4–1.6</td>
<td>0.697</td>
</tr>
<tr>
<td>Placental malaria infections (histology)</td>
<td>1.33</td>
<td>0.6–2.5</td>
<td>0.400</td>
</tr>
<tr>
<td>Submicroscopic malaria infections</td>
<td>5.14</td>
<td>1.9–13.3</td>
<td>0.001</td>
</tr>
<tr>
<td>All malaria infections</td>
<td>2.63</td>
<td>1.4–4.9</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 5: Factors associated with low birth weight in Medani Maternity hospital, Central Sudan using univariate and multivariate analysis.
Microphotograph 4: Shows Placenta tissues negative for malaria

(H &E X 40) .
Microphotograph 5: Shows placental tissues with chronic malaria infection (H&E X40)
Microphotograph 6: Shows Fibrin and pigment in fibrin placenta

with chronic malaria infection (H & EX 100)
Microphotograph 7: Shows Schizont in inter-villous space of placenta with chronic malaria infection (Giemsa X100).
Microphotograph 8: An ultrasound image of a fetus at 24 weeks, evident effect of malaria on fetal growth
Figure 3: Distribution of study groups by maternal age, years
Figure 4: Distribution of study groups by parity
Figure 5: Distribution of study groups by gestational age in weeks
Figure 6: Distribution of study groups by maternal weight, Kg
Figure 7: Hemoglobin level in gm/dl in women with submicroscopic malaria infection and women who had no submicroscopic malaria infection.
Figure 8: Hemoglobin level, gm/dl in women with placental malaria infections (histology) and women who had no placental malaria infection (histology)
Figure 9: Percentage of submicroscopic parasitaemia
Figure 10: Percentage of malaria infection
Figure 11: Status of malaria in histology
Figure 12: Birth weight in women with submicroscopic malaria infection and women who had no submicroscopic malaria infection
Figure 13: Birth weight in women with malaria infection (histology) and women who had no malaria infection (histology)
Figure 14: Correlations between maternal hemoglobin level and birth weight
Chapter Four

Discussion

The main findings of the current study were as follows: While there was no significant difference in the prevalence of placental malaria by histological examination (31.0% vs. 25.3; P = 0.500) between the two groups, significantly higher numbers of the cases had submicroscopic malaria infections than the controls (27.6% vs. 7.0%; P < 0.001). This study showed that the percentages of prevailing of submicroscopic parasitaemia prevail in placental more than in the cord. Maternal with 12.1% compared with 3.4% in the cord, and with 2.3% in mother. The level of parasitaemia increase in placenta may be due to less amount of immune cell found in placenta in nature and this make the parasite become more increasing than in the cord and maternal blood leading to direct effect on baby weight which causes low birth weight.

The percentage of submicroscopic parasitaemia in cord blood also has strong positive relationship with low birth weight, on other hand the percentage of submicroscopic parasitaemia in maternal blood has relationship with low birth weight but with less amount of parasite presence and this may be due to strong presence of immune system (macrophage-B lymphocyte –T lymphocyte – and other immune system component) in blood which make strong effect on the number of parasite
found comparing with placenta and in the cord. Generally the result of level of submicroscopic parasitaemia prevails in placental more than in the cord. While placental malaria infections that were positive by histology were not associated with LBW, submicroscopic malaria infections were, and this was a statistically significant finding. In fact, the impetus for this study arose out of the knowledge that two cross-sectional studies in Eastern Sudan failed to show significant associations between LBW delivery and placental malaria infection, as diagnosed by histology (85, 89). The prevalence (28.0%) of histologically determined placental malaria infections in both groups (cases and controls) in the current study is similar to the prevalence of the placental malaria infections recently observed in Eastern and Central Sudan (85,89,90).

Interestingly, in the current study only six of the malaria infections (28.0%) had both placental malaria infections (histology) and submicroscopic malaria infections. The performance of PCR versus histology for diagnosing placental malaria infections was recently investigated in the same hospital (Medani Hospital, Central Sudan) (65). The low sensitivity and specificity was explained by the different nature of the infections detected by the different methods (histology and PCR) (65).
In the current study, while women with placental malaria infections (confirmed by histology) were not at risk of having a LBW delivery, women who had submicroscopic malaria infections had a seven-fold higher risk of such an event. In addition, birth weights were significantly lower in women who had submicroscopic malaria infections. Interestingly, it has been shown that pregnant women in Burkina Faso with submicroscopic malaria infections delivered infants that weighed significantly less than those delivered by women with no placental infections. Yet in the same study, delivery of LBW babies was not more common among women with submicroscopic placental malaria parasitaemia than those women without malaria (65). In Gabon, (66) and Kenyan (76) it has been found that same result with our result that women with submicroscopic *P. falciparum* infections had a higher risk of LBW delivery compared with non-infected pregnant women. Conversely, the current study identified an association between submicroscopic malaria and LBW; this contrasts with previous reports from Malawi (68-70), where no statistically significant association between submicroscopic *P. falciparum* infection and LBW was observed. This study observed that the age of infected woman have no any influence on the degree of infection with malaria in study area (Medani) and the statistical analysis, found no correlation between age of pregnant woman, and infection with any degree of malaria, This finding disagree
with Rogerson *et al.*, (5) as they reported that younger pregnant women have been found to be more susceptible to malaria in some settings or adolescent and young adult women are more commonly parasitaemic than older adults. Simply this may be explained by that, the infection with malaria in low endemic area or high endemic area depend on the presence of parasite.

One of the limitations of the current study was the inability, by its design, to investigate the other effects of submicroscopic *P. falciparum* malaria on pregnancy outcomes such as anaemia. This is because it was a case-control study; a cross-sectional study is needed if the effects of submicroscopic *P. falciparum* malaria on haemoglobin are to be investigated. The second limitation is the sample size, where the current study failed to yield enough maternal, placental and umbilical cord submicroscopic *P. falciparum* malaria infections to enable parasite genotyping to be conducted. Therefore, a larger cross-sectional study is needed to address the effect on haemoglobin and parasite genotyping.
4.1 Conclusion

In central Sudan, pregnant women were at higher risk to have LBW delivery if they have submicroscopic infection rather than the placental malaria on histology.

4.2. Recommendations

In pregnancy effect with the placenta malaria. The risk of parasitemia during the first years of life is higher among children born to multigravid women than primigravidae women, the uses of P C R to study submicroscopic parasitemia instead of other investigation method is expensive put it give good result to approve the relationship between Submicroscopic malaria and low birth weight. These future studies should use the genotyping of *plasmodium falciparum* malaria and immunologic basis for this observation and understand their exact role.
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Appendix

Questionnaire of Submicroscopic Malaria and Low Birth Weight

Village: ----------------Date:-------------------
Name------------------------------------------------------- Serial No. -------------
Age in Years------ Parity-------Gravidity --------------
Gestational age in weeks :----------------------
History of miscarriage: Yes ------/ No---------
Residence Rural:---------------Urban:----------------
Education none :---------------Primary and Secondary -------------
University and above---------------
ANC :nill---------------once and twice------------------more than two---------
H/O using bednet in the current pregnancy: Yes-----------------No
Wt-----------------Ht-----------------
Maternal Hb----------------BF----------------Filter paper---------------Serum---------
Cord BF----------------Filter paper---------------Serum
Placenta BF---------------Rapid test--------------Filter paper
Placenta tissue – cutting section
Birth weight----------------Gender------------------
Mode of delivery VD------------CS------------------
1. **Mayer's Hematoxylin:**
   Hematoxylin 1g.
   Distilled water 1000 ml.
   Potassium or ammonium alum 50 g.
   Sodium iodate 0.2 g.
   Citric acid 1g (99).

2. **Giemsa stain:**
   Giemsa stock: Giemsa stain powder 4g.
   Glycerol 250 ml.
   Methanol 250 ml.
   Working Giemsa for parasites:
   Giemsa stock 4 ml.
   Acetate buffered distilled water, (PH: 6, 8) 96 ml (96).
Monocytes and macrophages and placental malaria infections in an area of unstable malaria transmission in eastern Sudan

Magdi M Salih¹, Amal H Mohammed¹, Ahmed A Mohmmed², Gamal K Adam³, Mustafa I Elbashir⁴ and Ishag Adam⁴*

Abstract

Background: Maternal immunity is thought to play a major role in the increased susceptibility of pregnant women to Plasmodium falciparum malaria. Few studies exist on immunohistochemical characterization of the placental inflammatory infiltrate. The current study was conducted in Gadarif hospital in an area characterized by unstable malaria transmission in eastern Sudan.

Method: Ninety three placentae were investigated for malaria histological changes and immunohistochemical study for monocytes and macrophages (CD68).

Results: While 1(1.1%), 2(2.2%) and 20(21.5%) of the 93 placentae had acute, chronic and past malaria infections, 70(75.2%) had no malaria infections. Monocytes and macrophage (CD 68) were detected in 29 (31.2%) of these 93 placentae. Significantly higher rate of monocytes and macrophage were detected in placentae with malaria infections [11/23 (47.8%) vs. 18/70 (25.7%); P = 0.047] especially in placentae with past malaria infections. Placental malaria infections and monocytes and macrophages cells infiltration were not different between primiparae and multiparae. There was no significant difference in the birth weight between the women with placental malaria infections/monocytes and macrophages cells infiltration and those who had no placental malaria infections/cellular infiltrations.

Conclusion: Significantly higher rate of monocytes and macrophage were detected in placentae with malaria infections. Neither placental malaria infections nor cellular infiltrates were associated with parity or lead to reduction of birth weight.

Introduction

Malaria during pregnancy is a major public health problem in tropical and subtropical regions; each year 25 million African women become pregnant in malaria endemic areas [1]. Pregnant women are more susceptible to malaria than their non-pregnant counterparts [2]. Malaria infections are associated with poor maternal and fetal outcomes [3,4]. Malaria during pregnancy is a huge burden in Sudan [3,5] and it is one of the leading causes of maternal mortality [6].

During pregnancy, adhesion of Plasmodium falciparum-infected erythrocytes to syncytiotrophoblast leads to parasite sequestration in the intervillous space. The parasite adheres specifically to chondroitin sulfate-A expressed on syncytiotrophoblast [7]. The increased susceptibility of pregnant women to malaria was thought to result from pregnancy-related immunomodulation and Th1/Th2 shift to decreased Th1-type cytokines and increased Th2-type cytokines to prevent rejection of fetal allograft [8,9]. However, this modulation was proposed to result from a state of monocyte activation and lymphocyte inhibition [10], the immunomodulation is more important in placental than in the peripheral blood [11]. The inflammatory response is responsible for functional damage in placental villi, and disturbs feto-maternal exchange, leading to low birth weight [12,13]. Histological studies on malaria have shown that
P. falciparum-infected placenta are characterized by an increase in inflammatory cells in the intervillous space [13,14]. The placental malaria parasite-related cell infiltrates are mainly monocytes and macrophages, with a smaller population of granulocytes and lymphocytes [14-17]. Few studies addressed the characteristics of the immunological responses of these cell infiltrates [18]. In the present study, we identified monocytes and macrophages in the placenta immunohistochemically, using monoclonal antibodies to CD68, in samples from women in Gadarif hospital which is located in an area characterized by unstable malaria transmission in eastern Sudan [19].

Materials and methods
A cross-sectional study was conducted in Gadarif Maternity Hospital during October 2009. Ninety-three consecutive women with singleton pregnancy were approached to participate in the study. After signing an informed consent, obstetrical and medical history (age and parity) were gathered using questionnaires. The babies were weighed immediately following the delivery and parity) were gathered using questionnaires. The women in Gadarif hospital which is located in an area characterized by unstable malaria transmission in eastern Sudan [19].

Placental Histology
The details of this have been shown before [5,20]. In summary in all women, approximately a three cm³ sample was removed from the maternal surface in an off-center position, half the distance between the umbilical cord and the edge of the placenta. Once collected, each biopsy sample was placed in 25 mL of 10% neutral buffered formalin. All biopsy samples were kept at room temperature and were stored in Gadarif until transportation to Khartoum, where the histologic studies were performed. The placental biopsy samples were then processed and were embedded in paraffin wax, by standard techniques. In every case, paraffin sections 4 mm thick were stained with hematoxylin-eosin and Giemsa stain. Because the samples were fixed in buffered formalin, formalin pigment formation, which has similar optical characteristics and polarized light activity to malaria pigment was not detected [21]: Placental malaria infections were characterized based on the classification of Bulmer et al [16]: 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), Figure 1.

Immunohistochemical methods
Immunohistochemical analysis was performed in neutral formalin-fixed, paraffin-embedded tissue, using the IHC-Tek™ Avidin/Biotin Blocking Solution following the manufacturer’s instructions http://www.ihcworld.com/products/IHC-Tek-Reagent.htm. In summary, 4-mm sections were deparaffinized and were hydrated through xylene and graded alcohols, and peroxidase was blocked for 5 min in 0.03% H₂O₂ containing sodium azide. Then the slides were incubated with the primary antibody for 40 min against CD68. The peroxidase-labeled polymer was then applied for 40 min. After washing in TBS, the slides were incubated with the diaminobenzidine substrate chromogen solution, washed in distilled water, counterstained with hematoxylin, washed, dehydrated, and mounted. A pressure cooker was used for heat-induced epitope retrieval with antibodies [22]. Monocytes and macrophage inflammatory cells quantification was performed with an Olympus microscope at magnification of 40 x using an eyepiece with a field of view of 26.5 mm. The numbers of monocytes and macrophages CD68 cells results were expressed as geometric mean and standard deviation cell count per square millimeter.

Statistics
Data were entered in computer using SPSS for windows version 16.0 for analysis. Student’s t-test and X² were used to compare means and proportions between the groups, respectively.

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

Results
General characteristics
The age of these 93 women ranged from 13-44 with the mean (SD) of 25.9 (7.3) years. Of these 93 women, 34 (36.5%) were primiparae. Only 12 (13.0%) gave history of using bed nets and none of these 93 women used malaria prophylactic treatment during the index pregnancy. There was one blood film positive in maternal and placental set. Of these 93 women, 47 (50.5%) were anaemic (haemoglobin < 11 g/dl) and 7 (7.5%) had low birth weight deliveries.

Placental histology and malaria infections
Placental histology showed that 1 (1.1%), 2 (2.2%) and 20 (21.5%) had acute, chronic and past malaria infections, respectively and 70 (75.2%) had no malaria
infections. Of 34 primiparae, 8 (23.5%) vs. 15 (25.4%) of the 59 multiparae had placental malaria infection, \( P = 0.838 \). The rate of anaemia was not different between women with placental infections and those women who had no placental infections, 12/23 (52.2%) vs. 35/70 (47.3), \( P = 0.944 \). There was no significant difference in mean (SD) birth weight between the women with placental malaria infections and those who had no

**Figure 1** Placental histology, malaria infections and monocytes/macrophages infiltrates
placental malaria infections, 2962.5(455.5) vs. 3035.7 (538.4) gm; P = 0.576.

Immunohistochemical analyses
The monocytes and macrophage cell infiltrations were detected in 29 (31.2%) of the placentae. The number of monocytes and macrophages ranged from one to fifteen cell with the geometric mean (SD) of 3.1(3.3) cells/mm². Of 34 primiparae, 11(32.3%) vs. 18 (30.5%) of the 59 multiparae, P = 0.853 had placental monocytes and macrophage cell infiltrations.

Significantly higher rate of monocytes and macrophages infiltrates were detected in placentae with malaria infections, 11/23 (47.8%) vs. 18/70 (25.7%); P = 0.047, table 1. The majority of these monocytes and macrophages were detected in placentae with past malaria infections (10/20, 50%) than in placentae with acute (0/1, 0%) and chronic infections (1/2; 50%). The mean (SD) birth weight (3016.9 (441.3) vs. 3018.5(551.2) gm; P = 990) and the rate of low birth weight deliveries was not different between those who had placentae with monocytes and macrophages infiltrates and those who had placentae without cellular infiltrate, table 1.

Discussion
This is the first study to investigate malaria placental histology and monocytes and macrophage cellular infiltrations in an area with unstable malaria transmission in Africa. The main findings of the current study were; most (20/23, 87.0%) of the placental infections were past infections which affect pregnant women regardless to their parity and had no effects on birth weight. Monocytes and macrophages cellular infiltrations were detected in 32.2% of the placentae. They were more predominant among placentae with past malaria infections irrespective to parity, associated with maternal anaemia and had no effect on birth weight. We have previously shown that placental malaria infections, hormonal and cytokines levels were not different between the primigravidae and multigravidae among pregnant women in eastern and central Sudan [20,23,24]. This observation could be explained by the low immunity among pregnant women in an area of unstable malaria transmission.

In neighboring Tanzania, it has been shown that, malaria parasitized placentae, especially in primigravidae, had the most significant increase in all inflammatory cellular types -except NK cells-with monocytes and macrophages representing the major population of the infiltrate [18]. It has been previously shown that the inflammatory response was particularly marked in chronic placental malaria infections, no increase in inflammatory cell counts were observed in cases with past infection and these infiltrates were associated with reductions in birth weight [18]. Likewise, Ismail et al., [15] observed that primiparae had higher placental infections, chronic infections and inflammatory cell infiltration more frequently than multiparae. In their observation; chronic malaria infection had the significant inflammatory cell infiltration, acute infections showed a mild increase in inflammatory cell infiltration and those with past infections had no increase in the cell infiltration. However, the low prevalence of placental malaria in these women in the current study, the relatively small sample size and perhaps the size of placental tissue itself makes it hard to compare this study to other ones of placental malaria. Because malaria (past or present) was not very common, and because chronic infections were very uncommon (and these chronic infections are the ones associated with heavy monocyte infiltrates and poor outcomes in previous studies), the power of this study to examine malaria associated changes is rather limited. Furthermore we introduce the presence/absence of CD68 cells as another way of stratifying the data; it is not clear what finding these cells in low numbers means (the normal number of these cells is not known), especially in the absence of malaria. There is a certain percentage of CD68+ cells in the blood in normal subjects, so there will be some chance of finding one or more of these cells on a normal placental section. Many other studies have reported increased inflammatory cells infiltration mainly monocytes and macrophages in placental malaria

Table 1 Histology, immunohistology and pregnancy outcomes of 93 placentae in eastern Sudan

<table>
<thead>
<tr>
<th>Variables</th>
<th>Positive for cellular infiltrations (N = 29)</th>
<th>Negative for cellular infiltrations (N = 64)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primiparae</td>
<td>11(38.0)</td>
<td>23(36.0)</td>
<td>0.855</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>18(62.1)</td>
<td>52(81.2)</td>
<td>0.029</td>
</tr>
<tr>
<td>All infections</td>
<td>11(38.0)</td>
<td>12(18.8)</td>
<td>0.047</td>
</tr>
<tr>
<td>Acute infection</td>
<td>0(0)</td>
<td>1(1.6)</td>
<td>0.579</td>
</tr>
<tr>
<td>Chronic infection</td>
<td>1(3.4)</td>
<td>1(1.6)</td>
<td>0.579</td>
</tr>
<tr>
<td>Past infection</td>
<td>10(34.5)</td>
<td>10(15.6)</td>
<td>0.040</td>
</tr>
<tr>
<td>Anaemia</td>
<td>20(69.0)</td>
<td>27(42.2)</td>
<td>0.005</td>
</tr>
<tr>
<td>low birth weight</td>
<td>2(6.8)</td>
<td>5(7.8)</td>
<td>0.876</td>
</tr>
</tbody>
</table>
infections [17]. These inflammatory cells might have an important role in *P. falciparum* clearance and phagocytosis of the infected red blood cells. On the other hand these inflammatory cells might lead to functional damage in placental villi, and disturb feto-maternal exchange, leading to low birth weight [12,13]. The mean birth weight was not different between women with placental malaria infection/with monocytes macrophages infiltrates and those women without placental malaria infections/cellular infiltrates. This goes with the previous observations where the placental infections were not associated with low birth weight in eastern Sudan [5,20]. The lack of association between malaria infections and low birth weight might be explained by the small sample size of these studies and the lack of power. However, these cellular infiltrates were associated with reduction in birth weight [12] and malaria infections were known to be associated with low birth weight [12]. Due to fund constraints, only the CD 68 marker for monocytes and macrophages was investigated in the current study. The other marker e.g. CD20 and other inflammatory cells (B, T lymphocytes were not investigated. However, Ordi et al. [13] reported that, malaria parasitized placentae, especially in primigravidae, had the most significant increase in all inflammatory cellular types (except NK cells) and these infiltrates were associated with reduction in birth weight.

**Conclusion**

Significantly higher rate of monocytes and macrophage were detected in placentae with malaria infections. Neither placental malaria infections nor cellular infiltrates were associated with parity or lead to reduction of birth weight.

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**Author details**

1. Faculty of Medical laboratory Sciences, University of Khartoum, Khartoum, Sudan.
2. Faculty of Medicine, Ribat University, Khartoum, Sudan.
3. Faculty of Medicine, University of Khartoum, Khartoum, Sudan.

**Authors’ contributions**

MMS and IA designed the study, GKA and MIE conducted the clinical work. MMS, AHM, MIE and AAM conducted the lab work. IA and GKA participated in the statistical analyses. All the authors approved the draft and the final paper.

**Competing interests**

The authors declare that they have no competing interests.

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Submicroscopic *Plasmodium falciparum* malaria and low birth weight in an area of unstable malaria transmission in Central Sudan

Amal H Mohammed1, Magdi M Salih2, Elhassan M Elhassan3, Ahmed A Mohmmed4, Salah E Elzaki5, Badria B El-Sayed5 and Ishag Adam1*

**Abstract**

**Background:** Malaria, which frequently occurs in pregnant women in the tropics, is a leading cause of maternal anaemia and low birth weight (LBW) in infants. Few data exist concerning malaria infections that are present at submicroscopic levels during pregnancy and their LBW delivery in babies.

**Methods:** A case–control study (87 in each group) was conducted at the Medani Hospital, Central Sudan. Cases were women who had LBW deliveries where the infants weighed < 2,500 g. Controls were parturient women without having LBW babies. Obstetrical and medical characteristics were gathered from both groups through structured questionnaires. Both cases and controls were investigated for malaria using microscopic blood film analysis, placental histology and polymerase chain reaction (PCR). Microscopic and PCR analyses were conducted on maternal peripheral blood, placenta, and umbilical cord samples. Infant weights were recorded immediately after birth.

**Results:** *Plasmodium falciparum*-positive blood films were not obtained from any of the women (cases or controls). Twenty-seven (31.0%) versus 22 (25.3%) (P = 0.500) of the cases and controls, respectively, had placental malaria infections as determined by histological examination. In comparison to the controls, the submicroscopic malaria infection prevalence rates were significantly higher in the cases; 24 (27.6%) vs six (7.0%), P < 0.001. Multivariate analysis showed that while malaria infection of the placenta (based on histology) was not associated with LBW, submicroscopic *P. falciparum* infection (OR = 6.89, 95% CI = 2.2–20.8; P = 0.001), or a combination of histologically determined and submicroscopic infections (OR = 2.45, 95% CI = 1.2–4.9; P = 0.012), were significantly associated with LBW.

**Conclusion:** In Central Sudan, pregnant women were at a higher risk of having an LBW delivery if they had submicroscopic infections rather than a histological diagnosis of placental malaria.

**Keywords:** *Plasmodium falciparum*, Submicroscopic infection, Pregnancy, Placenta, Parasite, Low birth weight

**Background**

Malaria is a big public health problem in tropical countries, especially sub-Saharan Africa. Around 125 million pregnant women live in malaria-endemic areas and 32 million of these are at risk of malaria in sub-Saharan Africa [1,2]. Malaria during pregnancy can lead to maternal anaemia and low birth weight (LBW) delivery, the latter of which is the main risk for neonatal and infant morbidity and mortality [3-5]. Malaria during pregnancy is caused by parasites sequestering in the placenta where selection of pregnancy-associated *Plasmodium falciparum* erythrocyte membrane protein-1 (*PfEMP-1*) variant surface antigen occurs [6]. Thus, plental malaria infection (especially in areas of unstable malaria transmission) may be detected in the absence of peripheral blood parasitaemia [7,8]. Sequestration of malaria parasites in the placenta may lead to functional damage of placental villi, disturb the foetomaternal compartment and lead to LBW [9,10].
Although placental histology is the ‘gold standard’ for malaria diagnosis during pregnancy, it is often not available in most settings where malaria is endemic such as sub-Saharan Africa [11]. Polymerase chain reaction (PCR) is an alternative diagnostic tool that is widely used to diagnose malaria infection during pregnancy [12,13]. However, there are few published studies, as well as inconsistent findings on associations between submicroscopic malaria infections and LBW [14-16].

In Sudan, malaria during pregnancy is a big health problem because women are more susceptible to malaria (peripheral, placental and submicroscopic infections) during pregnancy regardless of their age or parity and severe cases of malaria have been observed [7,12,17-19]. Malaria has many adverse effects on pregnancy and its outcome and it is a leading cause of maternal and perinatal mortality in Sudan [20-22]. The aim of this study was to build upon the previous work on placental malaria and LBW in Sudan [23-25]; specifically, to investigate the effect of submicroscopic levels of malaria parasites during pregnancy on birth weight. The study took place in the Medani Maternity Hospital in Central Sudan.

Methods
A case–control study was conducted during the post rainy season (September to November) 2010 at the labour ward of the Medani Maternity Hospital, Central Sudan. Central Sudan is characterized by unstable malaria transmission and *P. falciparum* is the sole malaria parasite species in the area and the transmission during the rainy (July –September) and post-rainy season [26]. Medani Maternity Hospital is a tertiary hospital for women who receive antenatal care at the hospital or are referred from other clinics and hospitals, and women who live close to the hospital facility. Women with high-risk pregnancies are referred to the hospital. However, the referral criteria are not strictly adhered to and many women without a high-risk pregnancy deliver at the hospital.

A total sample size was calculated to provide 80% power to detect the difference of 5% at α = 0.05 and assumed 10% of women would not respond or have incomplete data. In this study, a case represents a woman who had an LBW delivery (<2,500 g). A consecutive woman who delivered next to the case was taken as control for each case. Controls were parturient women who have no LBW delivery (≥ 2,500 g). Women pregnant with twins and those with hypertension, diabetes mellitus or antepartum haemorrhage were excluded from the study in both case or controls groups. After obtaining a signed informed consent, women in the case and control groups were enlisted to participate in the study. Information on socio-demographics, obstetrics history, medical characteristics and antenatal attendance were gathered through structured pretested questionnaires. Women in both groups were asked if they used bed nets and if they had experienced malaria infections in the index pregnancy. Body mass index was calculated by measuring maternal weight and height, which was expressed as weight (kg)/height (m)². Babies were weighed immediately following birth to the nearest 10 g on a Salter scale. Scales were checked for accuracy on a weekly basis. The gender of each baby was recorded.

Giems-stained blood smears and light microscopy
Peripheral blood films were prepared from the mother, along with placental and umbilical cord samples. Both thick and thin blood films were prepared and stained with10% Giemsa and the parasite counts were obtained by counting the number of asexual parasites per 200 leukocytes assuming a leukocyte count of 8,000 leukocytes/μl (for thick films) or per 1,000 red blood cells (for thin films); blood films were considered negative if no parasites were detected in 100 oil immersion fields of a thick blood film.

Placental histology
Full thickness placental blocks around 3 cm were taken from the placenta and kept in neutral buffered formalin for histopathological examination. Buffer was used to prevent formalin pigment formation, which has similar optical characteristics and polarized light activity as malaria pigment [27]. Placental malaria infections were characterized as previously described by Bulmer et al. [28]: 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), and past (no parasites and pigment confined to fibrin or cells within fibrin). The slide was read by a pathologist (AAM) who remained blind about the clinical characteristics and the arms of the study.

Parasite DNA extraction and PCR
*Plasmodium falciparum* DNA extraction and PCR assays were performed as described in the recent work [12,24]. In brief, three drops of blood were collected onto a piece of filter paper from maternal peripheral blood, the maternal side of the placenta, and the umbilical cord. These samples were air-dried and stored at ambient temperature in individual sterile plastic bags. The specimens were transported for processing and analysis in the lab in Khartoum. Approximately 25 μl (corresponding to approximately one third of a spot) of blood was punched out from the dried blood spots. The filter paper piece was washed with distilled water and placed directly in a PCR reaction tube containing 25 μl of all the PCR reaction components. A negative control sample with no template DNA and an internal positive
control were used for quality control purposes. Genomic DNA was checked, in an assay based on a nested PCR, for DNA from *P. falciparum* [29]. PCR assays were performed by two of the team (HMI and MIE) who were both blinded to the clinical and the histology study data.

**Definitions**

The malarial infection status of a participant was defined as any sample positive by microscopic and submicroscopic analysis or placental histology. Microscopic *P. falciparum* infections were determined at delivery from peripheral blood, placenta, and umbilical cord blood smears. Submicroscopic infections were defined as those participants with negative thick blood smears, but who were positive for *P. falciparum* based on the PCR results for peripheral blood, placenta, and umbilical cord samples. The *P. falciparum* negative group was defined by the absence of *P. falciparum* in thick blood smears, placental histology and PCR of peripheral blood, placental and umbilical cord samples.

**Ethics**

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

**Statistics**

Data were analysed using SPSS for Windows version 16.0. Data means and proportions were compared by Student’s-t test, $X^2$ and Fisher’s exact tests as appropriate. Univariate and multivariate analyses were performed using LBW as the dependent variable. Maternal socio-demographic characteristics (age, parity, education, residence, antenatal care), malaria infection status (diagnosed by histology), and submicroscopic blood analysis (maternal peripheral blood, placenta and cord, or a combination) were included as possible influencing factors. $P < 0.05$ was regarded as significant.

**Results**

Out of 820 deliveries, 98 (12.0%) were of LBW babies. Eighty-seven of these women with LBW deliveries fulfilled the inclusion criteria and had complete data, including placental histology and PCR diagnoses, and were, therefore, included in the final analyses. Such data were compared with an equal number of controls with complete data. There were no significant differences between the two groups (case or control) in their level of education, residence, antenatal care attendance and other socio-demographic characteristics (Table 1). While the mean (SD) maternal age was significantly lower [25.5 (5.7) versus 27.6 (6.4) years; $P = 0.022$], there was no significant difference in the body mass index [23.8 (3.3) vs 24.3 (2.2) kg/m$^2$] in the LBW delivery vs the control women, respectively. The bed net coverage was low in both groups, but there was no statistically significant difference (Table 1).

**Malaria infections**

No *P. falciparum*-positive blood films were obtained from maternal peripheral blood, placenta or cord samples in either the cases or controls. Twenty-seven (31.0%) vs 22 (25.3%) ($P = 0.500$) of the cases vs controls had placental malaria infections on histological examination. Three (3.4%), one (1.1%) and 23 (26.4%) vs two (2.3%), two (2.3%) and 18 (20.7%) of the placentae showed acute, chronic and past infection on histopathology examination in the two groups (case–control), respectively, while 60 (69.4%) vs 65 (74.7%) of them showed no signs of infection; $P = 0.500$, (Figure 1 and Table 2).

In comparison to the controls, the prevalence of submicroscopic malaria infection was highly significant in the cases; 24 (27.6%) vs six (7.0%); $P < 0.001$. None (0%), 19 (21.8%) and six (7.0%) vs four (4.6%), two (2.3%) and none (0%) were maternal, placental or cord submicroscopic malaria infections in the two study groups, respectively. One case had a placental and umbilical cord submicroscopic malaria infection, Table 2.

Significantly higher numbers of the cases than the controls had malaria infections (placental malaria infections on histology/submicroscopic malaria infection); 46 (53.0%) vs 26 (30.0%), $P = 0.002$. Out of these malaria infections, six (five and one in the cases and controls, respectively) had histologically positive placental malaria infections as well as submicroscopic malaria infections (Figure 1).

### Table 1 Socio-demographic characteristics and malaria status in the cases and controls

<table>
<thead>
<tr>
<th>Number (%) of</th>
<th>Low birth weight ($N = 87$)</th>
<th>Controls ($N = 87$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primigravidae</td>
<td>31 (34.3)</td>
<td>22 (25.3)</td>
<td>0.186</td>
</tr>
<tr>
<td>Antenatal care ≤ three time</td>
<td>62 (14.7)</td>
<td>68 (23.1)</td>
<td>0.446</td>
</tr>
<tr>
<td>Educational level &lt; secondary</td>
<td>36 (25.2)</td>
<td>38 (16.8)</td>
<td>0.151</td>
</tr>
<tr>
<td>Rural residency</td>
<td>61 (70.1)</td>
<td>57 (65.5)</td>
<td>0.627</td>
</tr>
<tr>
<td>Bed net coverage</td>
<td>15 (14.7)</td>
<td>12 (16.1)</td>
<td>0.675</td>
</tr>
<tr>
<td>Male gender</td>
<td>47 (54.0)</td>
<td>45 (50.6)</td>
<td>0.760</td>
</tr>
<tr>
<td>Anaemia (haemoglobin &lt; 11 g/dl)</td>
<td>74 (85.1)</td>
<td>70 (80.1)</td>
<td>0.246</td>
</tr>
</tbody>
</table>
Effects of malaria infection on birth weight

The mean (SD) of birth weight was 2,387.2 (152) vs 3,319.2 (358) g, $P < 0.001$ in the cases and controls, respectively. While there was no significant difference in the mean (SD) of the birth weight between those who had placental malaria infections based on histology (in both groups, $N = 49$) and those who did not [2843.0 (601.0) vs 2834.7 (533.0) g], the mean (SD) of the birth weights was significantly lower in those who had submicroscopic malaria infection (in both groups, $N = 30$) than in those who had no evidence of a submicroscopic malaria infection [2551.7 (497.0) vs 2896.9 (545.0) g] (Figure 2).

Malaria infection as a risk factor for low birth weight infants

In this multivariate analysis, while the presence of placental $P. falciparum$ infection (by histology) was not associated with LBW, submicroscopic infections with this parasite ($OR = 6.89$, 95% CI $= 2.2–20.8$; $P = 0.001$) and all $P. falciparum$ infections (histological or submicroscopic) were significantly associated with LBW ($OR = 2.45$, 95% CI $= 1.2–4.9$; $P = 0.012$), (Table 3).

Discussion

The main findings of the current study were as follows. While there was no significant difference in the prevalence of placental malaria by histological examination (31.0% vs 25.3; $P = 0.500$) between the two groups, significantly higher numbers of the cases had submicroscopic malaria infections than the controls (27.6% vs 7.0%; $P < 0.001$). While placental malaria infections that were positive by histology were not associated with LBW, submicroscopic malaria infections were, and this was a statistically significant finding. In fact, the impetus for this study arose out of the knowledge that two cross-sectional studies in Eastern Sudan failed to show significant associations between LBW delivery and placental malaria infection, as diagnosed by histology [7,18]. The prevalence (28.0%) of histologically determined placental malaria infections in both groups (cases and controls) in the current study is similar to the prevalence of the placental malaria infections recently observed in Eastern and Central Sudan [7,18,23].

Interestingly, in the current study only six of the malaria infections (28.0%) had both placental malaria infections (histology) and submicroscopic malaria infections. The performance of PCR versus histology for diagnosing placental malaria infections was recently investigated in the same hospital (Medani Hospital, Central Sudan) [24]. The low sensitivity and specificity was explained by the different nature of the infections detected by the different methods (histology and PCR) [24].

In the current study, while women with placental malaria infections (confirmed by histology) were not at risk of having an LBW delivery, women who had submicroscopic malaria infections had a seven-fold higher risk of such an event. In addition, birth weights were significantly lower in women who had submicroscopic
malaria infections. Interestingly, it has been shown that pregnant women in Burkina Faso with submicroscopic malaria infections delivered infants that weighed significantly less than those delivered by women with no placental infections. Yet in the same study, delivery of LBW babies was not more common among women with submicroscopic placental malaria parasitaemia than those women without malaria [14]. In Gabon, it has been found that women with submicroscopic *P. falciparum* infections had a 13-fold higher risk of LBW delivery compared with non-infected pregnant women [15]. Remarkably, malaria infections in pregnant Kenyan women as estimated by real-time quantitative PCR were strongly associated with LBW delivery, but malaria detected by nested PCR showed a weaker association [30].

Conversely, the current study identified an association between submicroscopic malaria and LBW; this contrasts with previous reports from Malawi [16,31] and Ghana [32], where no statistically significant association between submicroscopic *P. falciparum* infection and LBW was observed.

One of the limitations of the current study was the inability, by its design, to investigate the other effects of submicroscopic *P. falciparum* malaria on pregnancy outcomes such as anaemia. This is because it was designed to investigate LBW through a case–control study; another study is needed if the effects of submicroscopic *P. falciparum* malaria on haemoglobin are to be investigated. The second limitation is the sample size, where the current study failed to yield enough maternal, placental and umbilical cord submicroscopic *P. falciparum* malaria infections to enable parasite genotyping to be conducted. Therefore, a larger cross-sectional study is needed to address the effect on haemoglobin and parasite genotyping.

**Conclusion**

In Central Sudan, pregnant women were at higher risk of having an LBW delivery if they had submicroscopic infections rather than a diagnosis of placental malaria based on histology.

### Table 2 Malaria status in the cases and controls

<table>
<thead>
<tr>
<th>Number (%) of</th>
<th>Low birth weight (N = 87)</th>
<th>Controls (N = 87)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All malaria infection (histology/submicroscopic)</td>
<td>46(53.0)</td>
<td>26(30.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>Placental malaria (histology)</td>
<td>27(31.0)</td>
<td>22(25.3)</td>
<td>0.500</td>
</tr>
<tr>
<td>Acute</td>
<td>3(3.4)</td>
<td>2(2.3)</td>
<td>0.999</td>
</tr>
<tr>
<td>Chronic</td>
<td>1(1.1)</td>
<td>2(2.3)</td>
<td>0.999</td>
</tr>
<tr>
<td>Past</td>
<td>23(26.4)</td>
<td>18(20.7)</td>
<td>0.474</td>
</tr>
<tr>
<td>Submicroscopic malaria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal</td>
<td>0(0)</td>
<td>4(4.6)</td>
<td>0.129</td>
</tr>
<tr>
<td>Placental</td>
<td>19 (21.8)</td>
<td>2(2.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cord</td>
<td>6 (7.0)</td>
<td>0(0)</td>
<td>0.037</td>
</tr>
<tr>
<td>Maternal, placental and cord</td>
<td>1(1.1)</td>
<td>0(0)</td>
<td>0.999</td>
</tr>
</tbody>
</table>

### Table 3 Factors associated with low birth weight in Medani Maternity Hospital, Central Sudan based on univariate or multivariate analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age</td>
<td>0.94</td>
<td>0.8–0.9</td>
</tr>
<tr>
<td>Primigravidae</td>
<td>1.64</td>
<td>0.8–3.1</td>
</tr>
<tr>
<td>Residence</td>
<td>0.81</td>
<td>0.4–1.5</td>
</tr>
<tr>
<td>Educational level &lt; secondary</td>
<td>1.79</td>
<td>0.7–4.1</td>
</tr>
<tr>
<td>Lack of antenatal care</td>
<td>1.47</td>
<td>0.7–2.7</td>
</tr>
<tr>
<td>Body mass index</td>
<td>1.06</td>
<td>0.9–1.1</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>1.28</td>
<td>0.9–1.7</td>
</tr>
<tr>
<td>Male gender</td>
<td>0.88</td>
<td>0.4–1.6</td>
</tr>
<tr>
<td>Placental malaria infections (histology)</td>
<td>1.33</td>
<td>0.6–2.5</td>
</tr>
<tr>
<td>Submicroscopic malaria infections</td>
<td>5.14</td>
<td>1.9–13.3</td>
</tr>
<tr>
<td>All malaria infections</td>
<td>2.63</td>
<td>1.4–4.9</td>
</tr>
</tbody>
</table>
Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AHM and IA coordinated and carried out the study, and participated in the statistical analysis and procedures. EME and BBE participated in the clinical work and statistical analysis. MMS, AAM and SIE conducted the laboratory work. All the authors have read and approved the final version of this manuscript.

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We wish to thank all of the patients for their cooperation in this study. We are very grateful to the local health authority in Geizera State and to the National Fund for the promotion of Medical Service, Khartoum, Sudan. 12. Adam I, A-Elbasit IE, Salih I, Elbashir MI, Mohammed AA, Salih MM, Ibrahuim SA, Ryan CA: Plasmodium falciparum infections during pregnancy, in an area of Sudan with a low intensity of malaria transmission. Am J Trop Med Hyg 2005, 73:359–344.

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