REPUBLIQUE DU CAMEROUN
Peace-Work-Fatherland

MINISTERE DE L'ENSEIGNEMENT SUPERIEUR

UNIVERSITE DE YAOUNDE I

FACULTE DE MEDECINE ET DES SCIENCES BIOMEDICALES

REPUBLIC OF CAMEROON
Peace-Work-Fatherland

MINISTRY OF HIGHER EDUCATION

UNIVERSITY OF YAOUNDE I

FACULTY OF MEDICINE AND BIOMEDICAL SCIENCES

AGE AND IMMUNITY TO MALARIA IN PREGNANT WOMEN

THESIS SUBMITTED AND DEFENDED PUBLICLY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTORATE IN MEDICINE CERTIFICATE

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2005/2006 Academic Year

DEDICATION

This piece of work is dedicated to

The LORD ALMIGHTY

In whom all things are possible

AKNOWLEDGEMENTS

I express profound gratitude to a number of persons whose contributions led to the realization of this piece of work:

- I am deeply indebted to my supervisor Prof. Rose Leke for providing me with an opportunity to love laboratory work and for reading and correcting this work despite her numerous responsibilities.
- I am equally indebted to Prof. Diane Taylor, who equally supervised this work. She found time during her short stay for the MIM conference to personally supervise the laboratory work; she also read and corrected this work despite her tight schedule.
- I heartily thank Dr. Nana Philip who also provided supervisory advice, read and corrected this piece of work.
- I thank the dean and the staff of F.M.B.S. for providing me with the academic foundation that supported me.
- I am grateful to the entire staff and students of the Immunology/Parasitology Laboratory of the Biotechnology Centre, University of Yaounde I: Nyonglema Philomina, Fogako Josephine, Grace Sama, Jean Bopda, Che Rymond, Acha Patrick, Diabe Godlove, Dr. Tchinda, Mbang Stephanie, Frida Bessem, Claude, Joshua and Oumarou. You all contributed to the realization of this work, please accept my gratitude.
- I am very grateful to my parents for their endless support through all these years in medical school.
- I thank all other family members (Derek, Uncle Patrick, Uncle Walter, Relindis, Jacky, Aunt Lucy, Oliver, Uncle Jerome, etc) for all the love, prayers and care.
- I thank Patrice for help in data analysis.
- I am grateful to my friends and classmates for all the love they showed me. You were a family to me and without you; these seven years would have seemed an eternity: Gwen, Doris, Princi, Sammy, James, Terry, Cavin, Aime, Lucy, Valery.

- I am equally grateful to all other friends for good company and encouragement all along: Jude, Taku, Rose Akwo, Rose Mary, Anastasie, André, Bih, Julius, Papi, Alphonse, Stella, Colette.
- I am heartily grateful to the ICIDR/HIRE malaria project (collaboration between University of Yaounde I and Georgetown University, USA), for financing this work.
- And finally to all those whose names are not mentioned, I sincerely thank you for your respective contributions in my training and the realization of this work. May the Lord bless you abundantly.

FINANCIAL SUPPORT

This work received financial support from the National Institute of Health (NIH) through Grant Number:

U01 A35839-04

to the ICIDR/HIRE malaria Project, an international research collaboration between the University of Yaounde I-Cameroon and the Georgetown University-USA.

THE PHYSICIAN'S OATH

[Declaration of Geneva adopted by the Geneva Assembly of the World Medical Association in Geneva, Switzerland, September 1948 and amended by the 22nd World Medical Assembly, Sydney, Australia (August 1968)].

On admission to the medical profession:

I will solemnly pledge myself to consecrate my life to the service of humanity;

I will give my teachers the respect and gratitude which is their due;

I will practise my profession with conscience and dignity; the health of my patients will be my first consideration;

I will respect secrets confided in me, even after the patient has died;

I will maintain by all the means in my power the honour and noble traditions of the medical profession; my colleagues will be my brothers;

I will not permit considerations of religion, nationality, race, party politics or social standing to intervene between my duty and my patient;

I will maintain the utmost respect for human life from the time of conception, even under threat I will not use my medical knowledge contrary to the laws of humanity;

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LIST OF ABBREVIATIONS

AMA-1:	Apical Membrane Antigen
BSA:	Bovine serum albumin
CD:	Cluster of Differentiation
CSA:	Chondroitin sulphate A
CSF:	Colony-stimulating factor
CSP:	Circumsporozoite protein
CTL:	Cytotoxic T lymphocytes
DNA:	Deoxyribonucleic acid
ELISA:	Enzyme-linked Immunosorbent Assay
FMBS:	Faculty of Medicine and Biomedical Sciences
G6PD:	Glucose-6-Phosphate Dehydrogenase
HIRE:	Human Immune Response to malaria in Endemic areas
ICAM:	Intercellular adhesion molecule
ICIDR:	International Collaboration in Infectious Diseases Research
IFNγ:	Interferon gamma
Ig:	Immunoglobulin
IL:	Interleukin
IPT:	Intermittent Preventive Treatment
ITNs:	Insecticide-treated bed nets
IRBCs:	Infected red blood cells
IUGR:	Intrauterine growth restriction
KDa:	Kilo Dalton
kg:	Kilogram
LBW:	low birth weight
M:	Molar
MA:	malaria extract antigen
MHC:	Major Histocompartibility Complex
mg:	milligram
ml:	millilitre
mmHg:	millimetre of mercury

mmol:	millimole
MSP:	Merozoite surface protein
NK cell:	Natural killer cell
nm:	nanometre
NMCP:	National Malaria Control Programme
NO:	Nitric oxide
NRBCs:	Normal red blood cells
OD:	Optical density
PCV:	Packed Cell Volume
PBS:	Phosphate Buffered Saline
PfEMP1:	Erythrocyte membrane protein 1
PMN:	Polymorphonuclear
RBC:	Red blood cell
RESA:	Ring Erythrocyte Surface Antigen
SP:	Sulfadoxine-pyrimethamine
TH:	T helper lymphocytes
TNFα:	Tumour necrosis factor alpha
T20:	Tween 20
WHO:	World Health Organization
%:	Percent
º C:	Degree Celsius
µl:	microlitre
≤:	less than or equal to
≥:	greater than or equal to

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SUMMARY

Background

Malaria remains one of the major public health problems in the world. According to the World Health Organization, 40% of the world population live in malaria-endemic regions. With an annual morbidity of 300-500 million people per year the world over, it causes 1-3 million deaths each year, especially in children under 5 and pregnant women.

Malaria during pregnancy is usually accompanied by poor pregnancy outcomes for both the mother and the baby, such as anaemia, abortions and low birth weight. Presently, there is no vaccine and control relies mainly on intermittent preventive treatment (IPT) with sulfadoxine-pyrimethamine (SP) and the use of insecticide-treated bed nets (ITNs) as prescribed by the National Malaria Control Programme, though other drugs are still being used. Parasite and vector resistance to drugs and insecticide are on the rise, hence the urgent need for the development of a vaccine. The development of a vaccine requires an in depth understanding of immunity to the disease, especially in the vulnerable groups.

Besides the well established parity effect on the susceptibility to malaria in pregnant women, an age effect has been reported. This study therefore sought to find out if the age effect is due to a difference in the immune status of the women.

The specific objectives included:

- To determine the plasma total IgG levels to malarial extract antigen (MA), the ring erythrocyte surface antigen (RESA) and the circumsporozoite protein (CSP) in women at delivery.
- To study the association between antibody levels and age.
- To study the association between antibody levels and placental parasitaemia.

Methods

A retrospective cross-sectional study was designed in which the plasma of 247 women were selected on the basis of age from several plasma samples previously collected under the ICIDR/HIRE malaria project (1996-2001). Available data included the age, number of pregnancies and outcome, the last menstrual period, the use of chemoprophylaxis and placental parasitaemias read from impression smears of the placentas collected at delivery.

Plasma from the 247 women were used in indirect ELISA assays for total IgG to the antigens of interest. Data was analyzed using the following computer software: MS Excel 8.0 and Epi Info 6.04. Student's t test was used to compare means between two groups and the Chi square test was used to compare proportions between the two groups. A confidence interval of 95% was considered for statistical significance.

Results and discussion

Of the 247 women selected, 108 were ≤ 20 and 139 were ≥25 years. The conception of age limits for the groups was based on two studies in Cameroon which independently reported age ≤ 20 and age ≤ 25 as risk factors for placental malaria. The mean age for each group was 18.4 and 29.9 years respectively.

The use of chemoprophylaxis was reported by 83.4% of the women compared to 16.6% who reported no use. Also, fewer younger women reported the use of chemoprophylaxis than older women (75% compared to 89.2%, p=0.003). Age did not influence levels of antibodies to MA (r= -0.06, p> 0.05) and to RESA (r= -0.005, p> 0.05). On the other hand, anti-CSP antibody levels increased with age (r= 0.21, p< 0.001). This could explain why younger women are more susceptible to malaria as these antibodies protect against re-infections.

Antibodies against asexual blood stage antigens are essential in controlling parasitaemia, however in this study, no association was found between anti-MA antibodies and placental parasitaemia (r=0.07, p>0.05), as well as between anti-RESA antibody levels and placental parasitaemia (r= -0.07, p>0.05). A positive correlation was found between anti-CSP antibody levels and placental parasitaemia. The positive correlation obtained in this study could be

explained by the fact that infected women would produce high levels of antibodies, and high antibody levels would rather reflect infection than protection, as antibodies to CSP are not effective against asexual blood-stage parasites.

Conclusions

From the above findings, the following conclusions can be drawn:

1. There is no association between age and anti-MA antibody levels as well between age and anti-RESA antibody levels.
2. Anti-CSP antibodies increased with age, and can possibly explain why younger women are more susceptible to malaria, as anti-CSP antibodies protect against re-infection.
3. There is no association between antibodies to asexual blood stage antigens tested (MA and RESA) and placental parasitaemia.
4. There is a positive correlation between anti-CSP antibodies and placental parasitaemia. These antibodies are indicative of infection and do not play a role in parasite clearance.

Recommendations

Based on the conclusions arrived at, the following recommendations are made:

1. CSP be considered as a vaccine candidate in a cocktail vaccine for pregnant women as this would prevent re-infection amongst the younger women.
2. Similar studies should be carried out using different asexual blood stage antigens to evaluate their role in parasite clearance, hence their potential use as vaccine candidates.
3. Longitudinal studies should be carried out to evaluate the kinetics of anti-MA and anti-RESA antibodies during pregnancy to better assess the benefit of these antibodies on the level of parasitaemia.

RESUME

Introduction

Le paludisme demeure un des problèmes de santé publique majeurs dans le monde. D'après l'Organisation Mondiale de la Santé, 40% de la population mondiale vit dans des régions d'endémie du paludisme. Avec une morbidité annuelle de 300 à 500 millions de personnes à travers le monde, cette maladie cause 1 à 3 millions de décès chaque année, dont la plupart surviennent chez les enfants de moins de 5 ans et les femmes enceintes.

Le paludisme, pendant la grossesse, s'accompagne habituellement d'un pronostic médiocre tant pour la mère que pour le fœtus, tels que les anémies, les avortements et les petits poids de naissance. Actuellement, il n'existe aucun vaccin contre cette parasitose et le contrôle du paludisme pendant la grossesse s'appuie essentiellement sur le Traitement Préventif Intermittent (TPI) avec la sulfadoxine-pyriméthamine (SP) et l'usage de moustiquaires imprégnées (MI), suivant les prescriptions de la politique du Programme National de Lutte contre le Paludisme, bien que d'autres médicaments soient encore utilisés. Les résistances du parasite et du vecteur aux médicaments et aux insecticides augmentent, d'où l'urgence du développement d'un vaccin. Le développement d'un vaccin nécessite une bonne compréhension de l'immunité à la maladie, surtout dans les groupes vulnérables.

A côté de l'effet de la parité sur la susceptibilité au paludisme chez les femmes enceintes qui est un fait bien établi, un effet de l'âge a été rapporté. Cette étude avait donc pour but de vérifier si l'effet de l'âge sur la susceptibilité au paludisme chez les femmes enceintes était dû à une différence dans le statut immunitaire de ces femmes.

Nos objectifs spécifiques étaient:

- De déterminer le taux d'IgG contre l'extrait d'antigène malarique (MA), l'antigène érythrocytaire de surface (RESA) et la protéine circumsporozoïte (CSP) dans le plasma des femmes à l'accouchement
- D'étudier l'association entre les taux d'anticorps et l'âge.

- D'étudier l'association entre les taux d'anticorps et la parasitémie placentaire.

Méthodes

Nous avons réalisé une étude transversale rétrospective, au cours de laquelle les plasmas de 247 femmes ont été sélectionnés sur la base de l'âge parmi plusieurs échantillons collectés antérieurement pendant le projet ICIDR/HIRE (1996-2001). Les données disponibles étaient l'âge, le nombre et le devenir des grossesses, la date des dernières règles, l'usage de la chimioprophylaxie antipalustre pendant la grossesse et la parasitémie placentaire obtenue par la lecture des lames d'apposition de placentas collectés à l'accouchement.

Les plasmas de ces 247 femmes ont été utilisés pour mesurer le taux total des différentes IgG par des tests ELISA indirects. Les données ont été analysées en utilisant les logiciels MS Excel 8.0 et Epi Info 6.04. Les moyennes ont été comparées par le test de Student et les proportions par le test de Chi carré. Un intervalle de confiance de 95% était considéré comme le seuil de signification statistique.

Résultats

Sur les 247 femmes sélectionnées, 108 avaient un âge inférieur ou égale à 20 ans et 139 avaient un âge supérieur ou égale à 25 ans. Le choix des limites d'âge pour les groupes était basé sur deux études antérieures menées au Cameroun , qui ont rapporté indépendamment qu'un âge ≤ 20 ans et ≤ 25 ans sont des facteurs de risque du paludisme placentaire. La moyenne d'âge pour chaque groupe était respectivement de 18,4 ans et de 29,9 ans.

Concernant la chimioprophylaxie, 83,4% des femmes déclaraient avoir l'utilisé et 16,6% disaient n'en avoir pas fait usage. Les femmes jeunes utilisaient la chimioprophylaxie moins souvent que les femmes plus âgées (75% contre 89,2%, p=0,003).

L'âge n'a pas influencé le taux d'anticorps anti-MA (r=-0,06, p>0,05), ni d'anticorps anti-RESA (r=-0,005, p>0,05). Par contre, le taux d'anticorps anti-

CSP augmentait avec l'âge (r=0,21, p<0,001). Ceci pourrait expliquer la susceptibilité au paludisme observée chez les jeunes femmes pendant la grossesse, car les anticorps anti-CSP protègent contre les réinfections.

Les anticorps contre les antigènes des stades sanguins asexués du parasite, sont importants dans le contrôle de la parasitémie, néanmoins, aucune association n'a été retrouvée aussi bien entre le taux d'anticorps anti-MA et la parasitémie placentaire (r=0.07, p>0.05), qu'entre le taux d'anticorps anti-RESA et la parasitémie placentaire (r= -0.07, p>0.05) dans cette étude. Une corrélation positive a été retrouvée entre le taux d'anticorps anti-CSP et la parasitémie placentaire. Les femmes infectées produisent les taux d'anticorps élevés et les taux élevés des anticorps indiquent une infection et non la protection contre ces stades du parasite pour lesquels ils sont inefficaces.

Conclusions

Les conclusions suivantes peuvent être tirés des résultats qui précèdent :

1. Il n'y a pas d'association entre l'âge et les taux d'anticorps anti-MA, ainsi qu'entre l'âge et les taux d'anticorps anti-RESA.
2. Les taux d'anticorps anti-CSP augmentent avec l'âge, ce qui peut probablement expliquer pourquoi les jeunes femmes sont plus susceptibles au paludisme car les anticorps anti-CSP protègent contre des réinfections.
3. Il n'y a pas d'association entre les anticorps dirigés contre les antigènes des stades sanguins asexués du parasite (MA et RESA) et la parasitémie placentaire.
4. Il y a une corrélation positive entre le taux d'anticorps anti-CSP et la parasitémie placentaire. Ces anticorps sont des indicateurs d'infection et ne jouent aucun rôle dans la clairance du parasite.

Recommandations

Sur la base des conclusions auxquelles nous sommes parvenus, les recommandations suivantes sont faites :

1. La CSP soit considérée comme un candidat vaccin pour un cocktail de vaccin pour les femmes enceintes car ceci pourrait prévenir les réinfections parmi les femmes plus jeunes.
2. Des études similaires soient conduites en utilisant d'autres antigènes parasitaires des stades sanguins asexués afin d'évaluer leur rôle dans la clairance du parasite, et donc leur usage potentiel en tant que candidat vaccin.
3. Des études longitudinales soient menées pour évaluer la cinétique des anticorps anti-MA et anti-RESA pendant la grossesse pour mieux mesurer le bénéfice de ces anticorps sur le niveau de la parasitémie.

CHAPTER ONE: GENERAL INTRODUCTION

1.1. Introduction

Despite considerable advances in malaria control and drug therapy, malaria still remains the number one tropical disease. It affects about 300 - 500 million people yearly, causing between one and three million deaths (The world health report, 2003). The disease is caused by protozoan parasites of the genus Plasmodium. Amongst the four species of Plasmodium that cause human malaria, *Plasmodium falciparum* is the most common and most dangerous. *Plasmodium falciparum* malaria is therefore a major public health problem in Africa and many other tropical countries around the world.

Malaria is endemic in the tropics, especially in sub-Saharan Africa. In populations in which malaria is endemic, immunity is acquired in an exposure-related manner. Acquired immunity is both cell-mediated and humoural, with the production of antibodies of all immunoglobulin classes to a wide range of antigens from the different developmental stages of the parasite.

The greatest burden of the disease is borne by children under five years of age and pregnant women. The vulnerability of children below five is explained by the fact that acquisition of natural immunity against developing severe malaria occurs only after repeated infections (Baird, 1995). Pregnant women are more susceptible compared to non-pregnant women of the same age (McGregor *et al.*, 1983). Despite previously acquired immunity, pregnant women are at risk of developing malaria. The mechanism(s) responsible for the increased susceptibility of pregnant women are still unclear.

Several hypotheses have been proposed to explain this susceptibility. The cortisol hypothesis suggested a down-regulation of type1 T helper cell (TH1) cytokines responsible for controlling the acute phase of infection. This hypothesis however, fails to explain the increased susceptibility in the second trimester of pregnancy reported in several studies (Brabin, 1983; Zhou *et al.*, 2002), given that levels of cortisol increase through out pregnancy (Mattelli *et al.*, 1997).

Another hypothesis set forth to explain the increased susceptibility of pregnant women is that of “housing and shielding”, whereby the dense

vasculature of the placenta traps infected red blood cells (RBCs). These trapped RBCs multiply greatly, free from the immune system. This hypothesis also fails to explain the increased susceptibility in the second trimester of pregnancy, as one would expect women to be more susceptible in the third trimester, when the placenta attains its largest volume.

The most recent hypothesis is the "placenta parasite" hypothesis. This holds that there is a pregnancy-related receptor for infected RBCs. Parasites adhering to the placenta have a distinct adhesion phenotype, binding to chondroitin sulphate A (CSA) but not to CD36 (Fried *et al.*, 1996). These findings suggest the expression of unique proteins on the surface of infected RBCs by placental parasites. Serological studies confirmed this: males or primigravid women at early gestation lack antibodies that inhibit the adhesion of placental parasites to CSA; multigravid women from the same area manifest these antibodies, indicating exposure to new malaria antigens during previous pregnancies (Fried *et al.*, 1998a; Ricke *et al.*, 2000). This hypothesis explains the susceptibility in the second trimester: pregnant women are more susceptible in the second trimester because of the development of the placenta, which expresses a new receptor for infected RBCs, and because of the lack of immunity to the pregnancy-specific strain of *P. falciparum*. This immunity becomes effective after about 20 weeks and 12 weeks of gestation in primigravidae and multigravidae respectively (O'Neil-Dunne *et al.*, 2001).

Several studies show primigravidae at higher risk of malaria infection (Nair *et al.*, 1993; Menendez, 1995; Silver *et al.*, 1997; Miller *et al.*, 1998; Zhou *et al.*, 2002). The decrease in susceptibility with subsequent pregnancies suggests a gravidity-dependent acquisition of placental malaria specific immunity (antibodies that block the adhesion of infected RBCs to the placental chondroitin 4-sulfate). The early onset of efficient antibody production in multigravidae and the delayed production of anti-adhesion antibodies in primigravidae appears to account for the gravidity-dependent susceptibilities, as both primigravidae and multigravidae have similar levels of antibodies at term (O'Neil-Dunne *et al.*, 2001).

A significant effect of age on the prevalence of malaria in pregnancy was reported by Zhou and others in 2002 when they found that 74% of women ≤20 years old versus 43% of women >20 years old were slide-positive at ≥1 antenatal visits, in Etoa, a rural setting around the city of Yaounde and 39% versus 14% of younger to older women respectively were slide-positive at ≥1 antenatal visits in the city. The age effect has also been reported in Mali (Dicko *et al.*, 2003). Previous studies did not find age to be a risk factor in susceptibility (Brabin, 1983, McGregor, 1984). Although a decrease in microscopic slide positivity with age and gravidity is well established, no age and gravidity effect was found on the prevalence of sub microscopic infections in Cameroonian women (Walker-Abbey *et al.*, 2005).

1.2. Rationale

Individuals living in malaria-endemic areas require many years of continuous exposure to malaria parasites in order to acquire immunity. It is likely that younger women have a lower level of immunity to malaria than older women. Zhou and others in 2002 reported results from a previous prevalence study conducted in one of their study sites in the period 1996-1999 in which data showed that 35% (n=25) of non-pregnant women 15-20 years old, compared to 18% (n=79) of women 21-50 years were slide positive for malaria. They concluded that, the age effect observed in pregnant women does not seem to be pregnancy-specific.

The age effect may be due to a difference in the immune status of these women. This study therefore sought to evaluate the effect of age on the levels of antimalarial antibodies in women with and without placental malaria at delivery.

1.3. Hypotheses

- The acquisition of antibodies to malaria antigens is age-dependent. Younger women have lower antibody levels than older ones, as it is likely that they have had fewer encounters with the parasite.
- Antibody levels to asexual blood stage antigens vary with the degree of placental parasitaemia. It seems that women with high percentage placental parasitaemia are those who could not control the initial parasite population, leading to a larger number of parasites sequestering in the placenta.

1.4. Objectives

1.4.1. Overall objective

To assess the influence of age on antibodies to malarial extract antigen (MA), the ring erythrocyte surface antigen (RESA) and the circumsporozoite protein (CSP), in women at delivery.

1.4.2. Specific objectives

- Determine the total IgG antibody levels to MA, RESA and CSP in the plasma of women at delivery.
- Study the association between antibody levels and age.
- Study the association between antibody levels and placental parasitaemia, among the placental malaria positive women.

CHAPTER TWO: REVIEW OF LITERATURE

2.1. The History of Malaria

Malaria has been noted for more than 4000 years. From the Italia for “bad air”, malaria has probably influenced to a great extent human populations and human history. The symptoms of malaria were described in ancient Chinese medical writings. In 2700BCE, several characteristic symptoms of what would later be named malaria were described in the *Nei Ching* (The canon of Medicine).

Malaria became widely recognized in Greece by the 4th century BCE. It was responsible for the decline of many of the city-state populations. Hippocrates noted the principal symptoms. In China, during the 2nd century BCE, the Qinghao plant (*Atemisia annua L*) was described in the medical treatise, *52 Remedies*. In the United States, this plant is known as the annual or sweet wormwood. In 3400CE, Ge Hong of the East Yin Dynasty described the anti-fever property of Qinghao.

Following their arrival in the New World in the early 17th century, the Spaniard learned of a medicine used for the treatment of fevers. Spanish Jesuit missionaries in South America learned of a medicinal bark from indigenous Indian tribes. With this bark, the Countess of Chincón, the wife of the Viceroy of Peru was cured of her fever. The bark of the tree was then called *Peruvian bark* and the tree named Cinchona after the Countess. The medicine from the bark is now known as the antimalarial Quinine.

In November 1880, Charles Louis Alphonse Laveran, a French army surgeon stationed in Constantine, Algeria, was the first to notice parasites in the blood of a patient suffering from malaria. He was awarded the Noble Prize in 1907 for his discovery.

Quinine could prevent the worst consequences of pregnancy malaria such as maternal anaemia, stillbirth and preterm deliveries; but Goth in 1881 and Chiarleoni in 1886 determined that babies brought to term from infected mothers still suffered in the form of low birth weight. Camillo Golgi an Italian neurophysiologist, established that there were at least two forms of the disease in 1886: one with the tertian periodicity (fever every other day); and one with

quartan periodicity (fever every third day). He also observed that the different forms produced different number of new parasites upon maturity and that fever coincided with the rupture and release of merozoites into the blood stream. He was awarded the Noble Prize in Medicine for his discoveries in neurophysiology in 1906.

Up to this time, it was believed that there was only one species, *Oscillaria malariae*, according to Laveran. Italian scientists were leaders in the field at the time. The Italian investigators Giovanni Batista Grassi and Raimando Filetti first introduced the names *Plasmodium vivax* and *Plasmodium malariae* in 1890 for two of the malaria parasites that affect humans. An American, William H. Welch, reviewed the subject and in 1897, he named the malignant tertian malaria parasite, *Plasmodium falciparum*. In 1922, John William Watson Stephens described the fourth human malaria parasite, *Plasmodium ovale*.

On August 20th 1897, Ronald Ross, a British officer working in the Indian Medical Service was the first to demonstrate that malaria parasites could be transmitted from infected patients to mosquitoes by demonstrating the presence of malaria cyst in the stomach wall of anopheles mosquitoes. He showed in further work with bird malaria that mosquitoes could transmit the parasite from bird to bird.

In 1899, a team of Italian investigators led by Giovanni Batista Grassi, traced the course of the parasite through the mosquito, establishing the complete sporogonic cycle of plasmodium. Mosquitoes infected by feeding on a patient in Rome were sent to London, where they fed on two volunteers, both of whom developed benign tertian malaria, confirming the mosquito-transmission hypothesis of malaria.

Around 1900, Bignami and Sereni found that *P. falciparum* multiplied to a great density in the placenta and in 1915, Clark in Panama observed that indigenous women could carry heavy placental parasitaemias asymptomatically.

A German, Hans Andersag discovered chloroquine, an antimalarial in 1934. He named his compound resorchin. British and American scientists recognized chloroquine as an effective and safe antimalarial in 1946. A German

chemistry student, Orther Zeidler, synthesized dichloro-diphenyl-trichloroethane (DDT) in 1874, for his thesis. The insecticide property of DDT was discovered in 1939 by Paul Müller in Switzerland. It was used for malaria control at the end of World War 2, after it had proven effective against malaria-carrying mosquitoes by British, Italian and American scientists. Müller won the Nobel Prize for Medicine in 1948.

With the advent of less toxic, more effective antimalarial drugs and the success of DDT, the World Health Organization (WHO) submitted a proposal for the eradication of malaria worldwide, at the World Health Assembly in 1955. Eradication efforts included house spraying with residual insecticides, antimalarial drug treatment and surveillance.

In the 1960s, chloroquine-resistant strains of *Plasmodium falciparum* had arisen as a result of over usage and probably under dosage. At the time there was no drug except the ancient antimalarial quinine, to treat chloroquine-resistant malaria. WHO realized that the global eradication of malaria was impossible for several reasons and the completion of the eradication campaign was abandoned to one of control in 1967.

In 1971, Chinese scientists isolated the active principle of the plant Qinghao and named it Artemisinin. It is today a very potent and effective antimalarial drug (especially in combinations) against chloroquine-resistant malaria. In 1972, the Global Eradication of Malaria Programme was formally declared dead. Successes of the Global Eradication Programme include eradication in nations with temperate climates and seasonal malaria transmission. Some nations like those of sub-Saharan Africa were excluded from the eradication campaign; malaria is still therefore a major problem in these countries.

The new focus for eradication today is probable vaccine development. Since research began in this area, several vaccine candidates have been identified and tested but no practical vaccine is in clinical use.

2.2. Taxonomy of the human malaria parasite (Bruce-Chwatt, 1985)

Kingdom:	Animalia
Phylum:	Apicomplexa
Sub-phylum:	Sporozoa
Class:	Telosporida
Sub-class:	Haemosporidida
Order:	Coccidida
Sub-order:	Haemosporidiidea
Family:	Plasmodidae
Genus:	Plasmodium
Species:	falciparum, malariae, ovale, vivax

2.3. Life cycle of the malaria parasite

The life cycle of human *Plasmodium* spp. involves two hosts, man and the female mosquito of the genus Anopheles. Asexual reproduction otherwise known as schizogony occurs in man while the sexual reproduction also known as sporogony occurs in the mosquito. The schizogonic cycle is further divided into exo-erythrocytic schizogony and erythrocytic schizogony.

2.3.1. Schizogonic cycle

2.3.1.1. Exo-erythrocytic schizogony

It starts when a blood-feeding female Anopheles mosquito injects sporozoites into the sub-cutaneous tissue and less frequently, directly into the blood stream. Sporozoites briefly circulate in the blood stream and then invade hepatocytes within 5 to 30 minutes. The co-receptor on sporozoites that mediates invasion involves in part, the thrombospondin domains on the Circumporozoite Protein (CSP) and the thrombospondin-related adhesive protein (TRAP). These domains bind specifically to heparan sulfate proteoglycans on the

hepatocytes (Frevert, 1993). Sporozoites develop into hepatic schizonts, which contain thousands of merozoites. This liver stage is not responsible for any illness (Miller *et al*. 1994). *P. vivax* and *P. ovale* sporozoites may persist in liver cells as hypnozoites, capable of developing into merozoites later. This is responsible for recrudescence, relapses or epidemics, especially in travellers from endemic areas

2.3.1.2. Erythrocytic schizogony

Invasion of red blood cells (RBCs) by merozoites from ruptured infected hepatocytes occur a few minutes following release. Invasion is receptor-mediated. Receptors are found on the cell surface and on apical organelles (Chitnis, 2001), Within the RBCs, the merozoites develop over 48 hours, producing about 20 merozoites per mature parasite, with each merozoite ready to invade another RBC on release. A small proportion of asexual parasites converts to gametocytes that are essential for transmitting the infection to others through anopheles mosquitoes, but cause no disease. Here the strategy of *P. vivax* differs from that of *P. falciparum*. *P. vivax* develops into gametocytes soon after the release of merozoites from the liver; *P. falciparum* gametocytes develop much later, after several repeated erythrocytic cycles.

2.3.2. Sporogonic cycle (Beir *et al*., 1998)

After a blood meal of a female anopheles mosquito on an infected person, mechanical rupture, enzymes and the emerging gametes lyse RBCs in the gut. Gametogenesis occurs in the gut, induced by a drop in temperature from the human host to the mosquito and mosquito enzymes. The male gametocyte's (microgametocyte's) nucleus divides into 8 nuclei, each of which develops into a flagellum that is liberated as a gamete when the gametocyte membrane splits. This process is called exflagellation. The female gametocyte (macrogametocyte) gives rise to a single macrogamete. One microgamete fertilizes one macrogamete to give a diploid zygote.

The zygote transforms into a mobile ookinete within 12-24 hours, which escapes the acid- and enzyme-rich midgut by passing through the stomach mucosa. The ookinete penetrates the epithelium and basement membrane, residing between the basement membrane and the lamina. It grows into an oocyst. A sporoblast develops within the oocyst and gives several haploid sporozoites by meiosis. A single oocyst gives hundreds of sporozoites.

Mature sporozoites break off from the oocyst and invade the salivary glands. The cycle restarts when the sporozoite-infected mosquito bites man during a blood meal.

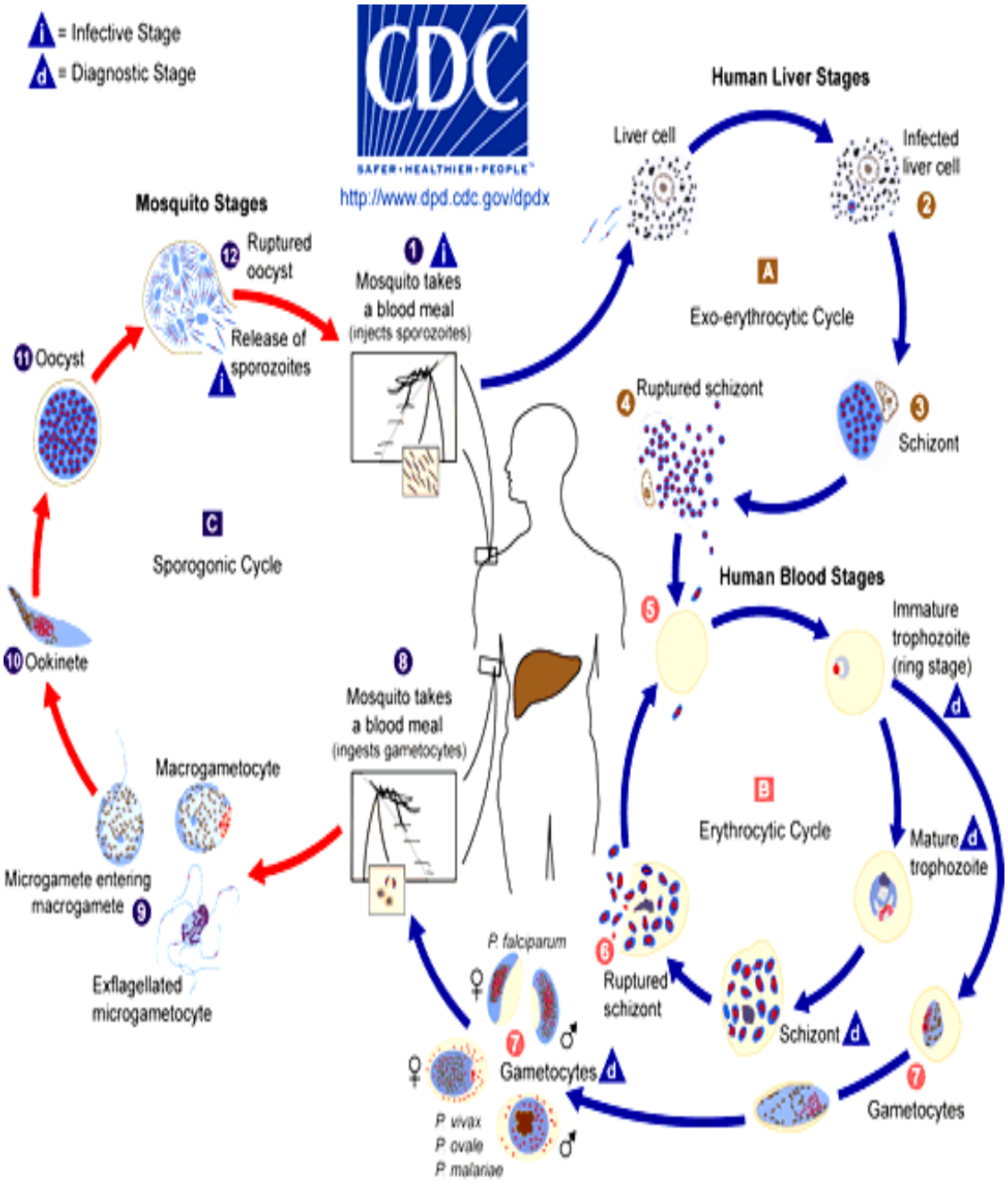

Figure1: The life cycle of Plasmodium

2.4. Pathogenesis of malaria

Pathogenesis relates to the various factors that are responsible for causing pathology. By understanding the pathogenesis, better strategies would be developed to prevent severe forms of malaria, since the clinical outcome of an infection depends on these factors. The factors could be grouped into parasite factors, host factors, geographic and social factors.

2.4.1. Parasite factors

2.4.1.1. Cytoadherence

Cytoadherence refers to the binding of infected RBCs to receptors expressed on the vascular endothelium (Udeniya *et al.*, 1981). Receptors for parasite adhesion include: CD36, intercellular adhesion molecule-1 (ICAM-1), thrombospondin (TSP) and chondroitin sulfate A (CSA), amongst others. The main parasite adhesion ligand is *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP1). Cytoadherence allows for sequestration in deep organs, a mechanism for the parasite's evasion from destruction by the spleen. It is also a critical event in disease and as such, sequestered parasites cause considerable obstruction to tissue perfusion and/or local production and deposition of pro-inflammatory cytokines.

2.4.1.2. Rosetting

Parasite rosetting is the binding of infected RBCs to uninfected RBCs (David *et al.*, 1988). It has been suggested to be an additional mechanism facilitating parasite sequestration and the development of cerebral malaria (Calson *et al.*, 1998). This phenomenon can facilitate the invasion of non-infected RBCs by merozoites released from the infected RBCs.

2.4.1.3. Agglutination

This is the binding of infected RBCs to other infected RBCs (Roberts *et al.*, 1992) or the agglutination of infected RBCs by immune sera in vitro (Marsh and Howard, 1986). In vivo agglutination may have the same pathological significance as rosetting, creating a focal concentration of infected RBCs that block the flow of blood. Agglutination mediated by immune sera is strain-dependent and the same serum sample can contain various strain specificities, agglutinating different parasite isolates to different degrees (Marsh and Howard, 1986).

2.4.1.4. Antigenic polymorphism

The different stages of the parasite express different antigens. Many parasite proteins exhibit polymorphism, which potentially accounts for immune system evasion, hence growth restriction and not parasite clearance results. Each successive wave of parasitaemia expresses a new variant surface antigen, thus allowing parasite multiplication despite the presence of antibodies directed against the preceding wave (Saul, 1999).

Other parasite factors affecting the clinical outcome of infection include: the redundancy of invasion pathways in *P. falciparum* as compared to the others and the development of drug resistance.

2.4.2. Host factors

2.4.2.1. Genetic factors

Certain genetically determined parameters such as receptor expression and red cell metabolism might influence the pathogenesis of malaria. *Plasmodium vivax* invades only Duffy blood group positive RBCs (Miller *et al.*, 1976). In West Africa where people are Duffy blood group negative, *Plasmodium vivax* has essentially disappeared.

A common haemoglobin disorder in Africa, is the sickle cell disease. Heterozygotes of the disease (carriers of the sickle cell trait) have been shown to suffer less frequently from malaria than normal individuals. Other haemoglobin disorders known to confer protection are the existence of haemoglobin C and the thalassemias, by impairing parasite growth in the RBCs.

Red cell membrane disorders such as hereditary ovalocytosis and red cell enzyme disorders such as glucose-6-phosphate dehydrogenase deficiency are thought to reduce cytoadherence and impair intracellular parasite growth respectively.

2.4.2.2. The pro-inflammatory response

The pro-inflammatory response together with antibodies protect against the asexual blood stages. This may relate to the cytokines tumour necrosis factor-α (TNF-α), interferon-γ (IFN- γ) and mediators such as nitric oxide (NO). It has been hypothesized that mediators such as NO are also central to disease (Clark and Cowden, 1999). Coma might be caused by local increased concentrations of NO. TNF-α has also been incriminated in pathology; one hypothesis suggests that it induces brain endothelial cells to express ICAM-1, hence, favouring cytoadherence.

2.4.2.3. Age

Age is known to affect the clinical outcome of infection in endemic areas. In areas of low endemicity, the disease affects all age groups to an equal extent. In endemic areas like Cameroon, mostly children less that five are affected and more complications are seen in this age group. This is due to the weak immunity at this age as immunity is acquired following at least one exposure (Gupta *et al.*, 1999).

2.4.2.4. Pregnancy

Pregnancy is a predisposing factor to malaria infection; pregnant women are more susceptible than non-pregnant women or adult males of comparable age (Dolan *et al.*, 1993). This is due to the sequestration of parasitized RBCs in the placenta, as placenta expresses specific receptors for binding. Several studies have noted an increased parasite prevalence and density in primigravidae compared to secundi- and multigravidae (Nair *et al.*, 1993; Menendez, 1995; Silver, 1997; Miller *et al.*, 1998).

Zhou *et al*. (2002) while working in Etoa, a village near Yaounde, showed that primigravid women were 2.1 times more likely to be infected than multigravid women. The observed difference in prevalence between parity groups is due to the degree of immunity developed as a result of infection in previous pregnancy. Primigravidae lack antibodies that block the adhesion of infected erythrocytes to placental CSA. Other studies showed that the majority of women lack antibodies in early gestation irrespective of gravidity, and that 88% of pregnant women produce anti-adhesion antibodies during the second trimester. The differences in the rate of production of antibodies in these women during the second trimester are suggested to contribute significantly to the difference in susceptibility (O'neil-Dunne *et al.*, 2001).

2.4.2.5. Immunity

Clinical disease also changes with immunity. Immunity to malaria has a major role in controlling disease and pathogenesis. The adhesion protein, PfEMP1, plays a role in immune evasion by clonal antigenic variation, leading to chronic infection. Humans are not refractory to malaria parasites even after several exposures, but develop clinical immunity that prevents symptomatic disease. This type of immunity limits disease. Though the individual may carry low number of parasites, they do not develop symptomatic infection (Snow *et al.*, 1998). The role of protection of anti-PfEMP1 antibodies is highlighted in placental

malaria. Exposure to parasites that sequester in the placenta during pregnancy induces immunity that stops infected RBCs from adhering to CSA. This may protect the mother and foetus from placental malaria in subsequent pregnancy (Fried *et al.*, 1998b; O'Neil-Dunne *et al.*, 2001). Pre-pregnancy immunity also determines the outcome of infection as non-immune women usually present with severe form of the disease. Exposure to pathogenic forms of *P. falciparum* can protect against these parasites leading to the selection of possibly less virulent parasites in subsequent infections.

2.4.3. Geographic factors

Factors such as the bio-ecology of the environment, the Anopheles species, seasonability of transmission, the number of infectious mosquito bites per year all play important roles in determining the clinical outcome of an infection.

2.5. The epidemiology and outcomes of maternal malaria

2.5.1. Epidemiology of maternal malaria

Individuals living in areas endemic for malaria acquire substantial immunity by the age of the first pregnancy (Menende*z et al.*, 1995). Primigravidae are more susceptible to malaria. This has been reported in Cameroon where at delivery, 22.8% of women were found to be infected with *P. falciparum* (Leke *et al*., 2002). Primigravidae were more likely to be infected than multigravidae at the time of delivery (24.9% compared to 16.4% respectively). They were also more likely to suffer from anaemia.

Zhou *et al.*, (2002) reported that besides the gravidity effect, there is also a trimester effect on the prevalence of malaria during pregnancy. The study reported that Cameroonian women experienced the highest prevalence of asymptomatic infection during the second trimester of pregnancy, with peak

prevalence occurring during the fourth month of gestation (i.e. 15 – 18 weeks). Prevalence peaks have also been reported in early or mid-pregnancy in other African countries (Brabin, 1983; Nair *et al.*, 1993; Diagne *et al.*, 1997)

Several studies have shown that age is not a risk factor to susceptibility (Brabin, 1983; McGregor, 1984). The effect of age was reported in Malawi (Steketee, 1996). A significant effect of age on prevalence has also been reported in Cameroon (Zhou *et al.*, 2002). Women less than or equal to 20 years were 1.8 times more likely to have malaria than women above 20 after adjusting for other factors such as gravidity and trimester. People living in malaria-endemic areas usually acquire full immunity against the parasite only after several years of continuous exposure to the parasite. Probably, younger women have a lower level of immunity to malaria and therefore are more prone to infection than older women, independent of gravidity, trimester and other factors.

Besides the above host factors discussed, other factors such as vectorial, environmental and social factors also play a role in disease epidemiology. Zhou and others in 2002 compared the prevalence of *Plasmodium falciparum* infection in pregnant women from Etoa, a rural setting about 20km from Yaounde and Biyem Assi, within the city of Yaounde. The prevalence of malaria during the first three months of pregnancy was 26% in the village and 9% in the city. The prevalence increased to 50% and 20% respectively in the two sites during the fourth month of pregnancy and then decreased progressively, that of the village remaining superior to that of the city all through pregnancy. In Etoa, malaria transmission was high and women had limited access to antimalarial drugs.

2.5.2. Outcomes of maternal malaria

Malaria in pregnancy has been associated with poor pregnancy outcomes for both mother and foetus. Outcome will depend on such factors as previous history of malaria exposure, malaria endemicity, age of pregnancy, the use of

chemoprophylaxis or intermittent preventive treatment and the general maternal health and nutrition of the mother.

2.5.3. Maternal outcomes

Outcome might range from asymptomatic infection through clinical disease (mild or severe malaria), to death. In the mild form of the disease otherwise referred to as uncomplicated malaria, the main symptoms are: fever, chills, malaise, headache and vomiting. There may be jaundice due to haemolysis. The liver and/or spleen may be enlarged. These symptoms could be present in varying associations, in the absence of criteria defining severe malaria.

In severe malaria, any of the following could be present (WHO, 2002):

- Neurological impairment, coma, convulsions
- Hypoglycaemia (blood glucose< 2.2mmol/l)
- Acute severe anaemia (hematocrit< 15%)
- Renal failure (urine output< 400ml/24hours and serum creatinine> 265µl)
- Circulatory collapse or shock (systolic blood pressure < 70mmHg, clammy skin, core-skin temperature difference > 10°C
- Acidaemia (arterial pH< 7.25)
- Spontaneous bleeding and/or disseminated intravascular coagulation
- Acidosis (plasma bicarbonate< 15mmol/l, serum lactate> 5mmol/l)
- Pulmonary oedema or adult respiratory distress syndrome

Death can result from severe malaria and is more common with non-immune women. In immune women, death will usually be accounted for by such factors as delayed hospitalization, inadequate or no treatment, young age and no antenatal care. Given the complications usually associated with maternal malaria, malaria in pregnancy is considered severe and managed as such.

2.5.2.2. Foetal outcomes

Poor foetal outcomes have been associated with maternal malaria. They include abortions, pre-term births, stillbirth and low birth weights. A study on the effects of malaria and the outcome of pregnancy done by Nair and others in 1993 revealed that infection during pregnancy caused complications like abortions (9.7%); preterm labour (59.6%) and still births (5.7%) which were all higher with *P. falciparum* infection.

The pathophysiology responsible for abortions and preterm labour are still unclear. It is thought that TNF-α produced during a malaria infection and the accompanying fever lead to the induction of premature labour (Raghupathy, 1997; Gucer *et al.*, 2001).

Low birth weight (LBW) could either be due to prematurity or to intra uterine growth restriction (IUGR). Several studies have reported a decrease in the mean birth weight of children born to malaria-positive women at delivery compared to malaria-negative women. Leke and others in 2002 found that in Cameroonian infants, there is an increased risk of LBW with placental malaria. Infants born to malaria-negative women averaged 3261g while those of malaria-positive mothers averaged 3077g, a mean depression of 184g. The mechanism(s) responsible are not quite clear. IUGR may result from slow placental development resulting in small placentas with reduced transport capabilities or from reduction in materno-foetal transport owing to inflammatory destruction of the syncytiotrophoblast. Inflammation within the placenta occurs in placental malaria leading to the recruitment of mononuclear cells (Walter *et al.*, 1982; Ordi *et al.*, 1998; Leke *et al.*, 2002) and the production of TNF- α.

Increased prevalence of TNF-α production has been found in women delivering LBW infants (Fried *et al.*, 1998c; Leke *et al.*, 2002). LBW is more common in primigravidae (Kasumba *et al.*, 2000).

Congenital malaria may occur. This results from transplacental transfer of infected RBCs. Routine microscopy of thin and thick blood films of cord blood are usually negative for malaria parasites, however, when other techniques such as polymerase chain reaction to detect parasite DNA are used, cord infections could

be established (Tobian *et al.*, 2000). The presence of *P. falciparum* IgM in cord blood also suggests that the foetus was infected in utero and B-cell activation occurred (Achidi *et al.*, 1997; Xi *et al.*, 2003).

2.6. Placental malaria

Placental malaria is the result of the accumulation of infected RBCs in the placenta. How the infected RBCs adhere and multiply in the placenta has been the subject of many studies. An old school of thought holds that the accumulation is mechanical, due to the placental vasculature.

The hypothesis of placenta-specific receptor binding has been widely studied with the identification of Chondroitin Sulfate A, a glycan expressed on the surface of syncytiotrophoblasts. Because this receptor may not be accessible for parasite adhesion in the non-pregnant host, women may not have substantial exposure to the CSA-binding sub-population until their first pregnancy, rendering primigravidae naïve to infection. Over successive pregnancies, women develop specific anti-adhesion immunity that blocks parasite adhesion to CSA, such that multigravid women are infected less frequently and with lower parasite densities compared to primigravid women.

Several methods have been used to identify placental infections. Placental infections have been determined by impression smears, smears of extracted placental blood and by histology. The appearance of malaria parasites early in infection, and then pigments as infection resolves, has been used to characterize the chronology of infection in the placenta (Bulmer *et al.*, 1993). Ismail and others in 2000 classified placental malaria as follows:

Table 1: Classification of placental malaria (Ismail *et al.*, 2002)

Not infected	No parasite, no pigment
Acute infection	Parasites present; absent/minimal pigment or cells within fibrin
Chronic infection	Parasites present; significant pigment deposition in fibrin or cells
Past infection	Pigments, no parasites

Acute and chronic infections are referred to as “active” infections.

Placental parasitaemia (percentage of infected RBCs on a slide) and peripheral parasitaemia generally correlate well (Brabin, 1983); many studies have however showed that parasites can concentrate in vast numbers in the placenta of women with scanty peripheral parasitaemias. Leke *et al.* in 1999 reported that 20.1% of women with placental malaria were peripheral blood smear negative by microscopy, even women with placental parasitaemias as high as 10% were misdiagnosed. Some studies have shown peripheral parasitaemias in the absence of placental infection (Ismail *et al.*, 2000; Leke *et al.*, 2002).

Placental malaria is associated with several pathological changes in the placenta, infiltrates of immune cells and the deposit of pigments. The striking changes of placental malaria occur within the intervillous space and to a lesser extent the immediately adjoining syncytiotrophoblast. Enormous numbers of both parasite and macrophages can accumulate within the intervillous space. Inflammation does not usually extend to the villi, where more subtle changes occur including basement membrane thickening (Bulmer *et al.*, 1993) and immune complex deposition (Maeno *et al.*, 1993). The stroma generally appears unchanged and pigment deposition is less common in the cytotrophoblasts or Hofbauer cells than in the syncytiotrophoblast. Foetal cells often appear normal.

2.7. Diagnosis of malaria

2.7.1. Clinical diagnosis

The clinical diagnosis of malaria is a presumptive diagnosis made on the basis of signs and symptoms suggestive of malaria. In the simple uncomplicated form, the patient presents with a history of fever, with or without rigors, headache, and general body pains, associated or not to gastro-intestinal symptoms. The severe form presents with fever associated variably to any of the WHO criteria for severity (WHO, 2002). Exception is made in pregnancy because of the risk associated with pregnancy malaria, such that any all cases are considered severe and given the appropriate treatment.

2.7.2. Paraclinical diagnosis

The paraclinical diagnosis of malaria may be done using several methods. Light microscopy of thick blood film and placenta impression smears is the gold standard to detect malaria parasites in the peripheral blood and placenta respectively. The thick film concentrates the parasites and is effective in low-density infections. The thin film is important in parasite speciation and calculating the parasite density.

Another parasitological diagnostic technique is the Quantitative Buffy Coat, in which a capillary tube containing acridine orange is used to collect blood. The acridine orange stains the nuclei of the parasites. The tube is centrifuged and the parasitized RBCs come to lie at the plasma-RBC interface just beneath the buffy coat layer, because of their low density. A fluorescent microscope is then used to detect parasites with stained nuclei. This technique has specificity and sensitivity comparable to that of light microscopy but neither permits for species diagnosis nor parasite density to be made.

Genomic techniques like polymerase chain reaction can also be used. This is the most specific and most sensitive method to determine infection. It determines the presence of any parasite DNA in blood but does not correlate to

active disease or disease severity. These, and besides the fact that it is a costly technique, make its use in clinical medicine difficult.

Immunological techniques such as Enzyme-Linked Immunosorbent Assay (ELISA) and Immunoflourescent Assay (IFA) can be used to detect the presence of specific parasite antigens, using monoclonal antibodies. Test kits have been designed for some antigens such as Histidine-rich Protein 2 (HRP-2) and Plasmodium Lactate Dehydrogenase (PLDH).

Certain haematological and biochemical test are done to investigate disease severity. These include, the packed cell volume, the haemoglobin level, platelet count, blood glucose level, liver function test, serum creatinine, arterial blood pH and serum lactate.

2.8. Management of malaria in pregnancy

2.8.1. Curative management

All cases of malaria in pregnancy are considered severe and treated with quinine in a health care unit. 500mg of a quinine salt are given in a dextrose infusion thrice daily, each lasting 4 hours. Oral quinine can replace this as soon as the patient feels better and should be continued for up to seven days. In case of a contra-indication to quinine, an artemisinin derivative could be administered parenterally.

Symptomatic treatment is given for symptoms that can complicate pregnancy, such as vomiting and pyrexia.

2.8.2. Preventive management

Owing to the consequences of malaria in pregnancy, prevention is of prime importance. The main objective of the National Malaria Control Programme (NMCP), which is in line with that of the Roll Back Malaria Initiative, is to reduce

by 50% the morbidity and mortality due to malaria in the vulnerable population made up of pregnant women and children below 5 years of age.

2.8.2.1. Parasite control

Intermittent Preventive Treatment (IPT) of malaria in pregnant women is recommended in Cameroon using Sulfadoxine/Pyrimethamine (SP). This was as a reason of widespread resistance to Chloroquine and the encouraging results with the use of SP in other countries. Pregnant women should receive 3 doses of SP as from the second trimester, with an interval of at least one month between the doses, in the absence of any contra-indication to the drug. It should not be given in the first trimester as it is teratogenic.

Regular prophylaxis is not recommended for the general population as this could lead to a loss in premunition or the development of resistant strains of the parasite.

2.8.2.2. Vector control

Several tools for the control of the vector population exist. Control strategies recommended by the NMCP are environmental sanitation (involving the clearing of bushes and the drainage of stagnant water around homes, which serve as breeding sites for mosquitoes) and the use of insecticide-treated bed nets (ITNs). One of the specific objectives of the NMCP is to have 60% of pregnant women sleep under ITNs by the year 2006.

Other vector control tools are the use of biological and chemical larvicides in breeding sites, especially in water scheme and irrigation projects and the use of residual insecticides.

2.9. Immunity to malaria

Like in most infectious diseases, humans develop immunity to malaria. Certain conditions also confer innate immunity to malaria. The innate immunity maintains parasitaemia to a certain threshold and the acquired immunity clears parasites within the blood (Kwiatkowski, 1995). The immunity developed after a malaria infection is not a sterile one, hence the rising need of prevention. The search for a malaria vaccine has therefore been at the centre of research in malaria in the past two decades. An anti-morbidity vaccine for each of the different risk groups found amongst those living in endemic areas would be of great benefit. An anti-infection vaccine aimed at protecting malaria-naïve travellers would also be beneficial.

2.9.1. Innate immunity

Innate defence mechanisms are the first line of defence against microbial invasion. They do not depend on any classical immunological memory; they are mediated mainly by cells of the myeloid lineage: neutrophils, eosinophils and mononuclear phagocytes. These cells phagocytose circulating parasites. This form of immunity interacts strongly with the other forms of the immune response. Cytokines (TNF-α, IFN- γ) released by T lymphocytes during acquired cell-mediated immunity enhance the activity of these cells. Cytokines released by elements of the innate system such as interleukine-2 (IL-2) released by antigen-presenting cells lead to antigen-specific T helper cell differentiation towards TH1 cells.

Natural killer (NK) cells are also involved in the lysis and elimination of infected cells. Besides these, proteins such as the complement components also play a role in the innate defence mechanism.

2.9.2. Acquired immunity

2.9.2.1. Cell-mediated immunity

This involves mainly T lymphocytes, which act by antigen-specific cytotoxicity. Antigen-specific cytotoxic T lymphocytes (CTLs) recognize peptides presented by class I Major Histocompartibility Complex (MHC I) antigens on the surface of target cells. CD8+ CTLs respond by clonal expansion that requires help from TH1 cells, a subset of CD4+ T lymphocytes.

TH1 cells also release inflammatory mediators such as IFN- γ that activate macrophage and neutrophil killing. Macrophages, NK cells, neutrophils and eosinophils also exhibit acquired cell-mediated immunity through antibody-dependent cellular cytotoxicity (ADCC) by increased phagocytosis after recognition of the Fc fragment of the antibody bound to the antigen. TH2 cells secrete cytokines for the antigen-specific activation of macrophages and B cells.

The role of the TH1 response seems to reduce the parasite density to a low level that can be controlled by the TH2 response. Kouontchou and others reported this in 2002; they found low levels of TH2 cytokines and high levels of TH1 cytokines in association with malaria severity. A balanced level of TH1/TH2 cytokines is required for an optimal immune response, since TH1 cytokines participate in both pathogenesis and protection.

In malaria, cell-mediated cytotoxic immunity is acquired especially to hepatic stages of the parasite. T cell recognition of antigens expressed on the surface of infected hepatocytes leads to the release of soluble immune mediators such as IFN- γ, resulting in intracellular death and the direct lysis of infected hepatocytes by CTLs.

2.9.2.2. Humoural immunity

2.9.2.2.1. Active humoural immunity

This involves the recognition of specific extracellular antigens for the production of antigen-specific antibodies. This follows antigen presentation

through MHC class II molecules on antigen-presenting cells. Antibody production proceeds through clonal antigen-specific B cell expansion, that for protein antigens require help from TH2 cells (a subset of CD4+ T lymphocytes).

Antibodies can disrupt cellular or microbial function, enhance microbial or macromolecule uptake by phagocytic cells. They also mediate macrophage, NK cell and neutrophil killing through ADCC. In malaria infection, antibodies are produced to blood stage antigens. Antibodies to these antigens will prevent binding interactions required for RBC invasion and enhance splenic clearance or complement-mediated lysis.

Merozoite antigens such as the ring-infected erythrocyte surface antigen (RESA), merozoites surface protein (MSP1 and MSP2) and the apical membrane antigen (AMA1) stimulate the production of antibodies which block invasion into erythrocytes, facilitate phagocytosis and agglutinate merozoites (Saul *et al.*, 2001). Erythrocytic growth stages also have antigens that stimulate specific antibody response, like the PfEMP1. Such antibodies will enhance splenic clearance, interfere with parasite growth and prevent endothelial adhesion.

Antibodies are also produced to sporozoite stage antigens such as the circumsporozoite protein (CSP), the principal surface antigen of sporozoites and thrombospondin-related adhesion protein (TRAP); such antibodies block sporozoite invasion of hepatocytes.

2.9.2.2.2. Passive humoural immunity

Passive immunity to malaria is acquired in utero by the transplacental transfer of immunoglobulin G (IgG) from the mother to the foetus. IgG passes through two cellular barriers: the syncytiotrophoblasts and the foetal endothelium. The transfer of maternal IgG to the foetus is receptor-mediated. The Fc region required for transport is known as the neonatal Fc receptor (FcRn). It is not expressed in foetal capillary endothelia and the mechanism of transport across this second barrier remains unknown (Story *et al.*, 1994; Simister *et al.*, 1996).

Among the different sub-classes of plasmodium falciparum specific IgG, IgG1 is the most efficiently transported and IgG4 is the least (Deloron *et al.*, 1997).

2.10. The effect of pregnancy on maternal immunity

During pregnancy, the immune system must accept the foetal allograft while maintaining defences against other assaults. Because of the potential immunologic conflict between the mother and its antigenically different foetus, some sort of immunomodulation is essential for the survival of the conceptus. Research in the past decade suggests that pregnancy entails a shift in the balance between the different arms of the maternal immune response, leading to less aggressive, but nonetheless competent forms of immunity.

2.10.1. The effect of pregnancy on innate defence

2.10.1.1. Polymorphonuclear leucocytes

The number of circulating polymorphonuclear (PMN) cells slightly increases in pregnant women, and depending on the activity measured, PMN activity either increases such as CD14 and CD64 (Sacks *et al.*, 1999) or is reduces (adherence, chemotaxis, bacterial killing) (Crouch *et al.*, 1995).

2.10.1.2. Macrophages/monocytes

Peripheral blood monocyte count also increases. Oestrogen induces an increased sensitivity of the bone marrow to the haematopoietic growth factor, macrophage colony-stimulating factor 1 (CSF-1) (Maoz *et al.*, 1985). Constitutive uterine production of CSF-1 also accounts for the elevated counts of blood monocytes (Bartocci *et al.*, 1986).

2.10.1.3. Complement/acute phase proteins

Complement components including C3 and C4 increase in normal pregnancy. This parallels that of other acute phase proteins such as ceruloplasmin and α_1-antitrypsin. It has been suggested that increases in such innate effectors compensate for decreases in the cell-mediated arm of immunity during pregnancy (Sacks *et al.*, 1999).

2.10.2. The effect of pregnancy on antibody-mediated immunity

Humoural immunity to infections can be assessed by responses, number of circulating B cells and the levels of immunoglobulin. There is neither a significant alteration in response to vaccine (Gill *et al.*, 1983) nor change in percentage of circulating B cells in pregnant women (Falkoff, 1987). There is however a reduction in IgG levels that can be attributed to haemodilution (Pedler *et al.*, 1995)

2.10.3. The effect of pregnancy on cell-mediated immunity

Cell-mediated immunity is suppressed during pregnancy, as symptoms of cell-mediated immune diseases such as rheumatoid arthritis reduce and grafts are prolonged. It has been reported that pregnancy produces a shift towards TH2 cells/cytokines, thus reducing the activity of the cell-mediated arm of immunity which requires help form TH1 cells/cytokines for effector functions.

The Pregnancy hormone, progesterone, has been observed to strongly bias T helper development towards TH2 (Piccinni *et al.*, 1996). This suggests a mechanism for the TH2 shift. Both local and systemic TH2 responses to paternal antigens are important to a successful human pregnancy. Studies in women with spontaneous abortion revealed decreased TH2 cytokine production by peripheral lymphocytes and decidual T cells (Hill *et al.*, Piccinni *et al.*, 1998).

2.11. *Plasmodium falciparum* antigens

Plasmodium falciparum has several antigens, the multiplicity of antigens owing to the several developmental stages of the parasite. Some antigens are today potential vaccine candidates. The leading candidates include the circumsporozoite protein (CSP), merozoites surface protein 1 (MSP-1), the apical membrane antigen 1 (AMA-1) and the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1).

2.11.1. Pre-erythrocytic antigens

- Circumsporozoite protein (CSP)

  CSP is the main sporozoite coat protein. It has a central area consisting of repeat amino acid sequences that are highly immunogenic. The central repeat region can elicit a B-cell immune response, producing antibodies that block sporozoites in culture. CSP was the first protein identified as a target of protective immune response directed against the infected hepatocyte.
- Other pre-erythrocytic antigens include:
  - Liver stage specific antigen 1 (LSA-1)
  - Thrombospondin- related anonymous protein (TRAP)

2.11.2. Erythrocytic phase antigens

- Merozoite Surface Protein 1 (MSP-1)

  It is a major surface antigen of merozoite and it is the best studied merozoite stage protein. During the invasion process several proteins of the 195-kDa MSP1 are shed. The C-terminus from which MSP-1 $_{19}$ and MSP-1$_{42}$ are derived has been shown to be the target of protective immune response (Holder et al 1998); also, antibodies against MSP-1 $_{19}$ can inhibit parasite growth.
- Ring-infected Erythrocyte Surface Antigen (RESA)

RESA is expressed on the surface of erythrocytes infected with ring stage parasites. It is also known as Pf/I55 because it has a molecular weight of 155 kDa.

- *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1). This is a parasite-derived ligand which mediates most of the adhesion phenomena of the parasite to different receptors expressed on vascular endothelial cells RBCs, and platelets. The protein is expressed on the surface of RBCs infected with late developmental stages of *P. falciparum*. This protein is encoded by a family of 40-50 var genes, mediating antigenic variation. The protective effect of antibodies against PfEMP-1 may be due to blocking of cytoadhesion (Staalso *et al.*,1998)
- Apical membrane antigen 1 (AMA-1)
  AMA-1 is an integral membrane protein located in the rhoptries and on the merozoite surface. The AMA-1 of *P. falciparum* is an 83 kDa polypeptide. It may be involved in merozoite release and invasion of erythrocytes (Peterson *et al.*, 1989)
- Other erythrocyte stage antigens include:
  - Serine repeat antigen (SERA) or serine-rich protein (SERP)
  - Erythrocyte-binding antigen (EBA-175)
  - Histidine-rich protein 2 (HRP-2)
  - Rhoptry-associated proteins (RAP-1 and RAP-2)

2.12. Strategies for malaria vaccine development

The development of a vaccine towards malaria seems to be a definite possibility despite the fact that there are no vaccines in standard use against parasitic infections and the fact that malarial infection does not induce solid immune protection towards future exposure. During the late 1970's induction of solid immune protection had been achieved in humans through the use of irradiated sporozoites. However, the irradiated sporozoites must be delivered through irradiated, infected mosquitoes, and this was an impractical and too expensive method for widespread use. Even though natural infection of malaria does not produce complete immunity towards the parasite, long-term exposure to

malaria has been shown to induce partially protective immune responses to malaria. In the partially immune, there are fewer and less dense parasitaemias, a reduction in malaria-related illness, significant protection from death, and antibody production towards erythrocytic stage parasites. An effective vaccine will probably need to incorporate multiple components that will induce an immune response towards the different stages of the malaria infection.

2.12.1. Pre-erythrocytic phase

One strategy towards the pre-erythrocytic stage is to target the parasite during the short span of time that the sporozoites are in the bloodstream. This sporozoite vaccine must induce the production of protective antibodies that will block and neutralize the sporozoites from invading liver cells. The other strategy is to target the sporozoites once they are inside the liver cells through the induction of CTLs that will destroy sporozoite-infected liver cells.

2.12.2. Erythrocytic phase

Another approach is to induce blocking antibodies towards the circulating merozoites, preventing them from infecting red blood cells. Once inside the erythrocytes, CTLs cannot be generated against them since red blood cells do not express MHC molecules on their surface. However, some malaria antigens are expressed on the surface of the infected RBCs toward which antibodies can be directed against and be used for opsonization and complement-mediated lysis. Also, it may be helpful to induce antibodies that block the infected erythrocytes from adhering to the lining of blood vessels. It is during the erythrocytic stage that illness associated with malaria occurs. There are strategies, called 'anti-disease' vaccines, towards the toxic products produced during this phase.

2.12.3. Transmission phase

There are also attempts to produce a 'transmission-blocking' vaccine. This approach targets the sexual stage gametocytes of plasmodium. The goal is to prevent the gametocytes from producing more sporozoites within the gut of the

mosquito vector, thus blocking the transmission of malaria. This vaccine does not prevent illness in an infected host, but it may be important to reduce the spread of malaria.

CHAPTER THREE: METHODOLOGY

3.1. Study Design

Retrospective cross-sectional study

3.2. Study Period

The study was carried out between September and December 2005.

3.3. Study population

Samples of plasma were randomly selected from plasma samples collected for the ICIDR/HIRE malaria project, which studied placental malaria in pregnant Cameroonian women between 1996 and 2001. In this project, the purpose was explained to each woman at the time of admission to hospital for delivery, irrespective of term. Consenting women provided information on themselves and information relative to their pregnancies, including age, number of pregnancies and outcome, the last menstrual period and the use of anti-malarial chemoprophylaxis during pregnancy. Length of gestation was estimated based on the number of weeks between the self-reported date of last menstrual period and date of hospital admission.

Samples collected included: peripheral blood, placental blood by the pool-biopsy method (Walker-Abbey *et al.*, 2005), a small piece of placental tissue and cord blood. In the laboratory, placental impression smears were made; thick and thin films were made from the maternal peripheral blood and the cord blood. All slides were air-dried and stained with Field's stain after prior fixation of the thin films with alcohol. Thick films were examined for parasites, when parasites were seen; the percent parasitaemia was estimated by determining the number of parasites per 2,000 erythrocytes.

Heparinized microhematocrit tubes were filled with a sample of the peripheral blood, centrifuged and the packed cell volume was determined. Samples of the peripheral and placental blood as well as samples of the cord blood were centrifuged, plasma removed, so the plasma and erythrocyte pellet stored in separate tubes at -20˚C.

The plasma used for this study were selected from several others stored at -20˚C, after prior consultation of the database for information collected during the project on each woman.

3.3.1. Inclusion criteria

Were included in this study women enrolled in the above project:

- Less than or equal to 20 years old with term delivery
- Greater than or equal to 25 years old with term delivery

3.3.2. Exclusion criteria

Excluded were:

- Women without peripheral plasma samples
- Women whose plasma were poorly conserved
- Women with incomplete clinical data
- Women with placental malaria, having a negative peripheral blood film.

3.4. Ethical considerations

- The project received approval from the Institutional Review Board of Georgetown University and the Ethical Committee of the Ministry of Public Health of Cameroon.
- Permission was obtained from the Directors of hospitals in which subjects were recruited.
- Only subjects who gave their consent were recruited for the project.

3.5. Setting

The clinical data and blood samples were collected from pregnant women at delivery at the Biyem-Assi District Hospital and the maternity of the Yaounde Central Hospital.

The parasitological and immunological analyses were done in the Immunology/Parasitology laboratory of the Biotechnology Centre of the University of Yaounde I.

3.6. Sample size

To determine the sample size, Lorentz formula was applied for each subgroup of young women with and without placental malaria as well as for older women with and without placental malaria.

$n= (Z_\alpha)^2 pq/d^2$

Where, n= sample size

p= prevalence of placental malaria

q= 1-p

α= level of confidence

d= level of precision

For α=0.05, Z= 1.96 and considering d=0.1 and p=19.9% (Tako *et al.*, 2005), n=61 for each subgroup.

3.7. Selection of samples

Women enrolled in the above project and fulfilling the inclusion criteria were sorted by age. All women less than or equal to 20 years were selected and classified into two groups, one with placental malaria, and the other without placental malaria. All women greater than or equal to 25 years with malaria were selected while those without were randomly selected from all the malaria-negative women aged 25 and above (who were the most represented in the lot of samples).

3.8. Materials

- **Equipment**

Polystyrene flat-bottomed microtiter plates (MaxiSorp, NUNC); 200µl multichannel pipette (FINNIPIPETTE); 0.5µl, 50µl, 200µl and 100µl pipettes (FISHERBRAND and DAIGGER); specialty tips (FISHERBRAND); 1.5ml flat top micro centrifuge tubes; racks; 15 and 50ml beakers (BIOLOGIX); plate sealers (COSTAR); semi-automated plate washer (NUNC); incubator (REVCO); pH

meter (DAIGGER); scale balance (ADVENTURER), magnetic stirrer (CORNING), aluminium foil paper, gloves, paper towels.

- **Reagents**
  - Sodium carbonate (SIGMA)
  - Sodium bicarbonate (FISHER SCIENTIFIC)
  - Sodium azide (SIGMA)
  - Potassium phosphate (SIGMA)
  - Sodium phosphate (SIGMA)
  - Sodium chloride (FISHER SCIENTIFIC)
  - Sodium hydroxide (EM SCIENCE)
  - Sulphuric acid (FISHERBIOTECH)
  - Skimmed milk
  - Tween 20 (FISHERBIOTECH)
  - Distilled water
  - Conjugate antibody (Horseradish peroxidase-labeled goat antihuman IgG (Kirkgaard and Penny Laboratories)
  - TMB substrate reagent set (BD BIOSCIENCES) containing substrate reagent A (hydrogen peroxide) and substrate reagent B (3, 3', 5, 5'-tetramethylbenzidine in an organic solvent).

- **Antigens and control sera**
  - Malarial extract antigen (MA): percoll-enriched late stage infected RBCs at 48% parasitaemia, kindly provided for by the Biology Department, Georgetown University, USA; was used as the test antigen for the anti-MA antibody ELISA.
  - Normal uninfected RBCS from a Caucasian who has never been exposed to malaria before were used as control antigen for the anti-MA antibody ELISA.
  - A peptide containing 5 copies of the conserved B cell epitope, EENV (Glutamic acid-Glutamic acid-Asparagine-Valine) of the ring erythrocyte surface antigen (RESA) of *P. falciparum* coupled to

bovine serum albumin (ANASPERC Inc.) was used for the anti-RESA antibody ELISA.

- Bovine serum albumin (SIGMA) was used as the control antigen for the anti-RESA antibody ELISA.
- A purified recombinant protein (expressed in *Escherichia coli*) containing 30 copies of NANP (Asparagine- Alanine- Asparagine-Proline) and 2 copies of NVDP (Asparagine-Valine-Aspartic acid-Proline) tetra peptide repeats of the circumsporozoite protein (CSP) fused with Leucine- Arginine served as capture antigen for the anti-CSP antibody ELISA.
- Positive control sera for each of the antigens. Were pools of Cameroonian plasma previously tested positive in comparison to WHO positive controls.
- Negative control sera. From Caucasians who have never been exposed to malaria before.

3.9. Methods

3.9.1. Preparation of buffers

The coating, blocking and diluting buffers were prepared as in the annex.

3.9.2. ELISA for plasma anti-MA total IgG level

- Standardization assays

  Preliminary titration assays were done to determine the optimal working concentrations of the different reagents. The following were obtained:

  - Malarial extract, 1:2000 dilution, giving $2.4*10^3$ IRBCs/µl from a stock of $4.8*10^6$ IRBCs/µl.
  - Normal RBCs, 1:200000 dilution, giving 5*10 NRBC/µl from a stock of $1*10^7$ NRBCs/µl.
  - Plasma samples, 1:2000
  - Conjugate antibody, 1:4000

- Test assay
    1. The test and control antigens were diluted as above in coating buffer. 100µl were dispensed into each well such that the odd rows had the test antigen, while the even rows had the control antigen. Each test well contained $2.4*10^5$ IRBCs and each control well had $5*10^3$ NRBCs. The plates were sealed and incubated overnight at +4°C.
    2. Each well was aspirated and washed 3 times by filling with wash buffer (400 µl) using a semi-automated plate washer. After the last wash, inverting the plate and blotting it against clean paper towels removed the remaining wash buffer.
    3. Plates were blocked by adding 200µl of blocking buffer into each well and incubating at 37°C for an hour.
    4. Plates were washed three times as in step 2.
    5. Plasma samples (test and controls) were diluted in the diluent and 100µl were dispensed into wells in duplicates. The first four wells of column 1 were left blank, the last occupied by the positive control. Two negative controls were used and they occupied the second column. The rest of the columns took two test samples each, such that each sample occupied two test and two control wells. Each plate had 20 test samples. The plates were sealed and incubated at 37°C for an hour.
    6. Plates were washed as in step 2, but for a total of 5 times and blotted against paper towels at the end of the last wash.
    7. The conjugate was diluted as above and 100µl dispensed into each well (except the blanks). The plates were sealed and incubated at 37°C for an hour.
    8. Plates were washed as in step 6.
    9. The enzyme substrate A was mixed with an equal volume of enzyme substrate B and 100µl of the mixture dispensed into each well. Plates were covered with aluminium foil to allow for colour development. The amount of colour developed is directly

proportional to the amount of substrate hydrolyzed and hence to the anti-MA antibody levels in plasma.

10. The reaction was stopped by adding 50µl of the stop solution (1M H_2SO_4) into each well 15 minutes later.
11. The optical density (OD) of each well was read at 450nm in the ELISA plate reader.
12. The background absorbance (mean OD of the blank wells) was subtracted from the OD of all the other wells. The net OD of each sample was got by subtracting the mean OD of the control wells from the mean OD of the test wells for the respective sample.
13. Cut off for negativity was set at the mean of all the negative controls plus 2 times the standard deviation of these negative controls. For the categorization, ODs between 1 and <2 cut off were considered as low positive, those between 2 and <4 cut off were considered medium positive and those ≥4 cut off were considered as high positive.

3.9.3. ELISA for plasma anti-RESA total IgG level

- Standardization assays

  Preliminary titration assays were done to determine the optimal working concentration of the different reagents. The following were obtained:

  - BSA-conjugated RESA peptide (2mg/ml), 1:100 (20µg/ml)
  - BSA, 20µg/ml
  - Plasma sample, 1:500
  - Conjugate, 1:2000

- Test assay

  1. The test antigen was diluted as above in coating buffer. 100µl were dispensed into the wells of the odd rows, while the wells of the even rows each had 100µl the control antigen (BSA) prepared to the

above concentration. Each test well contained 2µg of RESA peptide and each control well control well contained 2µg of BSA. The plates were sealed and incubated overnight at +4°C.

2. Each well was aspirated and washed 3 times by filling with wash buffer (400 µl) using a semi-automated plate washer. After the last wash, inverting the plate and blotting it against clean paper towels removed the remaining wash buffer.
3. Plates were blocked by adding 200µl of blocking buffer into each well and incubating at 37°C for an hour.
4. Plates were washed three times as in step 2.
5. Plasma samples (test and controls) were diluted in the diluent and 100µl were dispensed into wells in duplicates. The first four wells of column 1 were left blank, the last occupied by the positive control. One negative control was used and it occupied the first four wells of column 2, the remaining four being occupied by a test sample. The rest of the columns took two test samples each, such that each sample occupied two test and two control wells. Each plate had 21 test samples. The plates were sealed and incubated at 37°C for an hour.
6. Plates were washed as in step 2, but for a total of 5 times and blotted against paper towels at the end of the last wash.
7. The conjugate was diluted as above and 100µl dispensed into each well (except the blanks). The plates were sealed and incubated at 37°C for an hour.
8. Plates were washed as in step 6.
9. The enzyme substrate A was mixed with an equal volume of enzyme substrate B and 100µl of the mixture dispensed into each well. Plates were covered with aluminium foil to allow for colour development. The amount of colour developed is directly proportional to the amount of substrate hydrolyzed and hence to the anti-RESA antibody levels in plasma.

10. The reaction was stopped by adding 50µl of the stop solution (1M H_2SO_4) into each well 15 minutes later.
11. The optical density (OD) of each well was read at 450nm in the ELISA plate reader.
12. The background absorbance (mean OD of the blank wells) was subtracted from the OD of all the other wells. The net OD of each sample was got by subtracting the mean OD of the control wells from the mean OD of the test wells for the respective sample.
13. Cut off for negativity was set at the mean of all the negative controls plus 2 times the standard deviation of these negative controls. For the categorization, ODs between 1 and <2 cut off were considered as low positive, those between 2 and <4 cut off were considered medium positive and those ≥4 cut off were considered as high positive.

3.9.4. ELISA for plasma anti-CSP total IgG level

- Standardization assays

  Preliminary titration assays were done to determine the optimal working concentration of the different reagents. The following were got:

  - CSP antigen (1mg/ml), 1:500
  - Plasma, 1:500
  - Conjugate, 1:2000
- Test assay
  1. The test antigen was diluted as above in coating buffer. 100µl were dispensed into the wells. Each well therefore contained 0.2µg of the antigen. The plates were sealed and incubated overnight at +4°C.
  2. Each well was aspirated and washed 3 times by filling with wash buffer (400 µl) using a semi-automated plate washer. After the last wash, inverting the plate and blotting it against clean paper towels removed the remaining wash buffer.

3. Plates were blocked by adding 200µl of blocking buffer into each well and incubating at 37°C for an hour.
4. Plates were washed three times as in step 2.
5. Plasma samples (test and controls) were diluted in the diluent and 100µl were dispensed into wells in duplicates. The first 2 wells of the first column were left blank. The positive and negative controls occupied two wells each on column one. One plate had 45 test samples. The plates were sealed and incubated at 37°C for an hour.
6. Plates were washed as in step 2, but for a total of 5 times and blotted against paper towels at the end of the last wash.
7. The conjugate was diluted as above and 100µl dispensed into each well (except the blanks). The plates were sealed and incubated at 37°C for an hour.
8. Plates were washed as in step 6.
9. The enzyme substrate A was mixed with an equal volume of enzyme substrate B and 100µl of the mixture dispensed into each well. Plates were covered with aluminium foil to allow for colour development. The amount of colour developed is directly proportional to the amount of substrate hydrolyzed and hence to the anti-CSP antibody levels in plasma.
10. The reaction was stopped by adding 50µl of the stop solution (1M H_2SO_4) into each well 15 minutes later.
11. The optical density (OD) of each well was read at 450nm in the ELISA plate reader.
12. The background absorbance (mean OD of the blank wells) was subtracted from the OD of all the other wells. The net OD of each sample was got by calculating the mean of the test wells.
13. Cut off for negativity was set at the mean of all the negative controls plus 2 times the standard deviation of these negative controls. For the categorization, ODs between 1 and <2 cut off were considered as low positive, those between 2 and <4 cut off were

considered medium positive and those ≥4 cut off were considered as high positive.

3.10. Data analysis

A spreadsheet was created in MS Excel 8.0 and data was manually entered. Means and proportions were calculated. Student's t test was used to compare means and Chi square test to compare proportions between two groups with the help of the statistical software, Epi Info 6.04. Scatter plot diagrams were plotted and correlation coefficients calculated with the help of MS Excel 8.0. A 95% confidence interval was considered for statistical significance.

CHAPTER FOUR: RESULTS

4.1. Description of the study population

In this study, the plasma of 247 women were selected, 108 were from women ≤20 years old and 139 were from those ≥ 25. Extreme ages were 13 and 42 years. The mean ages for the two groups were 18.4 and 29.9 respectively.

4.1.1. Population distribution with respect to age and malaria status

Table 2: Population distribution with respect to age and malaria status

	Mal negative	Mal positive	Total
≤ 20	70	38	108
≥ 25	69	70	139
Total	139	108	249

4.1.2. Population distribution with respect to gravidity

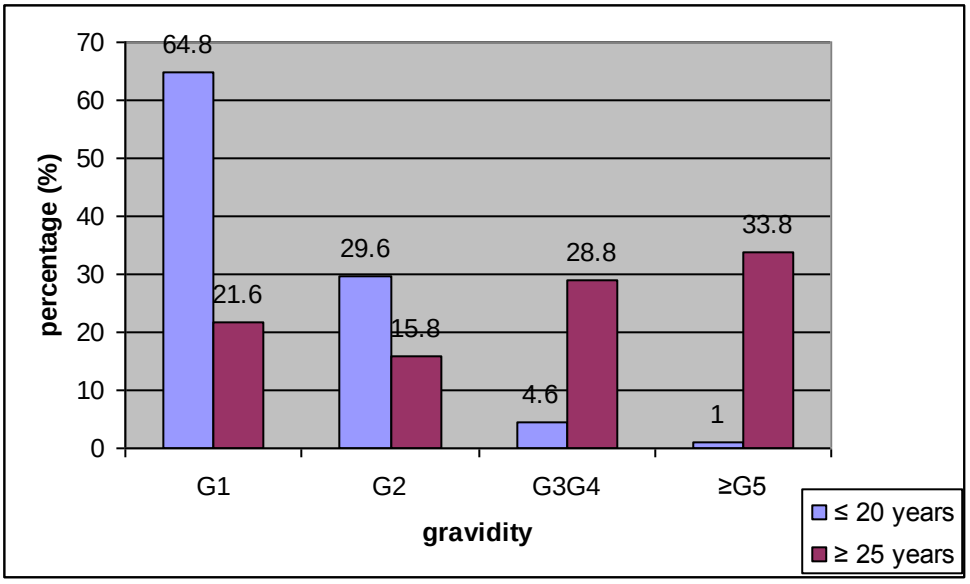

Figure 2: Population distribution with respect to gravidity

The age group of ≤ 20 was composed mainly of primigravidae (64.8%) while multigravidae were the most represented in the age group of ≥ 25; with women of gravidity 5 and above representing 33.8%.

4.1.3. Population distribution with respect to the use of chemoprophylaxis

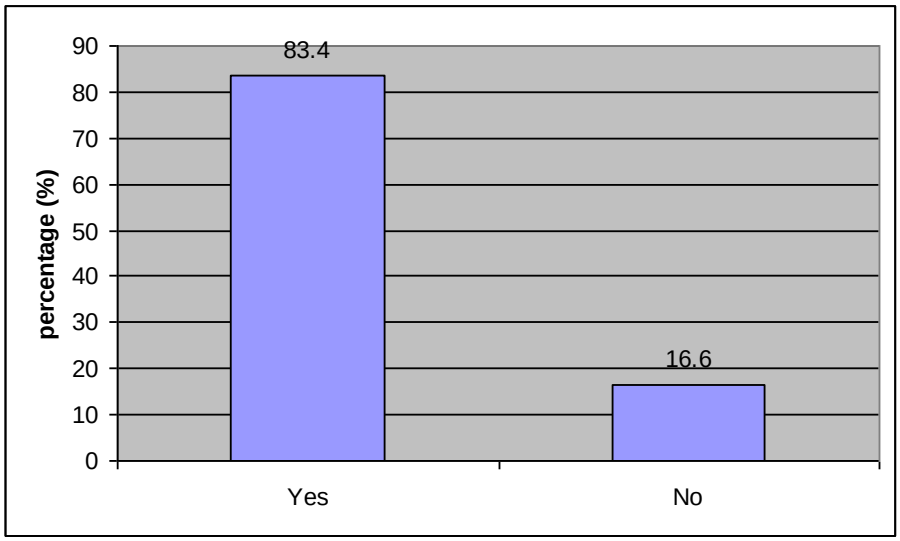

Figure 3: Population distribution with respect to the use of chemoprophylaxis

83.4% of the study population reported the use of a chemoprophylactic drug during pregnancy, while 16.6 reported no use.

Table 3: Drugs used for chemoprophylaxis in the study population

Drug	**Percentage** (%)
Chloroquine	45.6
Pyrimethamine	42.2
Proguanil	3.5
Others	8.7
Total	100

4.1.4. Distribution of chemoprophylaxis with respect to age group

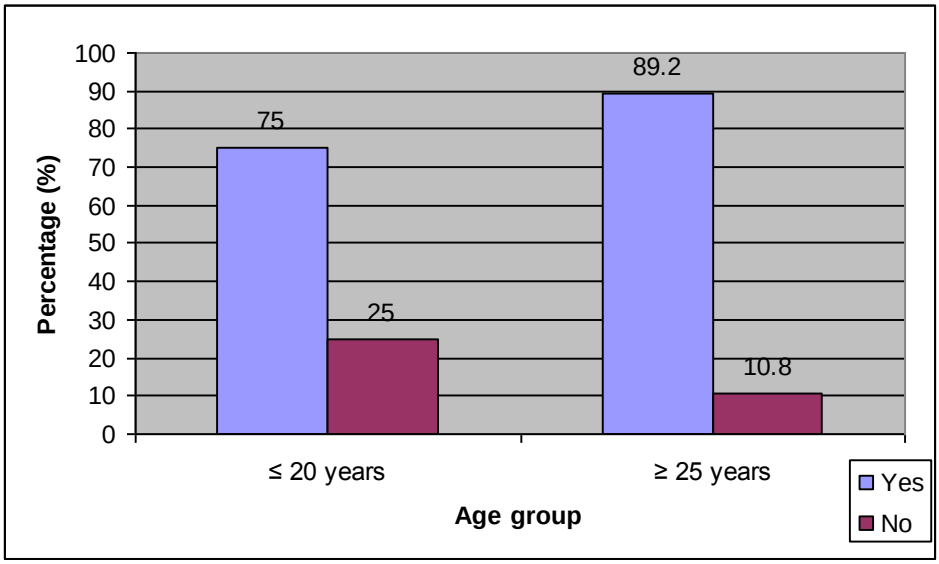

Figure 4: Distribution of chemoprophylaxis with respect to age group

The proportion of women who reported the use of a chemoprophylactic drug during pregnancy was higher in the age group of ≥ 25 years, n=139 (89.2%) compared to that in the ≤ 20 years, n=108 (75%). This difference had an X^2 value of 8.69 (p= 0.003).

4.2. Immunological data

4.2.1. Mean antibody level with respect to age group

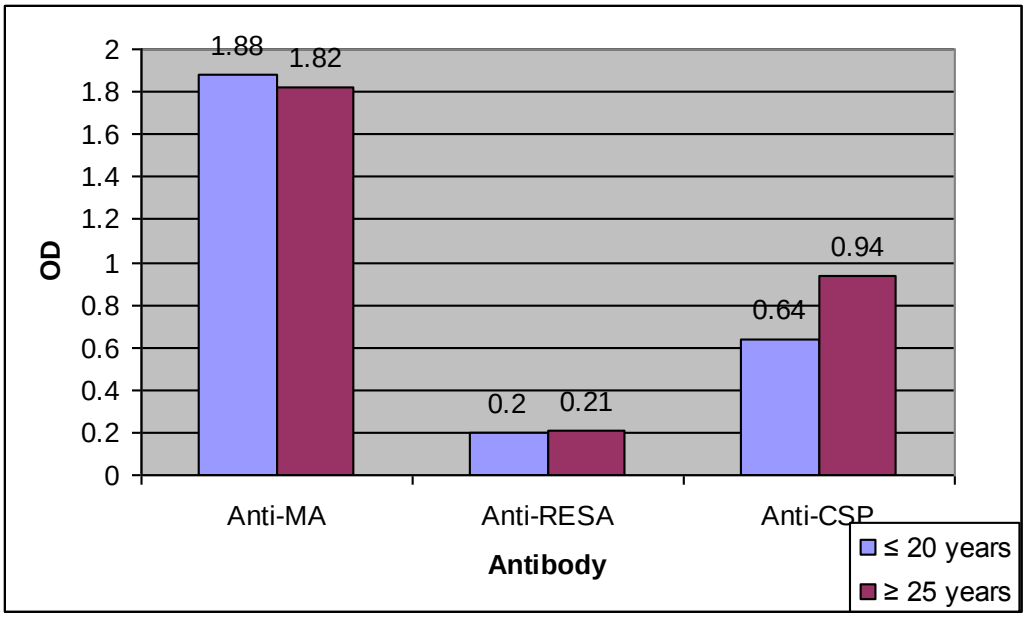

Figure 5: Mean antibody levels for the different antigens with respect to age group

There was no statistically significant difference between mean anti-MA and anti-RESA antibody levels between the ≤ 20 and the ≥ 25 age groups. For anti-CSP antibody levels, a statistically significant difference was observed (p=0.00006).

Table 4: Comparison of the mean antibody levels in the two groups

	≤ 20 (n= 108)	≥ 25 (n= 139)	p value
Mean anti-MA	1.88	1.82	0.398
Mean anti-RESA	0.2	0.21	0.604
Mean anti-CSP	0.64	0.94	0.00006

4.2.2. Correlation between anti-MA antibody level and age

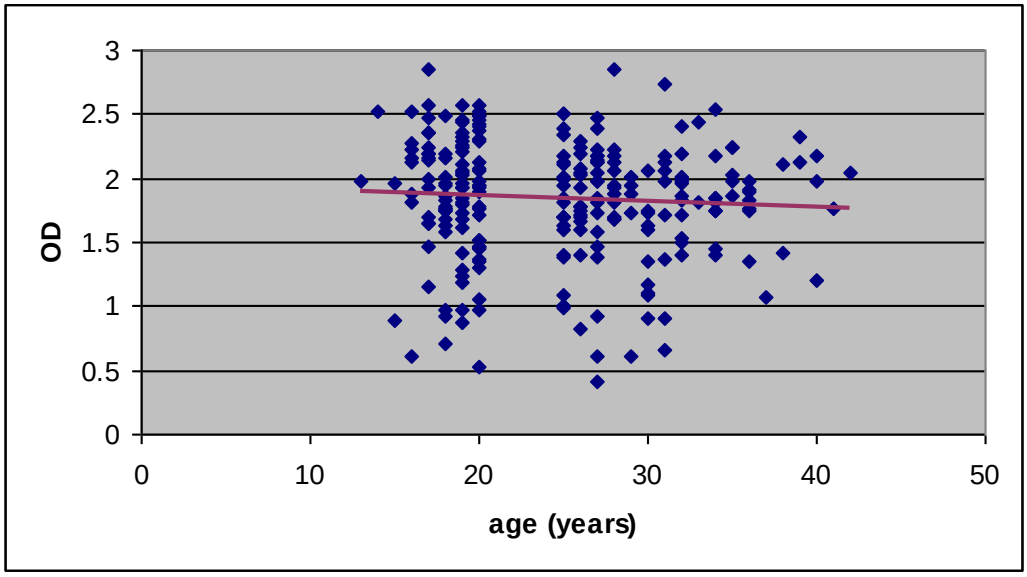

Figure 6: Scatter plot for anti-MA antibody level against age

Age had no effect on the levels of anti-MA antibodies. A non-significant ($p>0.05$) negative correlation was observed between anti-MA antibody levels and age ($r= -0.06$).

4.2.3. Correlation between anti-RESA antibody level and age

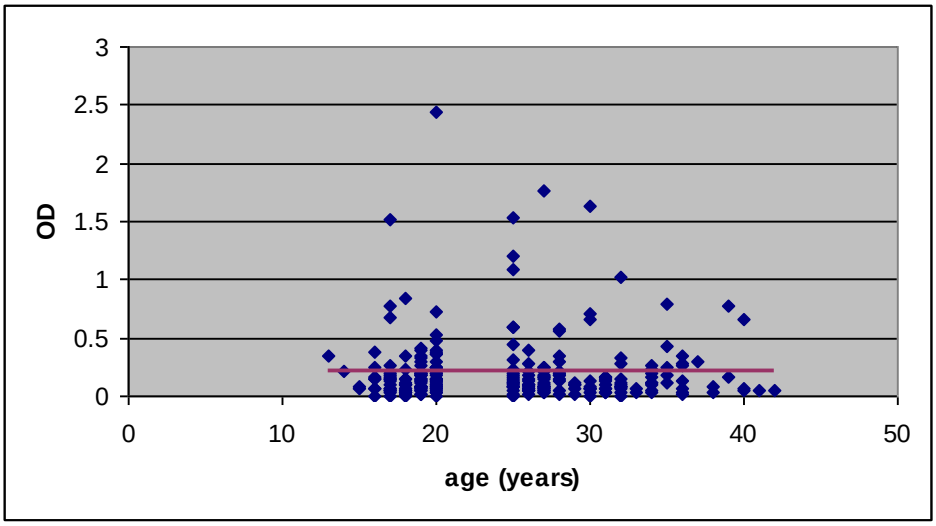

Figure 7: Scatter plot for anti-RESA antibody level against age

Age had no effect on the levels of anti-RESA antibodies. A non-significant ($p>0.05$) negative correlation was observed between anti-RESA antibody levels and age ($r= -0.05$).

4.2.4. Correlation between anti-CSP antibody level and age

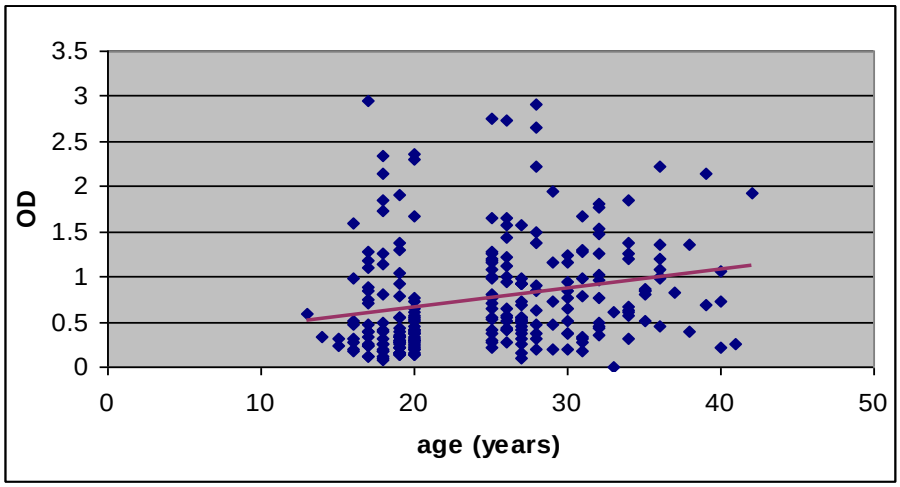

Figure 8: Scatter plot for anti-CSP antibody level against age

The immune response to CSP increased with age. A correlation coefficient of 0.21 was found. For a degree of freedom of 245, this positive correlation found was statistically significant ($p < 0.001$)

4.2.5. Correlation between anti-MA antibody level and placental parasitaemia

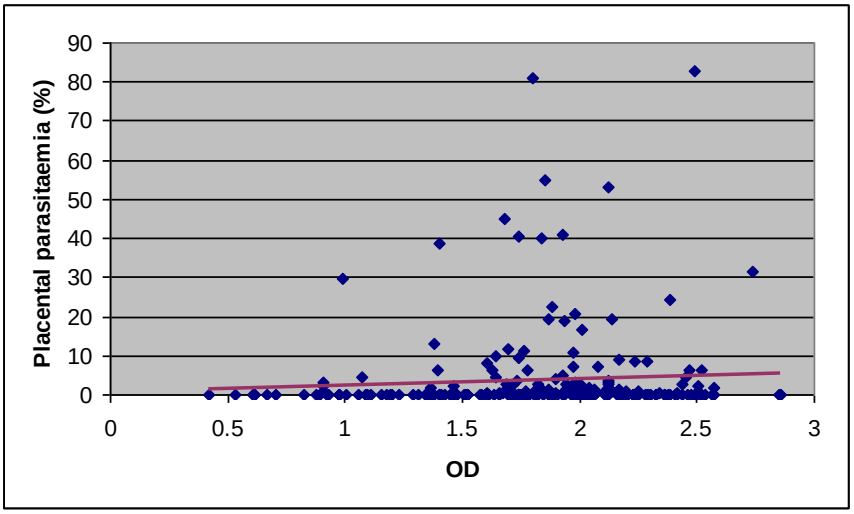

Figure 9: Scatter plot for placental parasitaemia against anti-MA antibody level

Antibody levels to MA had no effect on placental parasitaemia. A non-significant positive correlation was found between the two ($r= 0.07$, $p>0.05$).

4.2.6. Correlation between anti-RESA antibody level and placental parasitaemia

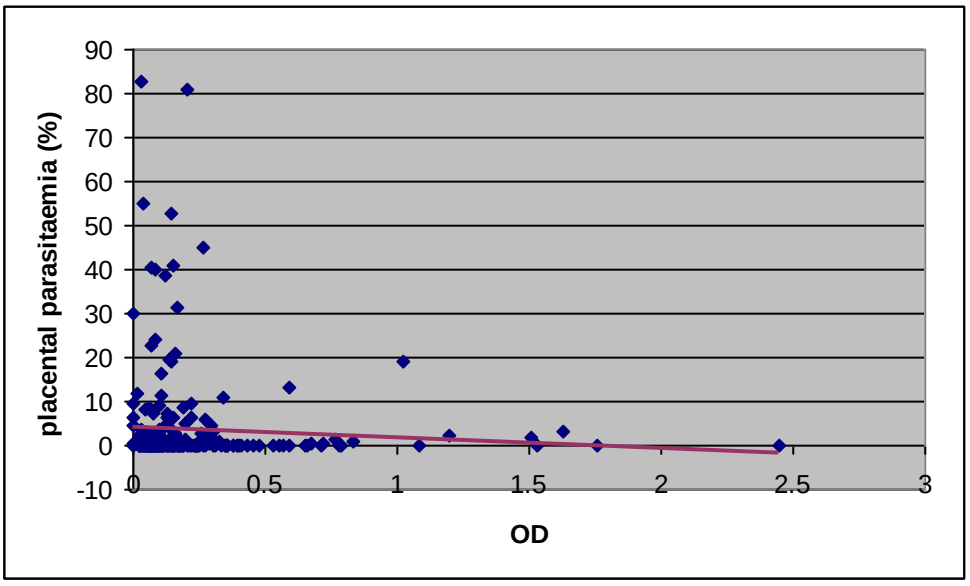

Figure 10: Scatter plot for placental parasitaemia against anti-RESA antibody level

Antibody levels to RESA had no significant effect on placental parasitaemia. A non-significant negative correlation was found between the two. ($r = -0.07$, $p > 0.05$).

4.2.7. Correlation between anti-CSP antibody level and placental parasitaemia

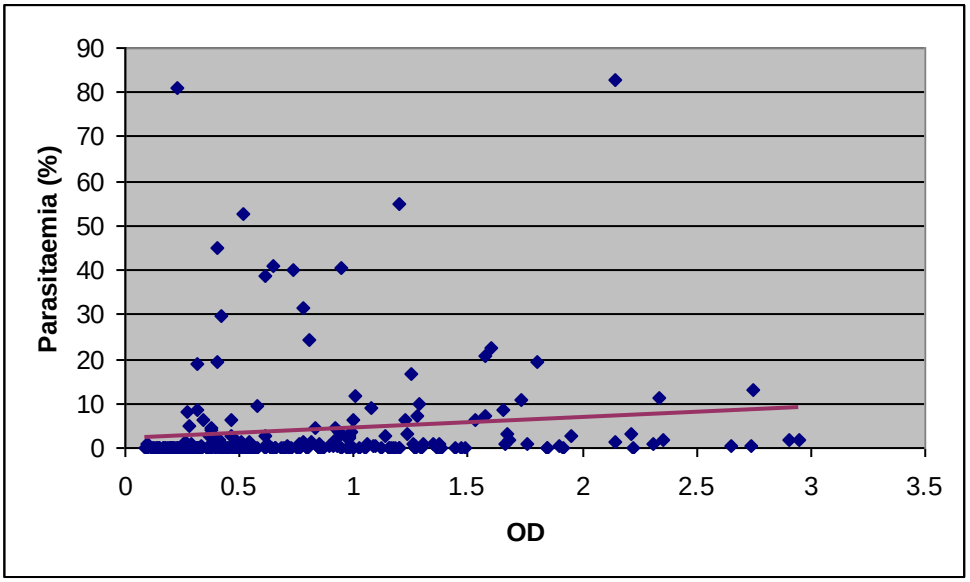

Figure 11: Scatter plot for placental parasitaemia against anti-CSP antibody level

A significant positive correlation was observed between placental parasitaemia and anti-CSP antibody level ($r= 0.13$, $p<0.05$).

CHAPTER FIVE: DISCUSSION

Malaria is endemic through out Cameroon, with the burden borne mostly by children below five and pregnant women. Pregnancy malaria is associated with poor outcome for both the mother and the baby. In areas where transmission is high, maternal death is rare, maternal consequences mostly represented by anaemia; the consequences for the baby being mainly IUGR and low birth weight.

There is a need for the prevention of the malaria in pregnancy, given the poor outcome usually associated with maternal malaria. Presently, preventive tools include environmental measures, the use of IPT and ITNs. However, these have their limitations and hence the need for the development of a potential vaccine against malaria cannot be over emphasized.

A detailed understanding of the mechanisms involved in the acquisition of natural immunity against the disease is primordial for the development of a vaccine. It was in the light of contributing to a better understanding of the acquisition of natural immunity against malaria that this study was carried out; to assess the influence of maternal age to humoural immunity against some malaria antigens.

5.1. The study population

A cross-sectional study was designed in which the plasma of 247 pregnant women were selected from previously collected samples, respecting the inclusion and exclusion criteria reported in the methodology. Two groups were formed on the basis of age, one less than or equal to 20 and the other greater than or equal to 25. Zhou et *al.* (2002) found women less than 20 to be more susceptible to malaria than older women; while Tako *et al.* (2005) found age less than 25 to be a risk factor for malaria in pregnant Cameroonian women.

5.1.1. The use of chemoprophylaxis

The samples used in this study were collected between 1996 and 2001 when chemoprophylaxis with chloroquine was recommended. However, not all the women reported the used of chemoprophylaxis: 83.4% reported the use as compared to 16.6%, who reported no use. Tincho in 2003 reported 74.1% use of chemoprophylaxis in Yaounde while Spencer and others (1987) reported 29.1% use in Kenya. These results show great disparity in the use of chemoprophylaxis. Spencer *at al.* conducted their study in a rural setting of Kenya, where the population is generally poor and access to health care and drug is limited; however, the use of chemoprophylaxis in cities is not 100%.

Faced with increasing resistance to chloroquine in several parts of Cameroon, the National Malaria Control Programme recommended the use of Sulfadoxine-pyrimethamine which even though not cheaper than chloroquine, has a better efficacy and less pill pressure. Besides, it is taken in the presence of the antenatal health care provider, assuring efficacy.

In this study, the proportion of women who reported the use of a chemoprophylactic drug during pregnancy was higher in the age group of ≥ 25 (89.2%) compared to that in the ≤20 years (75%). This difference was statistically significant ($p=0.003$, $\alpha=0.05$). Spencer *et al* (1987) also reported a similar disparity when they found that 43.9% of women 30 to 40 years of age were taking chemoprophylaxis compared to 25.1% of the younger women ($p<0.0005$).

5.2. Relationships between total IgG antibody response to MA, RESA, CSP and age

Indirect ELISA technique was used to determine the total plasma IgG to 2 asexual blood-stage antigens (MA and RESA) and 1 pre-erythrocytic stage antigen (CSP).

Mean antibody levels represented by their optical densities for each group were obtained. No statistically significant difference was observed when the means of anti-MA and anti-RESA antibody levels of the two groups were compared (p=0.398 and p= 0.604 respectively). Deloron *et al.* (1987) also found no relationship in antibody reactivity to the three synthetic peptides of the RESA molecule by age and gravidity.

Women ≤20 years had lower levels of anti-CSP antibodies compared to those ≥ 25 years (p=0.00006). Deloron *et al.* (1987) reported a similar increase with age.

Linear regression analysis of the respective optical densities against the age of each woman for the different antigens in this study revealed that age has no influence on levels of anti-MA and anti-RESA antibodies. A positive correlation coefficient was found between age and anti-CSP antibody levels (r= 0.12, p<0.001).

5.3. Antibody levels and placental parasitaemia

No association between anti-MA antibodies and placental parasitaemia was observed. Mvondo *et al.* (1992) reported similar results. However, Taylor *et al.* (2004) reported a significant correlation between high anti-MA levels to low placental parasitaemias, suggesting that unidentified antigens may play a role in parasite clearance.

There was no association between anti-RESA antibody levels and placental parasitaemia, a finding also reported by Taylor *et al.* in 2004. Previous studies have looked at the association between antibody levels to RESA and peripheral parasite densities. Deloron *et al.* (1989) in Kenya found no association between anti-RESA antibodies and peripheral blood parasite densities. This differs from the results obtained by Mvondo *et al*. (1992) who reported an inverse correlation between anti-RESA antibodies and peripheral parasitaemias in Cameroon (n=225). Astagneau *et al.* (1994) reported higher anti-RESA antibodies in women without parasites (n=235), compared to those with parasites (n=89) in Malawi, they concluded that antibodies to Pf155/RESA epitopes may

contribute to immune protection against the multiplication of blood-stage parasites.

A positive correlation was found between anti-CSP antibody levels and placental parasitaemia (r= 0.13). Studies have reported the absence of any correlation between anti-CSP antibody levels and parasitaemia in the placenta (Taylor *et al.*, 2004) and the peripheral blood (Deloron *et al.*, 1989; Mvondo *et al.*, 1992).

The positive correlation obtained in this study could be explained by the fact that infected women would produce high levels of antibodies, and high antibody levels would rather reflect infection than protection as antibodies to CSP are not effective against asexual blood-stage parasites.

CHAPTER SIX: CONCLUSIONS

At the end of this study which sought to assess the effect of age on immunity to malaria in pregnant women, the following conclusions can be drawn:

1. There is no association between age and anti-MA antibody levels as well between age and anti-RESA antibody levels.

2. Anti-CSP antibodies increase with age. Because anti CSP antibodies protect against re-infection, this variation with age observed, could explain why younger women are more susceptible to malaria.

3. There is no association between antibodies to asexual blood stage antigens tested (MA and RESA) and placental parasitaemia.

4. There is a positive correlation between anti-CSP antibody levels and placental parasitaemia. These antibodies are indicative of infection and play no role in parasite clearance.

CHAPTER SEVEN: RECOMMENDATIONS

Based on the conclusions of this study, the following recommendations are made:

1. CSP be considered as a vaccine candidate in a cocktail vaccine for pregnant women as this would prevent re-infections amongst the younger women who have lower levels of antibodies to this antigen.

2. Similar studies be carried out using different asexual blood stage antigens to evaluate their role in parasite clearance, hence the potential use as vaccine candidates.

3. Longitudinal studies be carried out to evaluate the kinetics of anti-MA and anti-RESA antibodies during pregnancy to better assess the benefit of these antibodies on levels of parasitaemia.

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APPENDIX

Preparation of buffers

- Coating Buffer

  For 1000ml of distilled water

  Na_2CO_3= 1.59g

  $NaHCO_3$= 2.93g

  NaN_3= 0.20g

- 10X Phosphate buffered saline (PBS)

  For 10 litres of distilled water

  KH_2PO4= 56g

  Na_2PO4= 225g

  NaCl= 672g

  Adjust pH to 7.2 using 5M NaOH

- 1X PBS

  Mix 1 part of 10X PBS with 9 parts of distilled water.

- Blocking buffer (PBS/10%milk/0.05%Tween 20)

  For 1000ml of PBS

  Milk= 100g

  Tween 20= 0.5ml

- Diluent for antibodies (PBS/1%milk/0.05% Tween 20)

  For 1000ml PBS

  Milk= 10g

Tween 20= 0.5ml

OR dilute blocking buffer tenfold in 0.05% Tween20/PBS solution.

- Washing buffer (PBS/0.05%Tween 20)
  PBS= 1000ml

  Tween 20= 0.5ml

- Stop solution (1M H_2SO_4)
  10.24g of 95.7% H_2SO_4 was dissolved in 100ml of distilled water.