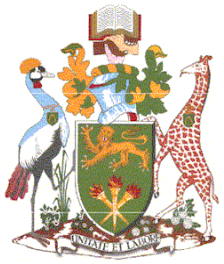

**UNIVERSITY OF NAIROBI**

# THE BABOON (*PAPIO ANUBIS*) –*PLASMODIUM KNOWLESI* MODEL FOR PLACENTAL MALARIA

**BY**

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A thesis in fulfillment of the requirement for the degree of Doctor of Philosophy in Biochemistry , School of Medicine, Biochemistry Department, University of Nairobi.

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## DECLARATION

This thesis is my original work and has not been presented in any other institution for any degree award or other qualifications.

**Onditi Faith Isdorah**

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## DEDICATION

This work is dedicated to my dear husband Abraham Agumba, daughter Joy Baraka and sons Isaac Jaden and Favor Nehemiah.

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Last but not least, special thanks go to NACOSTI and WHO for awarding grants that supported this study.

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LIST OF ABBREVIATION AND ACRONYMS

| | |
|---|---|
| ACT | Artemisinin-based combination therapy |
| ACUC | Animal care and use committee |
| ANOVA | Analysis of variance |
| CBA | Cytokine bead array |
| CD | Cluster of differentiation |
| CIDR1 | Cysteine-rich interdomain region 1 |
| $CO_2$ | Carbon dioxide |
| CS | Caesarian section |
| CSA | Chondroitin sulphate A |
| CSPG4 | Chondroitin sulphate proteoglycan 4 |
| DBL-γ | Duffy binding like-gamma |
| DC | Dendritic cells |
| EBP | Erythrocyte binding proteins |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme Linked Immunoabsorbent Assays |
| FACS | Fluorescent activated cell sorting |
| Fc | Fragment crystallization |
| FcRn | Neonatal Fc receptor |
| HAPLN1 | Hyaluronan and Proteoglycan Link Protein 1 |

| | |
|---|---|
| H&E | Haematoxylin and eosin |
| IEs | Infected erythrocytes |
| Ig | Immunoglobulin |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| IPR | Institute of primate research |
| IRC | Institutional review committee |
| IRS | Indoor residual spraying |
| IUGR | Intrauterine growth retardation |
| IVS | Intervillous space |
| Kg | Kilogram |
| LBW | Low birth weight |
| LLINs | Long lasting insecticide treated nets |
| mg | Milligram |
| MHC | Major histocompatibility complex |
| $N_2$ | Nitrogen |
| NaCl | Sodium chloride |
| NAI | Natural acquired immunity |
| NHP | Non-human primate |
| $O_2$ | Oxygen |
| OD | Optical density |

| | |
|---|---|
| Pan | *Papio anubis* |
| PBMC | Peripheral blood mononuclear cells |
| PCV | Packed cell volume |
| PD | Preterm delivery |
| PfEMP1 | *Plasmodium falciparaum* erythrocyte membrane protein 1 |
| PI | Post infection |
| PM | Placental malaria |
| PTD | Preterm delivery |
| RBCs | Red blood cells |
| RPMI | Roswell park memorial institute |
| SIV | Simian Immunodeficiency Virus |
| Tc | Cytotoxic T lymphocytes |
| Th | Helper T lymphocytes |
| Th1 | T helper 1 |
| Th2 | T helper 2 |
| TMB | 3,3',5,5'-Tetramethylbenzene |
| Trim | Trimester |
| uNK | Uterine natural killer |
| WHO | World Health Organization |

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## EXECUTIVE SUMMARY

Pregnant women and foetal well-being are compromised by malaria due to downregulation of normal maternal immune response during pregnancy. Consequently cell mediated immunity (Th1) is suppressed and as a result, the mother relies on humoral immunity (Th2) for protection against pathogenic infections like malaria. However, during placental malaria the maternal immune system is able to respond to the *Plasmodium* parasite infection.

This study sought to characterize the effect of placental malaria on pregnant baboons and infants born to placental malaria positive mothers experimentally infected with *Plasmodium knowlesi* blood stage parasites.

Chapters one, two and three of this thesis focus on general introduction, literature review and methodology respectively. Chapter 4 describes the methods for obtaining clinical, haematological and parasitaemia indices associated with placental malaria infection in non-immune baboons at different trimesters and the results obtained. Chapter five describes the methods for characterizing pathological features associated with placental malaria in *P. knowlesi* infected baboons and the results obtained. Chapter six describes the methods for determining the importance of placental malaria in protecting baboon infants against the progression of the disease via passive immunity while chapter seven describes the methods of quantifying T lymphocyte population and cytokine profile associated with *P. knowlesi* infection in pregnant baboons. Finally, chapter eight gives a general discussion and conclusions in the study while chapter nine describes the challenges of the study and the way forward.

Date generated from this study presents haematological and clinical indices associated with *P. knowlesi* malaria infection in pregnant Olive baboons. It also demonstrates pathophysiology of placental malaria, infant protection during *P. knowlesi* infection and cytokine profiles associated with placental malaria in Olive baboons. These features mimic placental malaria infection in *P. falciparum* pregnant women. Therefore, we propose that the baboon-*P. knowlesi* model is an ideal model for experimental therapeutics in management of malaria in pregnancy.

**ABSTRACT**

About 24 million pregnant women in sub-Saharan Africa are exposed to malaria in pregnancy, a condition referred to as placental malaria. This susceptibility is greatest in first and second pregnancies where malaria prevalence can be as high as 50% leading to low birth weight, intrauterine growth retardation, preterm delivery, anaemia and mortality. Comprehensive studies of placental malaria cannot be done in humans due to confounding variables that include mother's health status, inaccurate estimation of infection, inadequate tissue for analysis, patient compliance, socio-economic conditions and moral, ethical and financial limitations. Reproducible animal models are therefore required to overcome these challenges. The human-like structure of the baboon placenta and the cyto-adherent property of *Plasmodium knowlesi* have informed the choice for *baboon-P. knowlesi* model. Work in malaria laboratory at the Institute of Primate Research in Nairobi, Kenya, has demonstrated that baboons are susceptible to placental malaria and can therefore be useful a model for the study of placental malaria and pathophysiology of the disease in humans. This study sought to validate and apply baboon-*P.knowlesi* model of placental malaria. In order to determine haematological and clinical indices associated with placental malaria, twelve baboons were acquired and randomly grouped as control, first, second and third trimester with three baboons in each group. Apart from the controls, the rest of the animals were experimentally infected with $2x10^5$ *P. knowlesi* blood stage parasites, clinical changes observed, whole blood samples obtained and parasitaemia profiles observed from day two post infection. The animals were treated one week post infection with Pyrimethamine (10mg/Kg body weight) for three days and left to deliver at term. To determine pathogenesis of placental malaria, ten pregnant baboons were acquired and randomly grouped as

three controls and seven experimentals. Their placentas were harvested through caesarean section for histopathological analysis. Treatment was initiated on the day of caesarean section. To determine the effect of placental malaria on infant immunity to malaria, three infants born to placental malaria positive mothers and three from placental malaria negative mothers were experimentally infected with *P. knowlesi* malaria parasite, parasitaemia profile monitored and serum samples obtained for immunoglobulin G enzyme linked immunoabsorbant assay. Immunological mechanisms associated with *P. knowlesi* infection was obtained by analyzing mononuclear cells and serum samples obtained from peripheral blood, cord blood and placental blood using fluorescent activated cell sorting and cytokine bead array technologies. Data generated from this study demonstrated predictive values of clinical and haematological parameters associated with placental malaria in *P. knowlesi* infected baboons. Infiltration of infected *P. knowlesi* parasitized red blood cells and inflammatory cells in the placenta of non-immune baboons were associated with pathology of placental malaria. This study also demonstrated protective immunity against placental malaria in baboons. These findings validate the baboon-*P. knowlesi* model of placental malaria and we propose that this model be used for experimental therapeutics in management of malaria during pregnancy.

Key words: **Baboons model, Placental malaria, *Plasmodium knowlesi.***

# CHAPTER 1: GENERAL INTRODUCTION

## 1.1 Background information

Globally, malaria infection is one of the major public health concerns. As a disease it affects red blood cells (RBCs) and is transmitted by female *Anopheles* mosquitoes. In Africa, *Anopheles gambiae* and *A. funestus* are the main vectors of this disease. The protozoan parasite, *Plasmodium* is responsible for malaria. *Plasmodium falciparum, P. ovale, P. malariae* and *P. vivax* cause the disease in humans. *Plasmodium knowlesi*, a primate malaria causing parasite, has recently been reported to infect humans in Southeast Asia (Moyes *at al.* 2014; Cox-Singh and Singh 2008; Daneshvar *at al.* 2009).

As a disease, malaria is the leading cause of morbidity and mortality world-wide. In 2013, there were approximately 198 million cases and 584,000 malaria deaths worldwide. Of these estimated deaths, 90% occurred in sub-Saharan Africa with children under 5 years of age and pregnant women being most vulnerable ("WHO | Factsheet on the World Malaria Report 2014" 2015). Children are at risk because they lack developed immune systems to protect against the disease. Although clinical outcome of malaria infection depends on various factors attributed to parasite, host, geographical area and social factors (Weatherall *at al.* 2002), regions of stable malaria transmission are characteristic of asymptomatic infections that usually persist for long periods at low densities resulting in a protective partial-immunity against *P. falciparum* acquired during the first 10 to 15 years of life. ("WHO | World Malaria Report 2011" 2014; Rowe *at al.* 2006).

Pregnant women are vulnerable to malaria infection due to hormonal changes and reduced immunity during pregnancy (Nada K Bayoumi *at al*. 2009). Susceptibility is even higher if the pregnant woman is below the age of 20, primigravidae, infected with human immunodeficiency virus (HIV), or has never been exposed to malaria before such as pregnant travelers from non-endemic areas (Mount *at al*. 2004). The depression of cell-mediated immunity in pregnant women allows for the foetal allograft to be retained by the mother. This in turn interferes with resistance to various infectious diseases which include malaria (Meeusen, Bischof, and Lee 2001). Cellular immune responses to *P. falciparum* antigens are also depressed in these women leading to accumulation and survival of parasites in the intervillous space (IVS) of placenta (Seal, Mukhopadhay, and Ganguly 2010).

Globally, it is estimated that 25 million women are exposed to malaria infection every year, and therefore at risk (N K Bayoumi *at al.* 2009; Coulibaly, Gies, and D'Alessandro 2007; Desai *at al*. 2007; Steketee *at al*. 1996). Malaria during pregnancy leads to poor birth and maternal outcomes. Poor birth outcomes include low birth weight (LBW) that results from intrauterine growth retardation (IUGR), abortion and still birth while poor maternal outcomes consists of anaemia and mortality (Falade *at al*. 2010; Meeusen, Bischof, and Lee 2001; Steketee *at al*. 2001a).

Molecular and cellular events that occur during the life cycle of *Plasmodium* influence severity of disease. Simultaneous binding of parasites to several receptors, to uninfected erythrocytes (UEs) by rosetting, and clumping of infected erythrocytes through platelets are associated with pathogenesis of malaria, thereby affecting many tissues and organs such as brain, spleen, lungs,

liver, heart, and placenta. To date, various interventions have been used in the fight against malaria with the aim of controlling the impact of this disease. These interventions include: case management of infected patients through proper diagnosis and treatment, prevention of infection through vector control, and, disease prevention by prophylactic drugs. These interventions have had various shortcomings. In fact, the world health organization (WHO) recommends that persons suspected of having malaria be diagnosed and treated with effective drugs within 24 hours of the onset of symptoms. However, many patients in malaria endemic areas have no access to health care within the 24 hour period. This is mainly due to poverty, inadequate health, infrastructure and ignorance. On the other hand, vector control suffers from inadequate resources and larval resistance to larvicidal compounds. Similarly, the use of antimalarial drugs has led to resistance to affordable drugs ("WHO | World Malaria Report 2011" 2014; "WHO | World Malaria Report 2013" 2014a)("WHO | World Malaria Report 2011" 2014; "WHO | World Malaria Report 2013" 2014b).

In order to effectively manage this disease, placental malaria (PM) must be ultimately understood in humans. So far, characterization of this disease in adult humans and infants has been based on descriptive findings. Because of ethnical reasons, only a few reports have included a histopathological examination of the placenta from infected women (Davison *at al*. 2000). Research on PM in non-human primates (NHPs) has also been carried out over the years. Interestingly, these studies have been based on animal species whose reproductive system is different from that of humans. There is therefore a need to this condition in detail using an animal model whose reproductive system and menstrual cycle is similar to humans. The Olive baboon (*Papio anubis*) is an ideal candidate for this (Chai *at al*. 2007).

In this study, validation and application of the Olive baboon-*P. knowlesi* model of PM was explored by determining the pathophysiology of the disease through experimental infection of pregnant baboons and their infants with *P. knowlesi* malaria parasites. Since baboons have been used in biomedical research since 1927, there is enough baseline data which shows close parallelism to humans. The reproductive endocrinology of the female baboon is also similar to that of female humans. Its menstrual cycle is 28-30 days and its gestation period 27 weeks. Hormonal profiles between baboons and humans are comparable (Khan-Dawood and Dawood 1998). In addition, baboons are fully susceptible to experimental infection by *P. knowlesi* leading to either severe malaria or controlled parasitaemia that results in mild infection (Ozwara *at al.* 2003).

### 1.2 Problem statement

In order to develop an intervention strategy against PM, there is need to understand the underlying mechanism of the disease, including, normal physiology, immunology, biochemistry, impairment of maternal foetal exchange and poor birth outcomes which at the moment are not well understood. Although PM has recently attracted many research efforts, the studies have had shortcomings due to confounding variables such as mother's health status, inaccurate estimation of gestation age, inadequate tissues for analysis, socio-economic conditions, moral and ethical limitations (Matteelli *at al.* 1997). In addition, sequestration of *Plasmodium* infected erythrocytes in the placenta have not been convincingly demonstrated in the existing monkey models of malaria. Similarly, clinical and epidemiological observations suggest that the immunity of blood

stage *P. falciparum* is somehow initiated *in utero* but direct examination of the foetal immune responses to malaria antigens before birth is not feasible and is unacceptable in humans. On the other hand, studies of analogous responses in humans are logistically challenging and limited to examination of humoral and cellular immune responses in cord blood, which reflects the pool of circulating foetal lymphocytes at birth.

Therefore, if an ideal experimental system that would facilitate further studies of malaria in pregnancy is not developed, it will be relatively difficult to manage, control and prevent malaria associated complications and consequential social and economic burden (Sachs and Malaney 2002).

In this study, the baboon- *P. knowlesi* model of PM was validated and tested with the view to generate vital information that can be used in developing effective management strategies thereto.

### 1.3 Choice of the baboon as a study model

The ultimate goal of all biomedical research is to obtain a clear understanding of the normal physiology, immunology and biochemistry of disease processes that are usually of great relevance to humans, and malaria is not an exception. Although much has been learned from studies of malaria during pregnancy in humans, progress has been limited due to lack of a suitable animal model (Davison *at al*. 1998).

Baboons in this case are ideal because they have a similar host-pathogen interaction and a reproductive system that is physiologically similar to that of humans. They adopt well in captivity and have a menstrual cycle of 28 days and a gestation period of 180 ± 7 days (D'Hooghe *at al.* 2008; Chai *at al.* 2007). They are also susceptible to experimental infection by *P. knowlesi* parasites. This parasite is similar to *P. falciparum* with the exception of duration in the life cycle (48 to 72 hours for *P. falciparum*; 24 hours for *P. knowlesi*) (Weatherall *at al.* 2002). *Plasmodium knowlesi* is also a cytoadherent parasite (Ozwara and LUMC, 2005) and is likely to sequester in the placenta. Infection of pregnant baboons with *P. knowlesi* can therefore be expected to produce an experimental system that will facilitate further pathophysiological studies of malaria in pregnancy. This will undoubtedly contribute valuable data that can be used in the development of preventative, control and therapeutic measures against placental malaria in humans.

### 1.4 Justification of the study

Because of the social and economic problems associated with malaria globally, there is need to develop sound management strategies for the disease. This would be initiated by a clear understanding of the biochemical, physiological and immunological mechanisms associated with pathophysiology of malaria in pregnancy. Ethically, it is not possible to terminate pregnancy and perform continuous blood sampling in pregnant women for experimental purposes. In addition, the simian malaria parasite, *P. knowlesi* can infect humans via mosquito bites. People infected with the *P. knowlesi* parasite can suffer severe disease and death yet this disease has often been

misdiagnosed as a different malaria type and its geographical distribution is largely unknown. Therefore, this study sought to test and validate the baboon-*P. knowlesi* model of PM.

## 1.5 Research questions

1. What is the effect of PM in baboons infected with *P. knowlesi* H strain at different trimesters?
2. What are the underlying mechanisms leading to the pathogenesis of PM in *P.knowlesi* infected baboons?
3. Is malaria infection mediated *in utero*? If so, how?
4. What are the immunological mechanisms associated with *P.knowlesi* malaria infection in pregnant baboons and their infants?

## 1.6 Research hypothesis

### 1.6.2 The null hypothesis ($H_o$)

The baboon model of *P. knowlesi* PM infection is not similar to PM in humans in terms of pathology, immune profile, placental parasite sequestration and compromised foetal/infant development.

### 1.6.2 The alternative hypothesis ($H_a$)

The baboon model of *P. knowlesi* PM infection is similar to PM in humans in terms of pathology, immune profile, placental parasite sequestration and compromised foetal/infant development.

## 1.7 Objectives

### 1.7.1 General objective

This study sought to validate and apply the baboon-*P. knowlesi* model of PM by determining the physiological, biological, and immunological changes associated with PM in *P.knowlesi* infected pregnant baboons and their infants.

### 1.7.2 Specific objectives

The study aimed to accomplish the following objectives by December 2015;

1. To determine the clinical, haematological and parasitaemia profiles associated with PM in the baboon-*P. knowlesi* model at different trimesters.
2. To characterize the pathogenesis of PM in the baboon-*P. knowlesi* model.
3. To detrmine the effect of PM on infant protection againt *P. knowlesi* malaria infection.
4. To investigate the immonological mechanisms associated with *P. knowlesi* infection in pregnant baboons and their infants.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 Malaria epidemiology and disease burden

Malaria is a vector-borne infectious disease caused by unicellular parasites of the genus *Plasmodium* (Hafalla, Silvie, and Matuschewski 2011). These obligate intracellular parasites have the unique capacity to infect and replicate within erythrocytes, which are terminally differentiated host cells that lack antigen presentation pathways. Prior to the cyclic erythrocytic infections that cause the characteristic clinical symptoms of malaria, the parasite undergoes an essential and clinically silent expansion phase in the liver. By infecting privileged host cells, employing programs of complex life stage conversions and expressing varying immunodominant antigens, *Plasmodium* parasites have evolved mechanisms to down modulate protective immune responses against ongoing and even future infections. Consequently, anti-malaria immunity develops only gradually over many years of repeated and multiple infections in endemic areas. The identification of immune correlates of protection among the abundant non-protective host responses remains a research priority. Understanding the molecular and immunological mechanisms of the crosstalk between the parasite and the host is a prerequisite for the rational discovery and development of a safe, affordable, and protective anti-malaria vaccine (Hafalla, Silvie, and Matuschewski 2011).

Different species of *Plasmodium* infects a large range of vertebrate hosts (birds, reptiles, and mammals) and are largely distributed all over the world (Mayor 2008). *Plasmodium* species that infect birds such as *P. gallinaceum*, are found in much colder climates than human malaria.

*Plasmodium berghei*, *P. chabaudi*, *P. vinckei* and *P. yoelii* cause malaria in rodents. The five species of *Plasmodium* that infect humans are found mainly in the tropics, and seasonally in the sub-tropics. Four of the species (*P. falciparum, P. vivax, P. ovale, and P. malariae*) mainly infect humans only, but can also be found occasionally in other primates, such as chimpanzees and gorillas. The fifth species, *P. knowlesi*, is mainly considered a malaria of macaque monkeys in Southeast Asia, but can also infect humans ("WHO | World Malaria Report 2011" 2014; Mayor 2008; White 2008).

Malaria distribution is based on vector distribution and climatic factors such as temperature, humidity and rainfall. Although malaria is mainly transmitted in tropical and subtropical areas with the highest transmission occurring in sub-Saharan Africa, these mosquitoes are still found in many parts of the world. In cooler regions, transmission is less intense and more seasonal. In such climatic areas, *P. vivax* is more prevalent because it is more tolerant of lower ambient temperatures and is common in Asia, Latin America, and in some parts of Africa ("WHO | World Malaria Report 2009" 2014). *Plasmodium falciparum* is highly distributed in tropical and subtropical areas, *P. ovale* is found mostly in Africa (especially West Africa) and the Islands of the Western Pacific while *P. knowlesi* is prevalent in Southeast Asia ("WHO | World Malaria Report 2013" 2014a; "WHO | World Malaria Report 2011" 2014; "WHO | World Malaria Report 2009" 2014).

Malaria is possibly the most serious infectious disease of humans since it is a major cause of mortality and morbidity in both tropical and sub-tropical regions of the world (Figure 1). It

infects 5–10% of the world's population, with 300–600 million clinical cases and more than 2 million deaths annually (Figure 1)  (Murray *at al*. 2012). The overall pattern of this disease depends on the age and previous immunological experience of the host. In areas of high malaria transmission, the burden of disease in borne by infants, young children and pregnant women (Schofield and Grau 2005).

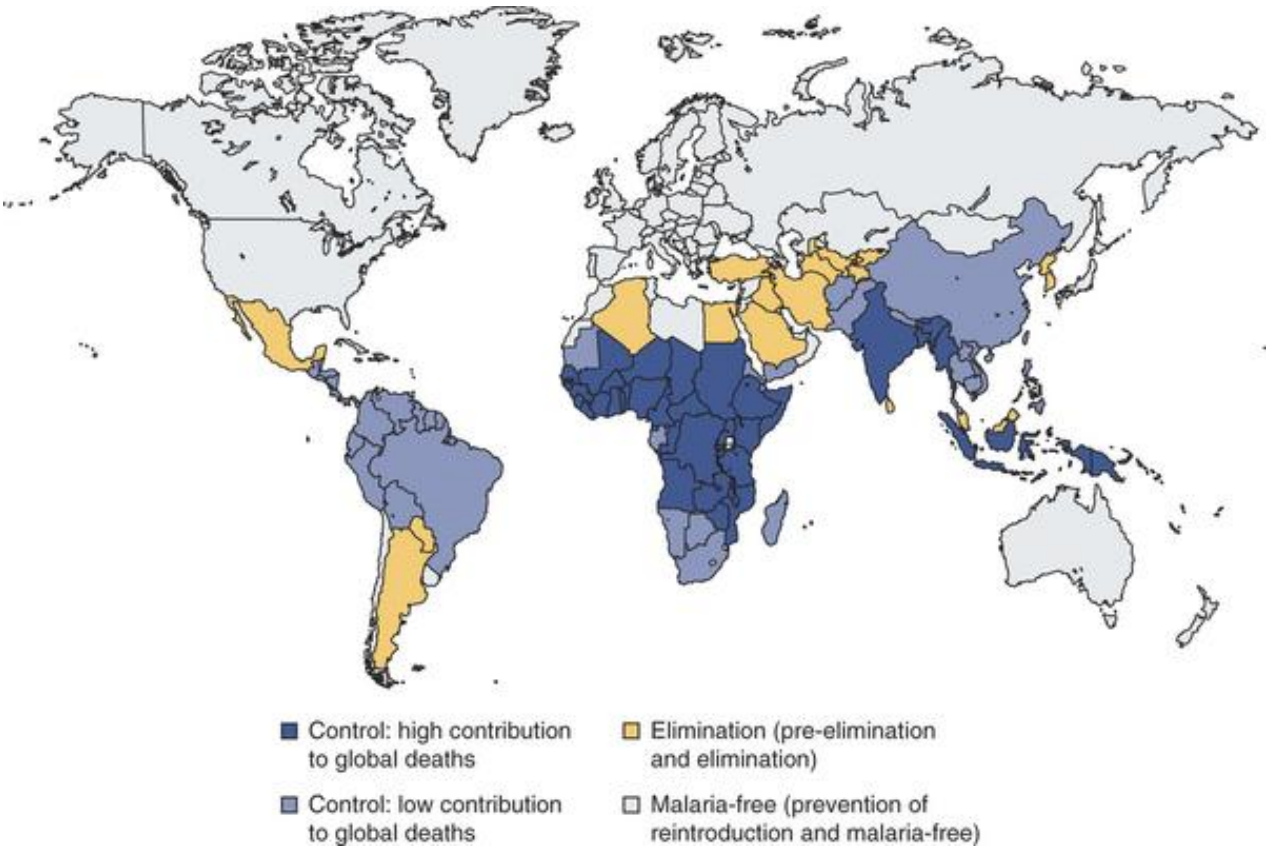


**Figure 1: The distribution of malaria worldwide (Alonso and Tanner 2013)**

## 2.2 Life cycle of *Plasmodium*

*Plasmodium* parasite encode for at least 5600 genes and have a complex life cycle that includes intracellular asexual growth within vertebrate hepatocytes (pre-erythrocytic stages) and erythrocytes ( blood or erythrocytic stages) ( Miller 2014). Sexual differentiation is initiated in the vertebrate host while fusion of gametocytes and further parasite propagation via sporogony occurs in the mosquito vector (Hafalla, Silvie, and Matuschewski 2011). The cycle begins when a female *Anopheles* mosquito bites a human host in search of a blood meal to produce eggs. The parasite is transferred through the mosquito saliva to the human host as the mosquito takes its blood meal. After infecting its human host, the parasite in sporozoites form travels through the blood stream to the liver and invade hepatocytes. They then mature into preerythrocytic (exo-erythrocytic) schizonts which rapture to release merozoites. This period varies from one species of parasite to another. Some species like *P. vivax*, *P. ovale* and *P. cynomolgi* have a dominant stage called the hypnozoite stage where parasites remain dormant in the liver for a while before they develop into mature schizonts. As a result relapses may occur by invading the bloodstream weeks or even years later. After this initial replication in the liver (preerythrocytic /exoerythrocytic schizogony), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic shizogony). The merozoites which are released into blood stream invade erythrocytes (red blood cells) where they develop through ring, trophozoite and erythrocytic schizont stages which then rapture to release merozoites that are capable of invading other erythrocytes. Erythrocyte invasion by merozoites is dependent on interactions of specific receptors on erythrocyte membranes with ligands on the surface of merozoites. These blood

stage parasites cause the illness and symptoms associated with malaria (Miller 2014; Hafalla, Silvie, and Matuschewski 2011).

Some of the merozoite infected blood cells leave the asexual replication and instead develop into sexual forms; macro-gametocyte (female) and micro-gametocyte (male) that circulate into the blood stream. When a mosquito bites the host for a blood meal, it ingests the gametocyte to begin the sporogonic cycle. Mature macro-gametocytes, taken into midgut of *Anopheles* mosquito, escapes from erythrocytes to form macro-gametes while the micro-gametocytes exflagellate to form motile micro gametes after a few minutes in the mosquito midgut. When the micro-gamete fertilizes a macro-gamete, a zygote is produced. The zygotes in turn become motile and elongated (ookinetes) as they invade the midgut wall of the mosquito developing into oocysts . The oocysts grow, rupture, and release sporozoites, which make their way to the mosquito's salivary glands (Figure 2). Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle (Miller 2014).

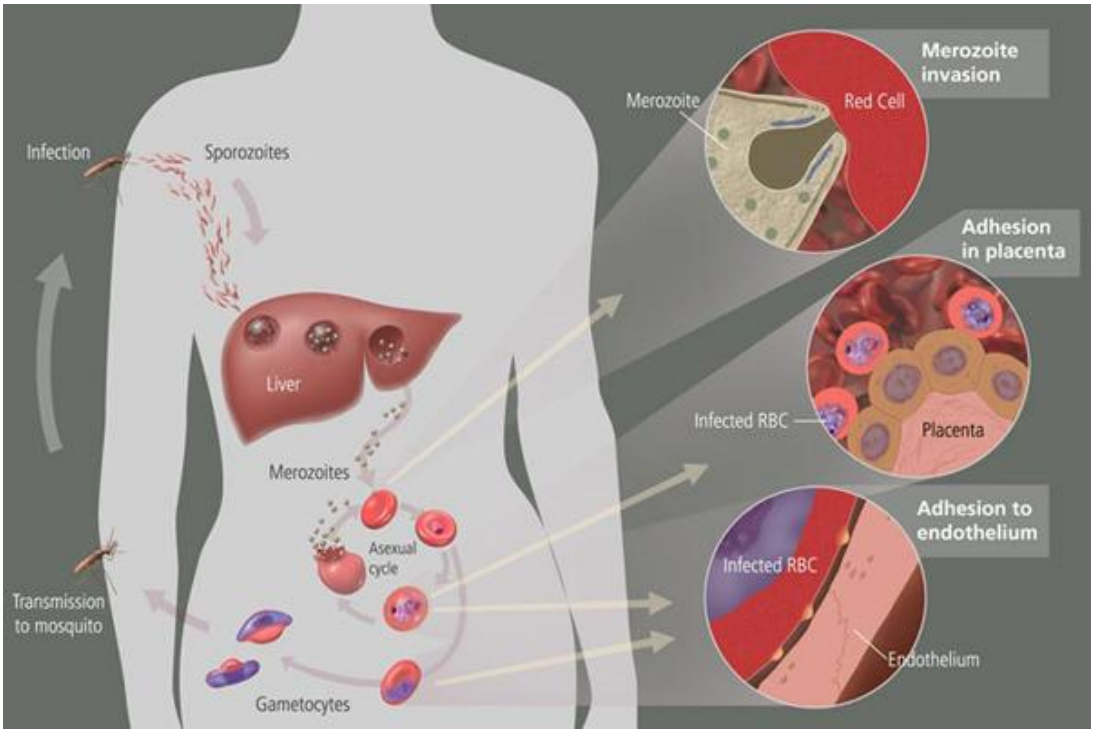


**Figure 2: The life cycle of *Plsmodium* in humans (K. Miller 2014)**

## 2.3 Pathogenesis of malaria

The outcome of an infection and progression into pathology depends on the specific and dynamic combination of host and parasite properties. Malaria is a multifactoral disease and its clinical outcome depends on many aspects such as parasite and host genetics, previous exposure to infection, age, nutritional status, and geographical and socio-economic factors (Schofield and Grau 2005).

The molecular and cellular events during malaria parasite life cycle influence severity of the disease. This occurs only as a result of the asexual blood stage (Miller, Good, and Milon 1994).

All human *Plasmodium* species invade by the same mechanism, but *P. falciparum* reaches higher parasitaemia because of greater flexibility in the receptor pathways that it can use to invade all erythrocytes. Infected erythrocytes (IEs) binds to endothelium or placenta for the parasite to avoid spleen-dependent killing mechanisms (Sim *at al.* 1994). The IEs remain attached until merozoites are released to invade other erythrocytes. As such, the predominant form in the peripheral circulation is the ring-infected erythrocyte which is the young form of the parasite (Miller, Good, and Milon 1994).

In non-immune individuals, the first symptoms of malaria which include fever, headache, muscle pain, chills, lethargy and vomiting are followed by malaria paroxysms between 7-15 days post infection. This is normally associated with the production of high levels of circulating cytokines. The activation of innate immune cells and consequent systemic inflammation lead to the initial signs of malaria, and can also influence the development of severe forms of the disease. If left untreated, the uncomplicated symptomatic malaria caused by *P. falciparum* can rapidly evolve into severe illness and mortality. Children with severe malaria may develop anaemia, jaundice, respiratory distress and/or cerebral disease. In adults, multi-organ involvement is also frequent and the impairment of kidney function may ultimately result in metabolic acidosis.

Individuals who have been previously exposed to malaria infection on multiple occasions develop natural acquired immunity to the disease over time. Malaria parasitism is low in hyper-immune individuals and with no deleterious activation of innate immune cells and as a result, the infection is asymptomatic (Gazzinelli *at al*. 2014).

The syndromes associated with malaria include systemic inflammation, anaemia, metabolic acidosis as well as cerebral malaria and PM. These result in connection with three main pathophysiological events that occur during malaria infection: the release of pro-inflammatory cytokines, adhesion of *Plasmodium* infected RBCs to capillaries and venules, and the rapture and removal of parasitized and altered RBCs by splenic macrophages ( Miller *at al*. 2013; Clark *at al*. 2006).

**2.4 Economic and social burden of malaria**

As far as malaria is concerned, the world can be divided into those regions that are malarious and those that are not (Malaney, Spielman, and Sachs 2004). Although malaria burden is not evenly distributed (Figure 1), observations indicate that areas where malaria prospers most, human societies have prospered the least (Sachs and Malaney 2002).

Malaria can impose great economic costs on the communities that it impacts. It affects economic development through changes in household behavior in response to the disease. This may lead to broad social cost influenced by factors such as schooling, demography, migration and saving. Macroeconomic costs which entail the impact of malaria on trade, tourism and foreign investment may arise as a result of the pandemic nature of the disease (Sachs and Malaney 2002).

Where malaria is highly endemic young children bear the burden in terms of morbidity and mortality. The overall impact of malaria on human capital development in children remains

largely unexplored and unquantified. In Kenya, an estimated 11% of school days are lost by primary school children every year, while secondary school students miss up to 4.3% school days because of malaria. Other studies have reported up to 50% of medically related school absenteeism to malaria (Sachs and Malaney 2002). In addition, malaria affects the cognitive development and learning ability since children with malaria have a poorer nutritional status than non-malarial children, an outcome that can impair brain development (Malaney, Spielman, and Sachs 2004).

Long term demographic impacts of malaria affect saving rates. The direct cost of prevention (buying of mosquito nets, insecticides and mosquito coils) and treatment of the disease eat into the disposable income of poor families, as do the cost of lost productivity (Sachs and Malaney 2002).

### 2.5 Malaria control

As malaria control intensifies, it is vital to monitor malaria burden and trends, and to track the coverage and impact of interventions. The government of every country affected by malaria has a national control policy covering prevention and care management. ("WHO | World Malaria Report 2013" 2014a). Current malaria control programs are aimed at malaria patients, exposed individuals and the mosquito vector , through parasite diagnosis and prompt treatment (Hafalla, Silvie, and Matuschewski 2011).

The objective of prompt anti-malarial treatment is to ensure cure of the infection, reduce morbidity and mortality of the disease, prevent progression of uncomplicated malaria into severe and potentially fetal disease, reduce the impact of malaria infection of the foetus during pregnancy and prevent drug resistance. Currently, this is achieved by use of atemisinin-based combination therapy (ACT), ("WHO | World Malaria Report 2013" 2014a; "WHO | World Malaria Report 2013" 2014b).

Vector control on the other hand is aimed at significantly reducing the incidence and prevalence of both parasite infection and clinical malaria. There are two main approaches to this; use of long lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS). These approaches can be complemented with larval control or environmental management. The LLINs are usually given free to children and pregnant women living in malaria endemic regions ("WHO | World Malaria Report 2009" 2014; "WHO | World Malaria Report 2011" 2014; "WHO | World Malaria Report 2013" 2014a; "WHO | World Malaria Report 2013" 2014b).

### 2.6 Placental malaria (PM)

Placental malaria (PM) is a common complication in malaria endemic areas. In Africa, where the burden of maternal and foetal morbidity is high, it accounts for 200,000 infant deaths annually ("WHO | World Malaria Report 2013" 2014b). Indeed pregnant women in these regions have higher levels of parasitaemia and parasite density compared to non-pregnant women (Nosten *at al.* 2004). As a result, the mother is susceptible to anaemia, cerebral malaria, pulmonary edema and kidney failure. Foetus is susceptible to abortion, stillbirth, premature delivery, low birth

weight (LBW) and intrauterine growth retardation (IUGR) (Steketee *at al*. 2001b; Shulman and Dorman 2003). Low birth weight in humans is defined as birth weight that is less than 2500g, and this is usually the most important risk factor for infant mortality associated with malaria (Greenwood *at al*. 1992; McCormick 1985).

Although studies have observed that some degree of pre-existing immunity is retained during pregnancy (Dorman and Shulman 2000), these women are still vulnerable to PM (Menendez *at al.* 2000; Tako *at al.* 2005). Placental malaria is also higher in primigravidae and secundigravidae than in multigravidae (Brown J Okoko *at al*. 2002). Several reports have shown that the risk and severity of PM decreases with increase in the number of pregnancies suggesting that immune built-up is finally achieved after several pregnancies and infections (Steketee *at al*. 1996; Shulman and Dorman 2003).

### 2.7 Characteristics of malaria infected placenta

Placental malaria is characterized by the accumulation of parasitized erythrocytes in the intervillious space (IVS), infiltration of inflammatory cells and release of pro-inflammatory mediators that cause pathologic alterations in the placenta (Walter, Garin, and Blot 1982; Fried *at al*. 1998; Ordi *at al*. 1998). These result in pathological characteristics that are manifested by presence of intravillous parasites and leukocytes, macrophages containing malaria pigments, fibrin deposits, proliferation of cytotrophoblastic cells, and thickening of the trophoblastic basement membrane (Yamada *at al*. 1989; Matteelli *at al*. 1997). Previous studies have shown presence of haemozoin (malarial brown pigments) that occur in the previllious deposit of

fibrinoid in macrophages and free in the intervillious space. Such studies have also revealed that PM leads to excessive fibrinoid deposits that are associated with syncytiotrophoblastic necrosis or ultra structural damage (Walter, Garin, and Blot 1982).

### 2.8 Parasite adhesion in the placenta

Red blood cells (RBCs) infected with malaria parasites are able to sequester in the placenta, a key phenomenon in the pathogenesis of PM (Fried and Duffy 1996). The syncytiotrophoblast cells in the placenta express different and variable amounts of host cell receptors onto which the parasites binds. This leads to the adherence process (Baruch 1999). The adhesion phenotypes are however not homologous and as a result, different parasites can bind to various numbers and a combinations of host receptors (Beeson *at al*. 1999). A single parasite protein- *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is usually expressed on the surface of the infected RBCs and mediates their binding to the different receptors (Baruch 1999).

Studies in humans have shown that parasitized RBCs isolated from placenta have a unique binding property compared to parasites isolated from non- pregnant individuals (Fried and Duffy 1996; Beeson *at al*. 1999). Hence, these parasites are capable of binding to chondroitin sulphate A (CSA) but not CD36 which is the main host receptor for sequestration in the microvasculature. This in turn allows the parasites to sequester in the placenta and not in the endothelium. Indeed, CSA binding parasites express PfEMP1 with a duffy binding like-γ (DBL-γ) domain that binds CSA and non CD36-binding cysteine-rich interdomain region 1 (CIDR1). On the contrary,

CD36-adherent parasites express a PfEMP1 with a CD36-binding CIDR1 (Buffet *at al*. 1999; Gamain *at al*. 2001).

An additional process through which the parasite sequesters in the placenta is by binding to non-immune immunoglobulin (Ig). A cloned parasite line selected from a high level of binding to Ig has been observed to bind to placental sections *in vitro*. Similarly, placental isolates from four infected Cameroonian women appeared to bind to IgG *in vitro*. This suggests that IgG could be acting as a bridge molecule between parasitized RBCs and the Fc receptors present on the syncytiotrophoblasts, thereby mediating parasite sequestration in the placenta (Flick *at al*. 2001).

## 2.9 The placental barrier

Structurally, the human placenta is a hemochorial villous organ that connects the developing foetus to the uterine wall. The processes involved in placental development are highly regulated to ensure normal growth of the developing foetal tissue and the maintenance of a healthy pregnancy. A unique function of the placenta as an interface link between maternal and foetal tissues is to prevent allograft rejection of the foetus and ensure sufficient foetal nutrient supply, gaseous exchange and transfer of foetal toxic metabolic waste into the maternal circulation for their elimination ( Malek 2013; Wang and Zhao 2010b; Martin and Ramsey 1970).

During the first few days of pregnancy, the developing foetus migrates from the fallopian tube to the uterus where it forms into a blastocyst. The outer membrane (chorion) and trophoblast layer of the placenta are formed from the trophoectoderm of the blastocyst cells. Other etra-embryonic

tissues including the amnion (placental inner membrane) develop from inner cell mass of the blastocyst. Placental vessels are derived from the mesoderm (Malek 2013).

The placental maternal surface is divided into cotyledons, each supplied by a major branch of the umbilical artery and drained by a major tributary to the umbilical vein (Figure 3). These vessels enter stem villi, which branch and rebranch like a tree to form microscopic terminal villi suspended within the intervillous space. Each cotyledon has several anchoring villi which extend into the decidua basalis and are anchored to it by syncytial cells and fibrin (Ahokas and McKinney 2009; Wang and Zhao 2010b).

When viewed from the fetal surface (amnion) it consists of the chorionic plate, its villi and the peripheral trophoblastic shell that surrounds the intervillous space and covers the maternal tissue. The anchoring villus contacts the decidua basalis (maternal portion) that is characterized by large, polyhedral pale blue stromal (decidua) cells (Walter, Garin, and Blot 1982; Wang and Zhao 2010a). Fetal vessels radiate from the umbilical cord between the amnion and chorion (Figure 3). The membranes on the surface of the placenta are continuous at its margin with the chorion and amnion lining the remainder of the uterine cavity (Ahokas and McKinney 2009; Wang and Zhao 2010a). The outermost layer is the chorion. It is in contact with the endometrium. The chorion further consists of two layers of cells, the inner cytotrophoblast and the outer syncytiotrophoblast (Wang and Zhao 2010b; Wang and Zhao 2010a).

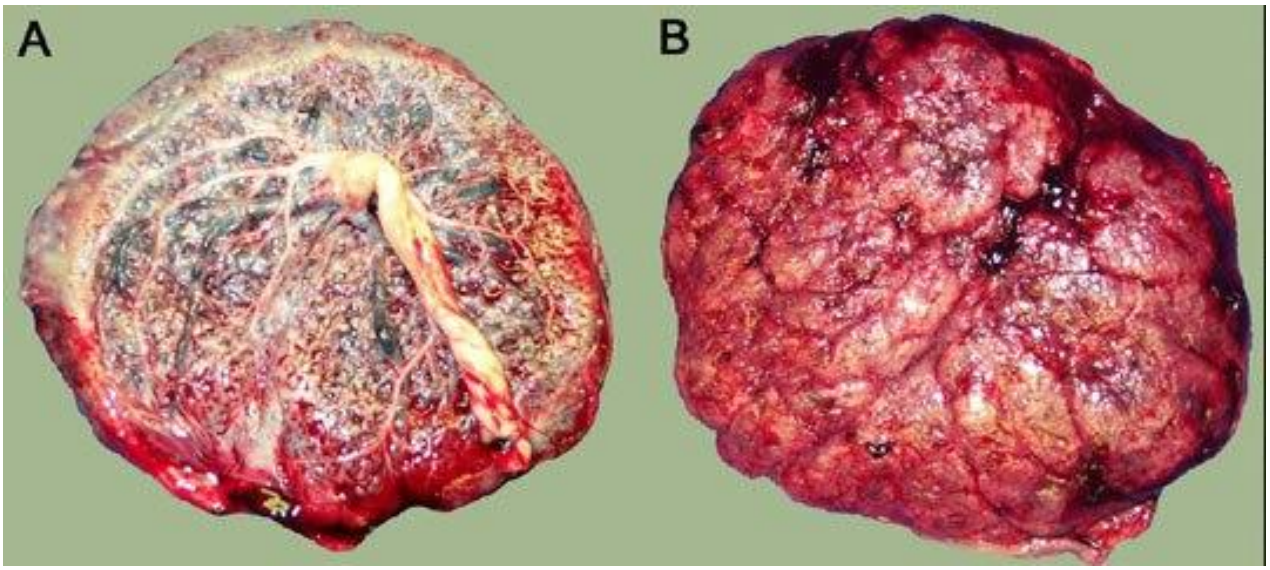


**Figure 3: Anatomy of placental explants showing foetal face (A) and maternal face (B) of a human placenta (Lecuit *at al*. 2004)**.

Foetal circulation enters the placenta via the umbilical arteries embedded within the umbilical cord. Once nutrients have been absorbed and waste products released in the placenta, the foetal blood ultimately collects into the umbilical vein, where it returns to the foetus via the umbilical cord. The main functional units of the placenta are the chorionic villi within which fetal blood is separated by only three or four cell layers (placental membrane) from maternal blood in the surrounding intervillous space. After implantation, trophoblast cells proliferate and differentiate as villous and extravillous. Non-migratory, villous cytotrophoblast cells fuse to form the multinucleated syncytiotrophoblast, which forms the outer epithelial layer of the chorionic villi. It is at the terminal branches of the chorionic villi that the majority of fetal/maternal exchange

occurs. Extravillous trophoblast cells migrate into the decidua and remodel uterine arteries. This facilitates blood flow to the placenta (Gude *at al*. 2004).

As there is no vascular continuity between the mother and fetus, the placenta must play an important role in acceptance of the fetus. Trophoblast cells are the most important fetal cells coming in contact with maternal cells, and three different trophoblast populations that are exposed to different maternal elements can be distinguished. The first population is the villous cytotrophoblast which form a pool of actively dividing trophoblast cells that remain in the villi. The second trophoblast population covers the first population and is called the syncytiotrophoblast and these float in the maternal blood. The third trophoblast population is the non-villous cytotrophoblast which are proliferating precursor trophoblast cells that migrate into the decidua and myometrium (Veenstra van Nieuwenhoven, Heineman, and Faas 2003).

### 2.10 Immunological aspects of pregnancy

According to Colbern and Main, reproductive immunology is defined as maternal-placental tolerance as opposed to maternal-foetal tolerance hence focusing on the interaction of maternal immune system on the placenta and not on the foetus (Colbern and Main 1991). This is because the foetus itself has no direct contact with the maternal cells. Instead, cells from the placenta are the only part of the foetus (per se) that interacts directly with the mother's immune system. This enables them to evade the mother's immune system and ultimately rejection as an allograft (Poole and Claman 2004).

Implantation and acceptance of the genetically and immunologically foreign foetus within the maternal body occurs in a controlled manner and must evade the mother's immune defence mechanism to avoid rejection. Because the foetus is not genetically identical to the mother having acquired "foreign" paternally derived histocompatibility genes, it is commonly referred to as an allograft (Poole and Claman 2004).

The human immune system includes many cellular patterns that constantly exchange information providing the body with the ability to recognize foreignness or non-self from self in the form of antigens that enter the body. The recognition of these antigens triggers inflammatory responses that must be balanced to minimize damage of host cells and at the same time act to eliminate the foreign material (Loke 1978). Antigens can be expressed by early human embryonic tissue, and as such, it would be expected that such an embryo would trigger inflammatory responses within the mother's system in order to eliminate the "foreign" foetus. However, the exposure to non-self paternal antigens on the foetus requires the adaptation of the mother's immune system to prevent its rejection without compromising on the mother's ability to fight infection (Loke 1978; Koch and Platt 2003).

There are multiple and diverse mechanisms involved in the immune regulation of pregnancy during gestation (Koch and Platt 2003). The immune system consists of an innate (humoral) and an adaptive (cellular) component. During pregnancy, the production of progesterone suppresses the humoral response. The adaptive response is hence the main immune response triggered by foetus, where there is antigen representation followed by responses instructed by helper T cells (Poole and Claman 2004).

Several studies on immunological tolerance during pregnancy have suggested several theories that work together to prevent immune rejection of the foetal allograft. First, it is hypothesized that a mechanical barrier forms between the mother and the foetus preventing exposure of the maternal immune system to allogenic antigens expressed by the foetal tissue. As a result the foetal and maternal circulations are segregated. Second, immunological immunity of foetal tissue exists. This causes the acting allogenic immunity to suppress the expression of antigens that would be recognized by the mother's immune system as foreign and hence target them for destruction. Third, the mother's immune system somehow "ignores" potentially immunogenic foetal tissue. Recent studies have further displayed that the mother's immune system not only recognizes foetal alloantigen but also responds to them. Foetal cells can be detected in maternal circulation and foetal tissues express antigenetically mature major histocompatibility complex (MHC) I and II which complex proteins coded by genes. These overlapping theories help to understand how rejection is avoided but fail to completely explain how the foetus evaded the maternal immune system. As a result focus has shifted to exploring the materno-foetal interface (placenta) which is thought to be an immunologically privileged site. Understanding immunological events and mechanisms occurring at the materno-foetal interface is likely to help understand the ability of the foetus to survive within the maternal body. This leads to the fourth theory of local suppression of maternal immune response at the materno-foetal interface. This negates systemic suppression which could be harmful to the mother (Koch and Platt 2003).

## 2.11 The effect of pregnancy on peripheral immune responses

Villous syncytiotrophoblast floating in maternal blood are in close contact with maternal peripheral leukocytes. Therefore, the peripheral immune response is adapted to the presence of the semiallogeneic syncytiotrophoblast cells. One of the first recognized changes in the peripheral maternal immune system during pregnancy is an increase in peripheral white blood cell count (Veenstra van Nieuwenhoven *at al*. 2002; Siegel and Gleicher 1981). Human deciduas contain abundant immune cells during gestation with more than 30% of stromal cells in the first trimester deciduas expressing leukocytes. There are four major populations of deciduas leukocyte associated with immunological balance during pregnancy: T lymphocytes, uterine natural killer (uNK) cells, macrophages and dendritic cells (DC) (Veenstra van Nieuwenhoven, Heineman, and Faas 2003; Chen, Liu, and Sytwu 2012).

### 2.11.1 T lymphocytes

Lymphocytes that mediate cellular immunity called T cells are the best studies peripheral immune cells in human pregnancy since they play a major role in sustaining pregnancy (Mellor and Munn 2004). Within the T-lymphocyte population, helper T lymphocytes (Th) and cytotoxic T lymphocytes (Tc) can be distinguished. Helper T lymphocytes provide help to other immune cells by producing cytokines, while Tc lymphocytes can directly kill foreign or infected cells. These T lymphocytes can also be classified into different functional subsets based on their profile of cytokine production. Type 1 T cells produce cytokines which promote cellular immune responses, while type 2 T cells produce cytokines that provide optimal help for humoral immune responses (Mosmann *at al*. 1986). Pregnancy is a type 2 phenomenon (Lin *at al*. 1993). The shift away from type 1 cytokine production during pregnancy is beneficial for pregnancy because

type 1 cytokines are harmful for pregnancy as they inhibit embryonic and fetal development and can terminate pregnancy when injected into pregnant mice (Chaouat *at al*. 1990; Haimovici, Hill, and Anderson 1991).

Maternal T lymphocytes are also present in the deciduas where they play a role in the immune response. Although these T cells are in close contact with the trophoblast, they do not attack the non-villous cytotrophoblast. This is because they do not recognize the MHC Ia-negative trophoblast as being foreign since cytotrophoblast cells do not express MHC 1a. The T lymphocytes in the decidua can produce a variety of type 1 and type 2 cytokines (Chaouat *at al*. 1990; Lin *at al*. 1993). Type 1 cytokines promote miscarriage by inhibiting trophoblast invasion. In fact, TNFα stimulates apoptosis of human trophoblast cells while IFNγ increases this TNF-α-mediated killing of trophoblast. Type 2 cytokines in general stimulate trophoblast outgrowth and invasion (Yui *at al*. 1994; Hill 1995).

### 2.11.2 Uterine natural killer (uNK) cells

The uNK cells have a natural killer cell-like function, but they are specific for the uterus as they show a different phenotype compared with peripheral natural killer cells (Ritson and Bulmer 1987). The number of uNK cells which are normally present in the endometrium increases remarkably during early pregnancy (Ozenci, Korgun, and Demir 2001). Their presence in the decidua may be explained by two mechanisms. First, peripheral blood uNK cells are selectively homing to the uterine mucosa since they can interact with adhesion molecules on the decidual blood vessels (Ruck *at al*. 1994). Second is *insitu* proliferation as uNK are actively dividing.

This proliferation can be stimulated by either cytokines produced by other decidual cells, or by steroid hormones (Gibson *at al*. 2015).

Knowledge of the function of uNK cells is still limited, and because they do not share all membrane expression markers with peripheral natural killer cells it is not clear whether these cell types have the same function. However, because of the large number of uNK cells in the uterus during early pregnancy, it is suggestive that they play an important role in protection against infections or in the regulation of immunity, whilst at the same time perhaps affecting implantation and placentation (Guimond, Wang, and Croy 1999). The uNK cells can also produce cytokines (Vince and Johnson 2000).

### 2.11.3 Macrophages

Macrophages are also immunoregulators of pregnancy. There is immune tolerance to the invading trophoblast and foetus. During implantation, apoptosis is necessary for tissue remodelling of maternal decidua and invasion of the developing embryo. Hence, apoptosis is active in the trophoblast layer throughout gestation resulting in a constant cell turn over at the site of implantation. During this process, a large number of macrophages are present in the maternal decidua and in tissues in close proximity to the placenta. Macrophage engulfment of apoptotic cell prevents the release of potentially pro-inflammatory and pro-immunogenic intracellular components. This is because trophoblast cells carry proteins that could initiate or accelerate immunological responses, with lethal consequences for the fetus (Palmeira *at al*. 2011).

Because of the relative absence of other immune defense systems within the placental tissue, decidual macrophages are thought to have an important role in non-specific host defense in the placenta. They also have a phagocytic role, as they may be involved in the removal of cell and tissue debris associated with trophoblast invasion (Bulmer and Johnson 1984). Macrophages are also suggested to have a role in placentation and production of cytokines. Beside cytokines, macrophages can also produce immunosuppressive prostaglandins, which may block the function of Tc lymphocytes and uNK cells (Hunt and Robertson 1996; Redline *at al*. 1990).

### 2.11.4 Dendritic cells

Dendritic cells (DCs) are known to be the most potent antigen presenting cells. Additionally, there is emerging evidence that DCs may be involved in the regulation of type 1/type 2 cytokine balances. Although little research has focused on these kind of cells and given contradictory results, some studies have demonstrated their important role in the immunological paradox of pregnancy (Steinman 1991; Nishioka *at al*. 2001).

## 2.12 Immune responses in placental malaria

With 50% of the genetic material derived from its father, the foetus's susceptibility to rejection by the maternal immune system is somewhat similar to the susceptibility of a transplanted organ (Jamieson, Theiler, and Rasmussen 2006). Cell-mediated immunity is particularly suppressed during pregnancy and therefore the mother is increasingly reliant on humoral immunity for protection. Initially, it was believed that this suppression was responsible for the high increase of malaria in pregnant women. However, it is now evident that despite the depression, the maternal immune system continues to respond to human malaria parasites. Indeed, antibodies

preventing the attachment of *P. falciparum* to the placenta can be produced and quantified (McLEAN *at al*. 2015; Duffy and Fried 2003). An increase in Th1 cytokines has also been observed during placental malaria infection in women (Fried *at al*. 1998).

Primigravidae women have been reported to be highly susceptible to malaria while multigravidae women have a reduced risk of infection and associated complications (Brabin 1983). This is so because when a woman is infected with the malaria parasite during her first or second pregnancy, she develops antibodies (Abs) against the parasite variants that adhere to the placenta. These Abs contribute to protection from malaria in subsequent pregnancies (Beeson *at al*. 2002). Initial studies have shown an association between reduced placental infection and the level of antibody primed against PfEMP1 *in vitro*. Such antibodies were also more prevalent in multigravidae women (Fried *at al*. 1998). A subsequent study carried out in Cameroonian women however, revealed the contrary. It showed that there was no clear association between infection (peripheral or placental blood) and adhesion-blocking Abs although these Abs were more common in pregnant women than in non-pregnant women (O'Neil-Dunne *at al*. 2001).

Immunomodulation also occurs in primates challenged with malaria during pregnancy. A study conducted by Billie *at al.*, (2005) showed that in pregnant monkey's leukocyte populations did not increase during *Plasmodium* infection. That study also concluded that the host immune system under the influence of pregnancy was responsible for the increased severity in clinical symptoms (Davison *at al*. 2005).

## 2.13 Pathology of placental malaria

In humans, PM is characterized by the accumulation of parasitized RBCs in the intervillous space (IVS), infiltration of inflammatory cells and release of pro-inflammatory mediators. Other pathological changes include detection of malaria pigment (haemozoin), fibrin deposits and leukocytes within the placental IVS, thickening of trophoblastic basement membrane and proliferation of cytotrophoblastic cells (Ismail *at al*. 2000; Uneke 2007).

Various factors may be responsible for placental pathology. Leucocytes, through the production of non-chemotactic cytokines, might be associated with the thickening of trophoblastic basement membrane that could cause mechanical blockage of oxygen and nutrient transport across the placenta. These changes have been associated with syncytiotrophoblastic damage and cytotrophoblastic proliferation. It has also been suggested that these placental lesions, especially the thickening of trophoblastic basement membrane may alter materno-fetal exchange and contribute to the deleterious effect of malaria-associated placental lesions on foetal growth (Galbraith *at al*. 1980; Walter, Garin, and Blot 1982; Yamada *at al*. 1989).

In addition, *P. falciparum* infection could disturb the folate-$B_{12}$-metabolic pathway, a mechanism contributes to IUGR (Bernard J. Brabin, Fletcher, and Brown 2003). Larger molecules such as IgG and transferrin-bound iron are usually transferred across the trophoblast by receptor-mediated endocytosis/exocytosis mechanisms. However IUGR reduces this transfer (Yang *at al*. 1999).

## 2.14 Adverse pregnancy outcome of placental malaria

Placental malaria is a major determinant of congenital malaria (Larkin and Thuma 1991). Congenital malaria is described as the presence of asexual *Plasmodium* parasites in cord blood smear at delivery or in foetal peripheral blood smear during the first seven days of life irrespective of clinical symptoms. Pregnant women exposed to malaria parasites may develop congenital malaria with prevalence ranging from 3% to 54.2% in sub-Saharan Africa (Uneke 2007; Piñeros-Jiménez *at al*. 2011b). This condition can be transmitted via several mechanisms such as maternal transfusion into foetal circulation at the time of delivery or during pregnancy, by direct penetration through chorionic villi or by penetration through premature separation of the placenta (De Silva *at al*. 1982). In spite of this, the placenta can effectively restrain the passage of malaria parasites (Miller and Telford 1996).

Effect of PM on perinatal mortality (foetal or infant death from 28 weeks of pregnancy up to the seventh day after birth) has also been reported. These include abortions and still births. In facts studies have revealed direct association of PM with increased risk of still births (Brown J Okoko *at al*. 2002; van Geertruyden *at al*. 2004).

Low birth weight (LBW) may also occur during PM. It is documented that malaria accounts for 3.5 million LBW (i.e., >2.5kg) babies born annually in sub-Saharan Africa (B. Brabin and Piper 1997). This occurs when parasites either mechanically compromise placental circulation through a widespread trophoblast basement thickening and increased fibrinoid necrosis or indirectly by interfering with placental functions and/or induce pathological lesions leading to malaria induced anaemia (Galbraith *at al*. 1980; Moshi, Kaaya, and Kitinya 1995; Kassam *at al*. 2006).

Preterm delivery (PTD) and IUGR are also adverse outcomes of PM (B. J. Brabin *at al*. 1990). Although the precise effect of malaria-parasitized placentas on PTD is uncertain, malarious placentas often carry antibodies, cytokines and macrophages which are indicative of an active immune response that is likely to stimulate early labor. Similarly, the biological processes that mediate IUGR due to PM are also unclear since placentas can only be studied after they have been delivered although it relates to insufficient nutrient transport to the foetus (Ismail *at al*. 2000; Guyatt and Snow 2004).

Neonatal anthropometric parameters such as neonatal length, head circumference, and placental weight have also been associated with PM. A study in Southeastern Tanzania has shown that chronic ongoing malaria infection of placenta is associated with significant reduction in mean head circumference, neonatal length and body index (weight/length), while past infection is associated with reduced mean length at birth (Menendez *at al*. 2000).

### 2.15 Use of non-human primate (NHP) models in biomedical research

The ultimate goal of all biomedical research is to get clear understanding of the normal physiology, biochemistry and disease process that are relevant to humans. Although some aspects of research can be directly carried out in humans, some experimental aspects are not ethically and practically feasible. Several animal models have so far been used (for example rats, dogs, cats, swine, sheep, goats, cattle etc) and have provided abundant data with significant correlation valuable in the field of medicine. However, because of biological differences between these animals and humans, the data has been inapplicable since they have failed to demonstrate sequestration in the placental IVS, a key phenomenon during PM in humans.

Non-human primates (NHPs) hold a prominent key position between these animal models and humans. The most commonly used primates are the Old World species like the rhesus monkeys, the long tailed macaque and the baboon (Firyal and Khan, 1998). The baboon (*Papio spp*) has been used in medical research for a very long time (since 1927) and therefore, a lot of baseline biomedical data exists. This data shows close parallelism to humans. As a result, the baboon has been extensively used in numerous studies on cancer, reproductive, cardiovascular, pulmonary and infectious diseases (Daadi *at al*. 2014; Bauer 2015).

### 2.16 Non-human primates as models for placental malaria

Current animal models that are commonly used in the study of malaria in pregnancy are pregnant mice and rats infected with *Plasmodium berghei* (Tegoshi *at al.,* 1992). However, their relevance to malaria in human pregnancy is questionable because of the many differences between rodent and human pregnancies. First, progesterone in rats is produced by the corpus luteum of the ovary, rather than the placenta as in humans and secondly because the rodent placenta is labyrinthine hemodichorial, rather than villous hemomonochorial, like the human placenta (Fischer 1996).

An effective disease model should closely mimic the pathogenesis of malaria in pregnant women. Ideal candidates are NHPs because they are susceptible to many species of *Plasmodium* and have humoral and cellular responses similar to those of humans (Coatney 1971). The macaques, great apes and baboons have discoid villous hemochorial placentas similar to those in humans (King 1993). Macaques are readily available and have been used frequently in malaria research. Macaques used in malaria research include pig-tailed monkeys *(Macaca nemestrina),*

cynomolgus or long-tailed monkeys *(M. fascicularis),* and rhesus monkeys *(M. mulatta).* Of these, according to Billie *at al.,* (1998) the rhesus monkey is the best characterized, most widely used, and most available. In addition, the rhesus placenta has been studied extensively (Davison *at al*. 1998).

Great apes cannot be used because they are an endangered species while baboons are expensive to house because of their size, although they are used in reproductive studies. In addition, baboon malaria models are less well-developed than macaque malaria models. None the less, the reproductive endocrinology of female baboons is similar to that of human females. Their menstrual cycle being 28-30 days with gestation period of 27 weeks (shorter than that of humans but with similar hormonal profiles) making them a unique NHP model for studies that involve reproductive physiology, perinatal biology and experimental embryology and PM (Research 2003; D'Hooghe *at al*. 2008).

### 2.17 *Plasmodium knowlesi* as a model for human malaria

Human malaria parasites are a major priority in research due to the association of malaria with mortality rates in the human population. However, because of host specificity of the malaria parasite to its human host, research on parasite biology is restricted. For this reason, different experimental systems have been employed to model the complex interactions between parasites and their hosts. The three *Plasmodium* groups that are mainly used in experimental studies on host-parasite interactions are rodent, avian, and primate *Plasmodia* (Snow and Omumbo 2006; Silvie *at al*. 2008).

The rodent malaria parasite *P. berghei* is commonly used to study parasite biology. However, it does not effectively allow investigations of natural-host parasite interactions because of its phylogenetic distance (Ozwara *at al*. 2003). Although the avian *Plasmodia* such as *P. gallinaceum* and *P. lophurae* are closely related to *P. falciparum*, differences in their life cycle, host specificity and immune system of the host limits their usefulness as models for human malaria. On the other hand, simian *Plasmodium* such as *P. knowlesi* has a comparable phylogeny and host-parasite relationships to the human malaria parasites in addition to its cytoadherance property. It is therefore capable of sequestering in the placenta (Coatney 1971; Ozwara and LUMC 2005).

The natural host of *P. knowlesi* parasite is the *Macaca fascicularis*. This parasite causes chronic infection in its definitive host. It can also experimentally infect and cause acute infection in other non-human primates such as *M. mulatta*, *M. radiata*, *M. assamensis, Presbytis entellus, Callithrix jacchus, Aotus trivigatus*, *Saimiri sciureus*, and baboons (Butcher 1996). In baboons, the infection has been induced in *Papio cynocephalus*, *P. doguera*, *P. jubileaus*, and *P. papio* (Coatney 1971). This parasite has also been confirmed as the fifth human malaria parasite, accounting for 70% of malaria cases in Southeast Asia where it is mostly found. It also has a close phylogenetic relationship with *P. vivax* (Vythilingam *at al*. 2006). To date, protocols have been developed for long term *in vitro* culturing of *P. knowlesi* (Kocken *at al*. 2002). This together with the availability of both natural and artificial hosts, plus primate phylogeny to humans make *P. knowlesi* infection in NHPs the ideal model to study malaria in humans including PM.

## 2.18 Life cycle of *Plasmodium knowlesi*

The natural vertebrate hosts of *P. knowlesi* are *Macaca fascicularis* and *M. nemestrina*. It can also infect other monkey species such as *M. mulatta, M. radiata, M. assamensis, Presbytis entellus, Callithrix jacchus, Aotus trivigatus, Saimiri sciurens* and Olive baboons (Butcher 1996).

Malaria infection due to *P. knowlesi* is initiated by the injection of sporozoites with saliva during the feeding process of the mosquito vector (*Anopheles latens, A. dirus*, *A. balabacencis* and *A. hackeri).* The sporozoites then enter the blood stream and are transported to the liver where they invade hepatocytes. Here, the parasites undergo asexual replication to form extraerythrocytic schizogony. These then culminate to produce merozoites which are released into blood stream and mature into schizont following the rapture of infected erythrocytes (Shulman and Dorman 2003). The merozoites differentiate into sexual forms called macro- or micro gametocytes (trophozoites). These are usually large parasites that fill up the erythrocytes but only contain one nucleus. In this form, the mosquito vector can ingest them and induce them to form gametes that eventually develop into oocytes that in turn enlarge to form sporozoites that lodge in the salivary gland of the mosquito. When the mosquito bites a new vertebrate host, the cycle continues (Ozwara and LUMC 2005) (Figure 4).

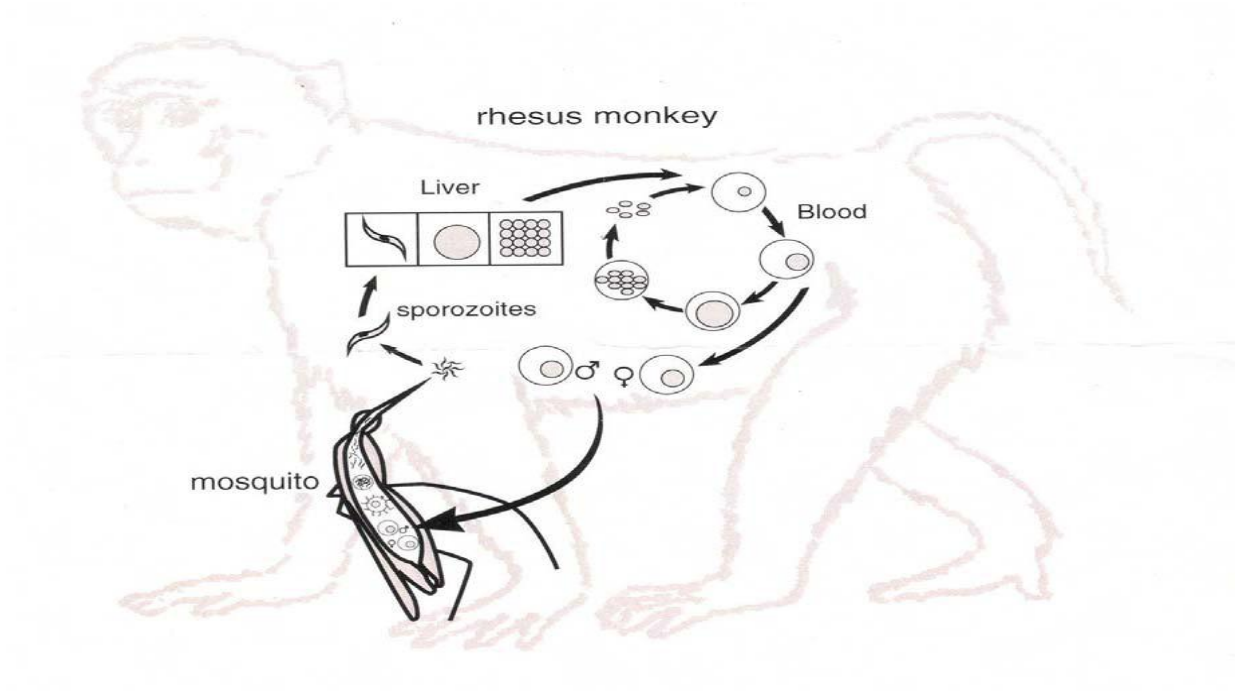


**Figure 4: The lifecycle of the simian malaria parasite, *P. knowlesi* (Ozwara, 2005).**

## 2.19 Importance of this study in placental malaria

Malaria affects almost every aspect of social and economic endeavour, including fertility, savings and investment rates, crop choices, schooling and migration decisions. Where transmission is intense, the disease creates a complex set of biological and behavioural responses with a long-term effect on economic growth and development. Further, malaria has consequential impact on social and economic growth and development: children miss school, working days are lost, and tourism suffers (Sachs and Malaney 2002).

Sensitive measures of malaria which include haematological and clinical signs lack predictive values during pregnancy which makes it very difficult to distinguish signs and symptoms of malaria during pregnancy from other bacterial and viral infections (Maina *at al*. 2010). In addition, pathogenesis of malaria is poorly understood due to inconsistent results in studies

conducted in humans because of differences in sample collection, sample analysis and definition of placental malaria (Brabin *at al.* 2004). Animal models that have been used in the past have failed to demonstrate parasite sequestration in the placenta, which is a key feature in placental malaria in humans (Tegoshi *at al.* 1992; Coatney 1971; Davison *at al.* 1998)

This study sought to help bridge the gap in pathophysiology of PM in human studies by validating and applying the baboon-*P.knowlesi* model. This model gives predictive values of clinical and haematological indices associated with placental malaria and demonstrate pathophysiology of this condition in *P. knowlesi* infected Olive baboons. It sheds light on the impact of disease at different gestation period, how infection during the different trimesters affects the foetus and pattern of disease in the semi-immune/immune state. So far, the baboon- *P. knowlesi* model is an ideal model that can be used for further studies in experimental therapeutic management of malaria during pregnancy.

# CHAPTER 3: MATERIALS AND METHODS

## 3.1 Study site

This study was conducted under the Malaria Research Program at the Institute of Primate Research (IPR) located in Karen, Nairobi, Kenya. The Institutional Review Committee (IRC) comprising of animal care and use committee (ACUC), scientific review and the research ethics and integrity team approved all protocols and use of animals in this study.

## 3.2 Study design

Ultrasound pregnancy test was done to confirm gestational periods after synchronized natural colony time mating sessions of 22 adult baboons. This was achieved by allowing female baboons to interact with male baboons in their breeding cages during the ovulation season of the female baboon. The animals were grouped and infected in first (day 40 of gestation), second (day 100 of gestation) and third trimesters (day 170 of gestation) as illustrated in figure 5. In the third trimester group, three animals were left to deliver at term while seven animals delivered via caesarean sections (CS) on day nine post infection (PI). From day two PI, daily parasitaemia and clinical conditions were monitored until treatment on day nine PI or at 1% parasitaemia levels. The animals that underwent CS were treated on the day the CS was conducted.

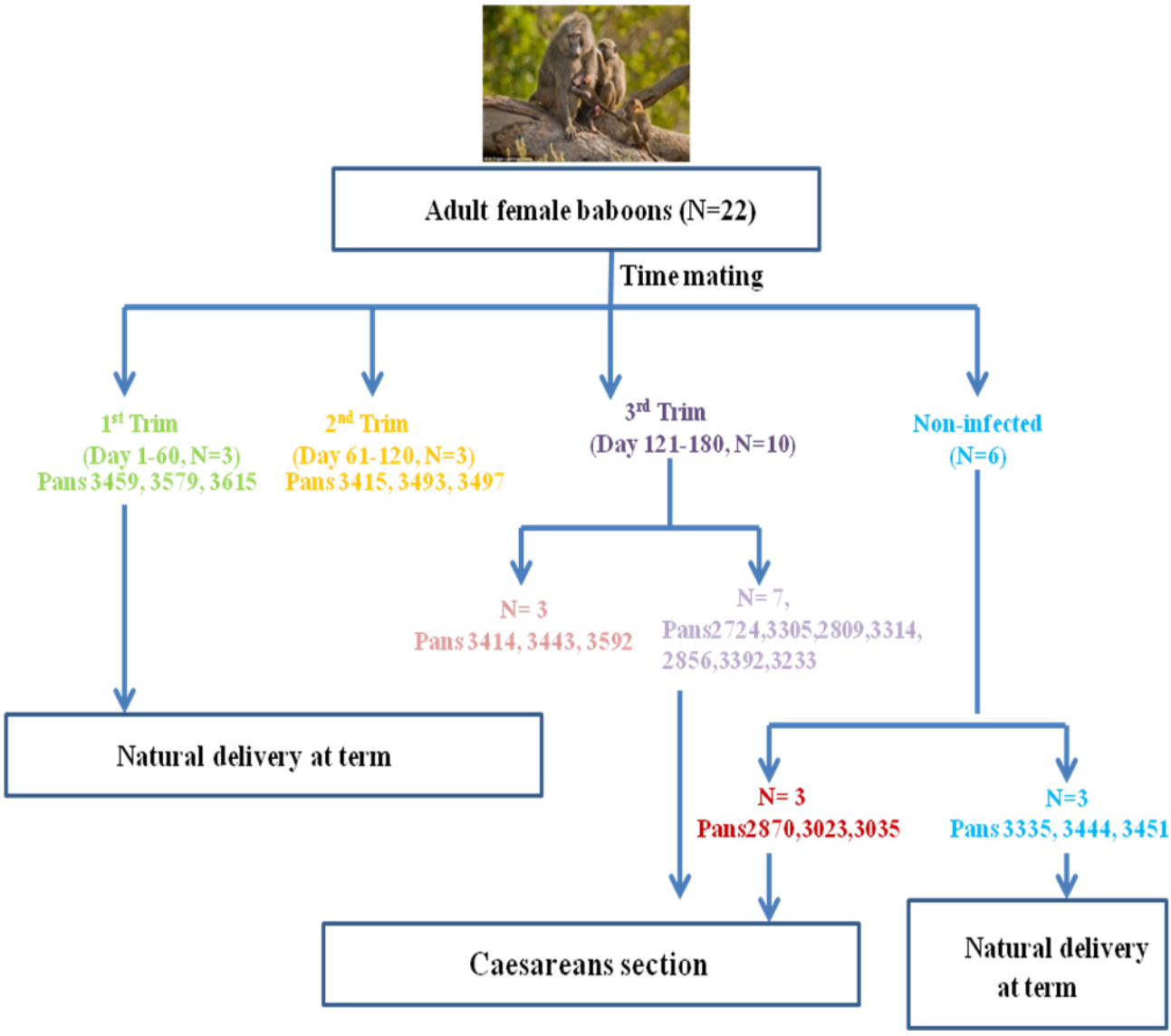


**Figure 5: Study design of adult baboons. Trim means trimester.**

Sterile placentas were collected during CS as described by Moore *at al.*, (Moore *at al*. 1999). The cord was cleaned with 70% alcohol to avoid maternal contamination and incised at 15 cm from its attachment to placenta with a fresh blade to obtain cord blood (Adebami *at al*. 2007). Placenta and cord blood samples were used to prepare thin blood smears in order to detect and quantify parasitaemia levels in placental maternal surface, placental foetal surface and cord

blood. Peripheral blood mononuclear cells (PBMC) were isolated and used for the detection of cell surface markers of B cells and T cells associated with malaria infection using Fluorescent activated cell sorting (FACS) technique. Peripheral, cord and placental serum samples were also prepared and used for measurement of IgG antibody responses in enzyme linked immunoabsorbent assays (ELISA) and cytokine detection using cytokine bead array (CBA). Peripheral whole blood was used for haematological assays. Placental tissues were processed using paraffin wax for histopathology.

The delivered infants (delivered via Cs and naturally at term) were assesses for birth weight, height and head circumference. The infants delivered at term to both PM positive and PM negative mothers as illustrated in figure 6 were further monitored for two months in order to determine their immunological response towards malaria infection. They were infected with P. knowlesi blood stage malaria parasite ($2x10^5$ parasites/ml incomplete RPMI)two weeks after delivery, parasitaemia levels observed for seven days before treating with Pyrimethamine (1mg/Kg body weight), and immunological assays performed.

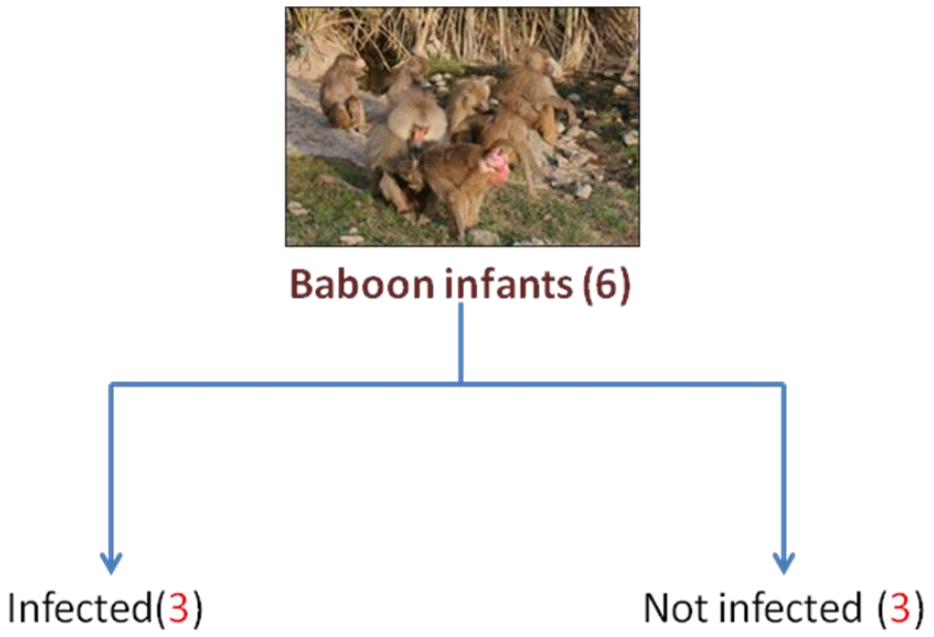


**Figure 6: Study design of baboon infants born at term from malaria infected and non-infected mothers**

### 3.3 Experimental animals and parasites

Experimental animals consisted of 22 adult female olive baboons (*Papio anubis)* weighing between 11-21 kg originating from Kajiado district of Kenya. These baboons were trapped and maintained in the quarantine facilities at IPR, Karen, for not less than 3 months. Prior to the experiment, all animals were screened and determined to be free of hemoprotozoan, gastrointestinal parasites and Simian Immunodeficiency Virus (SIV). Additionally microbiological examination of effusions, pus, ulcer material and skin specimens was done by the Animal Sciences Department (ASD) to detect pathogenic agents which cause infection in wounds and the skin. For all invasive procedures, baboons were anaesthetized with Ketamine hydrochloride.

Retrieval of *P. knowlesi* H strain blood stage parasites from liquid nitrogen was done and cultured overnight before inducing malaria infection in baboons. For overnight cultures, cryopreserved cultures of *P. knowlesi* were quickly thawed in water bath at 37$^{o}$C and transferred into 50ml centrifuge tubes. The parasites were washed twice in 3.5% Sodium chloride (NaCl) and twice with Roswell Park Memorial Institute (RPMI) 1640 culture medium with baboon serum at 400x *g* (Sorvall RT 6000 D; San Diego, USA) for 10 minutes. The pellet was transferred to an incubator set at 37$^{o}$C for about 18 hours. Cultured parasites were adjusted in injection solution (0.9% NaCl plus 0.2% D-glucose/dextrose) to 2 x $10^{5}$ ml $^{-1}$ before inoculation into baboons via the femoral vein (Ozwara *at al.* 2003).

### 3.4 Processing baboon red blood cells for parasite culturing

Alsever's diluted baboon whole blood was transferred from a 20ml syringe into a sterile 50ml tube and spun at 500x g for 10 minutes. Supernatant was discarded and cells resuspended in Alsever's solution twice the pellet's volume. Washing was done three times in twice the RBC pellet's volume of Alsever's solution and spun as before. Blood was mixed well to ensure proper washing. One final washing in RPMI 1640 was done as before. After the last wash, incomplete RPMI 1640 of equivalent volume to the pellet was added to the erythrocyte pellet resulting in 50% baboon RBC PCV solution. Baboon erythrocytes were stored at 4$^{0}$C and used within two weeks (Ozwara and LUMC 2005).

### 3.5 *In vitro* propagation of *Plasmodium knowlesi*

Long term *P. knowlesi in vitro* cultures were initiated with cryopreserved parasites stored in liquid nitrogen (-135 $^{o}$C). For retrieval, parasite vials were removed from liquid nitrogen and quickly thawed at 37$^{o}$C in a water bath. They were then transferred into 50 ml centrifuge tubes. Equivalent volume of 3.5% NaCl (at room temperature) was added and mixed with the parasites before centrifugation at 400x *g* at 24$^{o}$C for 10 minutes while the supernatant was sucked off and half the original volumes of 3.5% NaCl added before centrifugation as before. Next, RPMI 1640 with 10% baboon serum (equivalent volume to the first 3.5% NaCl) was added, mixed and centrifuged again. The final wash was done by adding 5x RPMI-1640 of the original volume, mixed and centrifuged as before. Parasites were grown in static cultures at 37°C under reduced oxygen conditions (5% $CO_2$, 5% $O_2$, 90% $N_2$) in RPMI 1640, supplemented with 20% pooled, heat-inactivated baboon serum, baboon erythrocytes at 2.5% haematocrit and 15µl/ml gentamycin solution. For starting and daily parasitaemia, 100-200 µl of culture was used for thin smear preparation. During the propagation period, medium was changed every 24 hours, and fresh erythrocytes added every 4 days to a maximum haematocrit of 5%. Once they had been established in culture, parasites were maintained under the same conditions except for medium changes every 48 hours and subculturing when parasitaemia exceeded 5%.

### 3.6 Cryopreservation of *in vitro* cultured *P. knowlesi*

For freezing, cultures were first centrifuged for 20 minutes at 400x *g* and the supernatant discarded. Cryoprotectant, containing 70 ml glycerol added to 180ml of 4.2% sorbitol in 0.85 % NaCl was filtered using 0.45µm pore size filters. The filtrate was added and allowed to equilibrate for 5 to 10 minutes at room temperature. Aliquots of 400µl volumes were transferred

into cryovials containing *P. knowlesi* parasites, kept at -70$^{o}$ C for one hour and then in liquid nitrogen for long term storage.

### 3.7 Preparation of *P. knowlesi* antigens for immunological assays

*Plasmodium knowlesi* H strain parasites from culture and infected baboons was used for the preparation of sonicated antigens for ELISA assays. The parasites were washed twice by centrifugation at 400x *g* for 10 minutes at 24°C with an equivalent volume of Alsever's solution. They were suspended at a final concentration of $10^{9}$ parasites/ml in incomplete RPMI 1640. This was followed by sonication at 14-18 amplitude microns for 5 periods of 45 seconds each in ice. The parasites suspension was centrifuged at 300x *g* for 30 minutes. Aliquots of the crude parasite antigens were stored at -70°C until use.

# CHAPTER 4: HAEMATOLOGICAL AND CLINICAL INDICES OF PLACENTAL MALARIA IN NON-IMMUNE PREGNANT BABOONS.

## 4.1 Introduction

Changes in haematological parameters can be influenced by infectious diseases such as malaria. Malaria is a major cause of mortality and morbidity in tropical areas of the world (Kotepui *at al*. 2014). Pregnant women are more likely to develop symptomatic malaria than their non-pregnant counterparts leading to several complications that affect both the mother and her infant. Malaria during pregnancy is therefore a serious problem in sub-Saharan Africa (Davison *at al*. 1998). Haematological and clinical changes are some of the major complications associated with the pathogenesis of malaria as a disease (Bakhubaira 2013). Fever (general increase in body temperature), weight loss, diarrhea and loss of appetite are some of the common signs and symptoms associated with the disease. These are sensitive measures of malaria but lack predictive values especially during pregnancy (Maina *at al*. 2010). This makes it very difficult to distinguish the signs and symptoms of malaria infection during pregnancy (i.e., placental malaria) from other viral and bacterial infections. Nonetheless, microscopic examination of peripheral blood remains to be the most common and widely used test since it is the gold standard for detecting malaria infection (Lathia and Joshi 2004).

For women living in endemic areas, high frequency and density of *P. falciparum* parasitaemia, morbidity, fever, severe anaemia and PM are a common phenomenon (Uneke 2007; Menendez 1995; Brabin and Diseases 1991). Although *P. falciparum* malaria is usually asymptomatic, it contributes to adverse perinatal outcomes with a high risk of infant death (Steketee *at al*. 2001b).

This chapter aims to assess the occurrence of clinical signs (fever, decrease in weight, loss of appetite) and changes in haematological parameters (Haemoglobin, red blood cells and white blood cells) associated with PM in non-immune baboons infected with *P. knowlesi* at first, second and third trimesters.

## 4.2 Materials and methods

Pregnant adult baboons in first trimester (Pan3459, Pan3579 and Pan 3615), second trimester (Pan3415, Pan3493 and Pan 3497) and third trimester (Pan3414, Pan3443, and Pan3592) were infected with *P. knowlesi* late in their respective gestational stages and analyzed for clinical, haematological and parasitaemia changes. In *P. anubis*, first trimester is from day 1 to 60; second trimester is from day 61 to 120 while third trimester is from 121 to 180±7 days of gestation. The animals were followed up and sampled bi-weekly. Whole blood was collected in EDTA tubes and used for heamatological analysis and compared with control (non-infected) pregnant baboons (Pan3335, Pan3444 and Pan3451).

### 4.2.1 Infection of baboons with *P. knowlesi* H strain parasites

Overnight cultured *P. knowlesi* parasites were used to initiate blood stage malaria infection in baboons. Two culture flasks were pooled for parasitaemia counts determined before spinning at 400x *g*, 24 $^{0}$C for 10 minutes. The parasites were resuspended to a population of (2.0x $10^{5}$ parasites/ml) in incomplete RPMI 1640.The baboons were anaesthetized and bled for baseline blood samples before infecting with 1 ml of the inoculum.

### 4.2.2 Observation of parasitaemia and giemsa staining

Parasitaemia was evaluated every 24 hours for every culture flask. Labeled duplicate slides were prepared. First, cultures were gently mixed and then centrifuged for 1 minute at 1200x *g* in a micro centrifuge. The supernatant was removed and the pellet mixed to dislodge it. A drop (5-10µl) was then placed on a microscope slide and a thin smear made. The smear was air dried for 5 minutes then fixed in 100% methanol. The fixed slides were stained for 10 min in 10% Giemsa solution. At least 2000 RBCs were counted in every parasitaemia count session. This was done by counting erythrocytes from a quarter of each field and multiplying by four. Parasitaemia was calculated as follows; the total number of infected erythrocytes counted in a minimum of 2000. Parasitaemia % = (number infected erythrocytes counted ÷ Total number of erythrocyte counted) x 100. To determine the levels of developmental stages of the parasites, differential count was also performed for rings, trophozoites and schizonts stages. Once the parasites were established in the culture, they were maintained under same conditions except for medium changes every 48 hours and sub culturing done whenever parasitaemia exceeded 5%. Parasites were preserved at the young ring stage of development.

### 4.2.3 Clinical monitoring of infected baboons

Pregnant baboons were monitored for changes in axilliary temperature, body weight, demeanor, stool and appetite. Daily parasitaemia was observed every 24 hours beginning on day 2 PI by finger prick method. Double smears were prepared for each thin smear preparation session. A baboon's finger was cleaned by alcohol swabbing followed by pricking with a sterile needle. A drop of blood from the pricked finger was transferred onto slides using capillary tube. Parasitaemia determination was done as described in section 4.2.2.

### 4.2.4 Determination of haemoglobin concentration

Two capillary tubes were filled with well mixed Ethylenediaminetetraacetic acid (EDTA) whole blood by capillary action to about three quarters of each tube. One end of the capillary tube was sealed with sealant until the plug is 4-6 mm long. Two tubes were placed in the radial grooves of the haematocrit centrifuge head exactly opposite each other with the sealed end facing away from the centre of the centrifuge. Centrifugation was done for three minutes. The tubes were removed from the microhaematocrit centrifuge as soon as it stopped spinning. The packed cell volume (PCV) of each tube was read using a PCV reader as follows: The centrifuged haematocrit tubes were first placed into the groove of the sliding capillary tube holder facing upwards. The haematocrit tubes were adjusted such that the bottom line (0%) ran exactly at the inter-phase of the sealant and the packed cells. The sliding tube holder was adjusted until the end of the plasma column touched the 100% mark of the PCV reader. The packed cell volume of the sample was read and reported as a percentage. Haemoglobin concentration was calculated by dividing PCV by three based on the formula 3Hb= PCV.

### 4.2.5 Red blood cell counting

Test tubes were labeled and in each tube 2mls of formol citrate added. A 10µl volume of well mixed whole blood was added and mixed well (a dilution of 1 in 200). After assembling the haemocytometer, the diluted blood sample was remixed. Using a capillary held at 45°on each of the grids of the chamber was filled with the diluted blood. The chamber was left undisturbed for 5 minutes for red cells to settle in the humid chamber. The underside of the chamber was wiped then mounted onto a microscope stage. Using 10 x objective, cells in the four corner squares and the central square were counted. The number of red cells per liter of blood was calculated as

follows: (N x D x $10^6$) ÷ V. Where N is the number of cells counted, D is the dilution factor and V is the volume of the area of the chamber counted. The results were reported as cells/$mm^3$.

### 4.2.6 Total leukocyte count

Test tubes were labeled with animal numbers and 380µl of Turks solution dispensed in each tube. A 20µl volume of well mixed whole blood was added and mixed well. The haemocytometer was then assembled as described in 4.2.5 above. The slide was moved on the stage to examine the smear's body and with the help of a tally counter; different types of leukocytes counted until a total of 100 leukocytes were counted. The morphology of different leukocytes, lobulation and appearance of granules were noted.

## 4.3 Results

### 4.3.1 Clinical outcome of non-immune baboons at different trimesters

All the infected animals remained active and appeared normal with bright demeanor from the point of parasite inoculation to day 3 and 5 post infection (PI) in early (first and second trimester) and late gestational age (third trimester) respectively. The animals became dull (low apathy) after infection but improved gradually after treatment with Pyrimethamine (dose of 1mg/Kg body weight). Control animals remained active throughout the experimental period.

Prior to infection, the baboons had good body condition characterized by healthy and shiny fur. Their body condition deteriorated following infection and but improved after treatment. Stool was normal in consistency but after *P. knowlesi* infection it changed to "loose". Following

treatment, their stool became normal. Control animals had good body condition and normal stool through the experimental period.

Their appetite was graded based on the daily portion of food they consumed from the total amount of food given (750g). Good appetite was considered if the animal consumed 75-100% of their food portion, fair if 50-74% of food was consumed and poor if 0-49% of food was consumed. Although there was a general decrease in appetite by day 2 PI in the infected animals (0.99, 0.79 and 0.46 fold decrease in first, second and third trimester infected pregnant baboons respectively), control, first trimester and second trimester baboons had good appetite throughout the experimental period (Figure 7) while third trimester animals had poor appetite that improved after treatment (day 7PI).

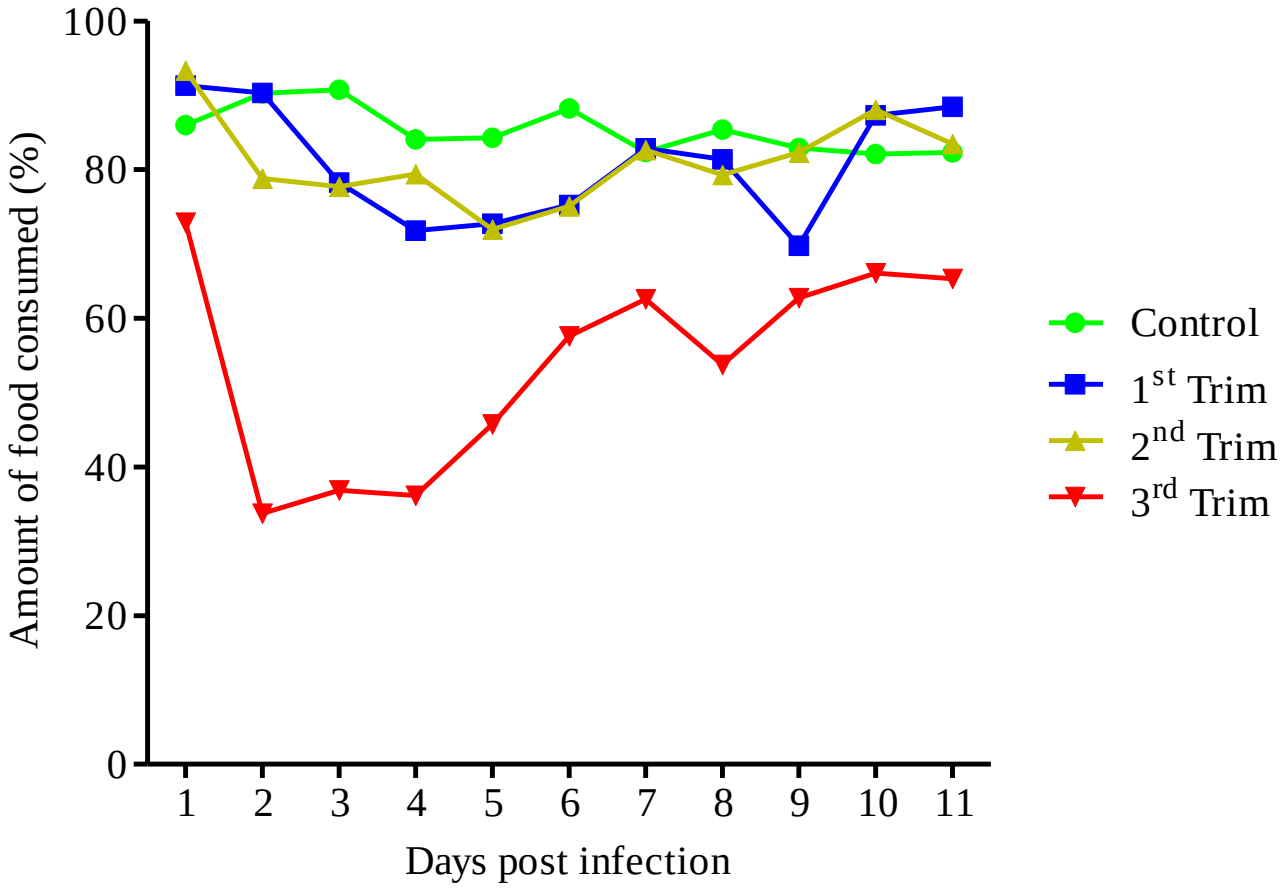


**Figure 7: Appetite levels in pregnant baboons. Appetite was measured based on the amount of food consumed by pregnant baboons infected at first (1st) second (2nd) and third (3rd) trimesters (Trim).**

Changes in clinical and haematological parameters were compared between baseline and one week post infection and presented as either percent decrease or increase (Table 1). There was a general weight decrease in the experimental animals. Third trimester animals had the highest percent decrease (11.3%) while first trimester animals had the lowest percent change (4.4%). Compared to controls, the observed weight decrease was significantly different at first (Student *t*

test, p= 0.0001), second (Student *t* test, p= 0.0037) and third (Student *t* test, p= 0.0061) trimesters (Figure 8)

**Table 1: Changes in clinical and haematological parameters in pregnant baboons observed during first, second and third trimesters at baseline (BS) and after *P. knowlesi* experimental infection (AI).**

| Animal Number | Group | Time point | Weight (Kg) | Temp (ºC) | RBC ($10^6$/µl) | Hb (gm/dl) | WBC (%) |
|---|---|---|---|---|---|---|---|
| Pan3459 | 1st Trimester | BS | 12.0 | 37.2 | 4.8 | 12.6 | 8.2 |
| | | AI | 11.5 | 37.6 | 3.12 | 12.2 | 12.6 |
| Pan3579 | 1st Trimester | BS- | 13.4 | 37.9 | 5 | 11.5 | 8 |
| | | AI- | 12.8 | 38.1 | 3.86 | 10.9 | 13 |
| Pan3615 | 1st Trimester | BS- | 14.6 | 37.0 | 2.5 | 13.4 | 9.2 |
| | | AI- | 13.9 | 37.7 | 3.6 | 12.8 | 9.4 |
| Pan3493 | 2nd Trimester | BS- | 11.7 | 37.2 | 5 | 13.7 | 12.5 |
| | | AI- | 10.5 | 37.6 | 4 | 12.9 | 12.8 |
| Pan3415 | 2nd Trimester | BS- | 13.6 | 37.4 | 4.6 | 14.2 | 9.4 |
| | | AI- | 12.7 | 37.8 | 3.9 | 13.5 | 9.6 |
| Pan3497 | 2nd Trimester | BS- | 14 | 37.9 | 4.6 | 13.9 | 6.7 |
| | | AI- | 12.9 | 38.1 | 3.58 | 13.4 | 9.3 |
| Pan3414 | 3rd Trimester | BS- | 14.6 | 37.0 | 3.8 | 11.8 | 13.8 |
| | | AI- | 13.4 | 37.6 | 2.9 | 9.9 | 17.1 |
| Pan3443 | 3rd Trimester | BS- | 15.1 | 37.0 | 3.5 | 11.4 | 9.6 |
| | | AI- | 13.5 | 37.7 | 2.7 | 10 | 13.4 |
| Pan3592 | 3rd Trimester | BS- | 13.3 | 37.2 | 4.1 | 12.4 | 12.4 |
| | | AI- | 11.4 | 37.7 | 3.0 | 10.5 | 16.9 |
| Pan3335 | Control | BS- | 11 | 37.6 | 3.6 | 13.6 | 10.2 |
| | | AI- | 10.8 | 37.5 | 3.3 | 12.8 | 11.1 |
| Pan3444 | Control | BS- | 13.2 | 37.2 | 2.8 | 12.1 | 16.9 |
| | | AI- | 13 | 37.3 | 2.6 | 11 | 19.5 |
| Pan3451 | Control | BS- | 12.4 | 37.4 | 4.1 | 11.8 | 10.4 |
| | | AI- | 12.2 | 37.6 | 3.7 | 11.2 | 12.5 |

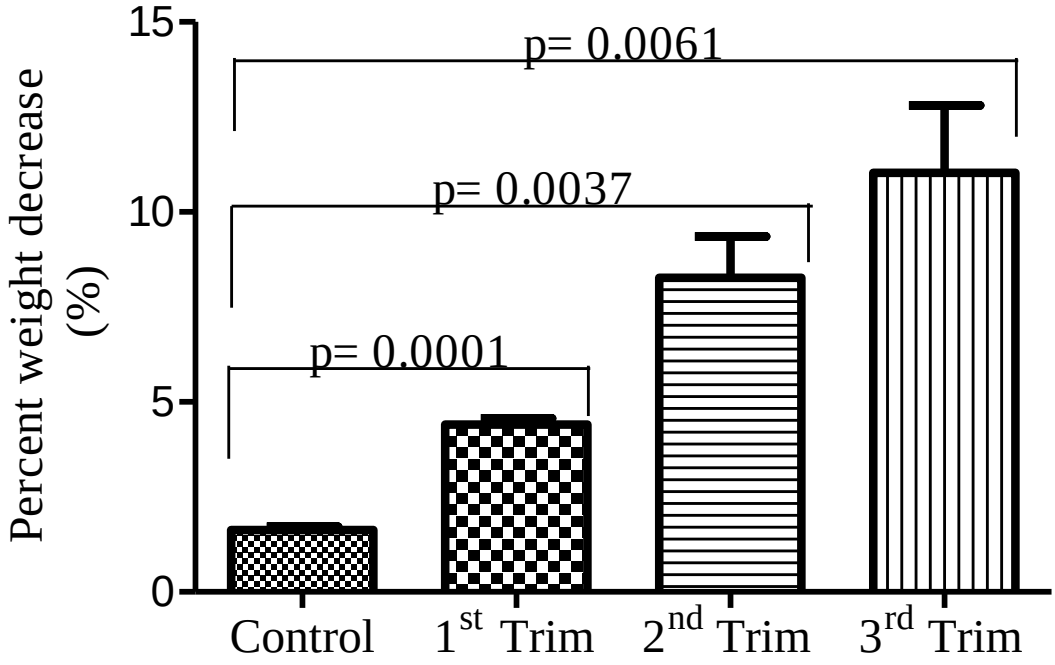


**Figure 8: Change in body weight of *P. knowlesi* infected pregnant baboons at different trimesters. Trim means trimester.**

A general increase in axilliary temperature was also observed (Table 1). On average, third trimester animals had the greatest percent increase (1.62%) while first trimester had the least (0.17%). The observed changes in *P. knowlesi* infected baboons was significantly different at second (Student *t* test, p= 0.0024) and third (Student *t* test, p= 0.0022) trimesters compared to non-infected controls (Figure 9).

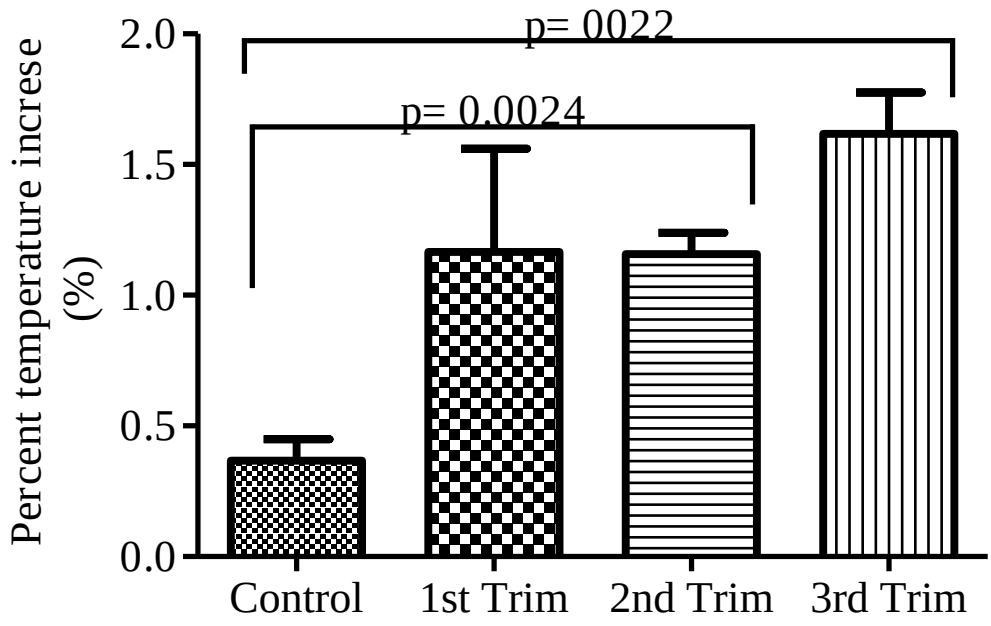


**Figure 9: Change in axilliary temperature of *P. knowlesi* infected pregnant baboons at different trimesters. Trim means trimester.**

### 4.3.2 Haematological changes in non-immune baboons at different trimesters

Haematological parameters like RBC, Hb and WBC indices in pregnant baboons experimentally infected with *P. knowlesi* at first, second and third trimester were analyzed and compared with non-infected controls. Levels of RBC and Hb indices reduced in the infected animals after experimental infection (T1), and then increased slightly after treatment (T2) as demonstrated in with *P. knowlesi* (Table 1). Decreased levels of RBC was significantly different in baboons infected at first (Student *t* test, p= 0.0004), second (Student *t* test, p= 0.0081) and third (Student *t* test, p= 0.0146) trimesters compared to non-infected pregnant controls. Haemoglobin (Hb) levels were significantly different in third trimester (Student *t* test, p= 0.0046) compared to controls. The observed changes in Hb at first and second trimester were not significant (Figure 10).

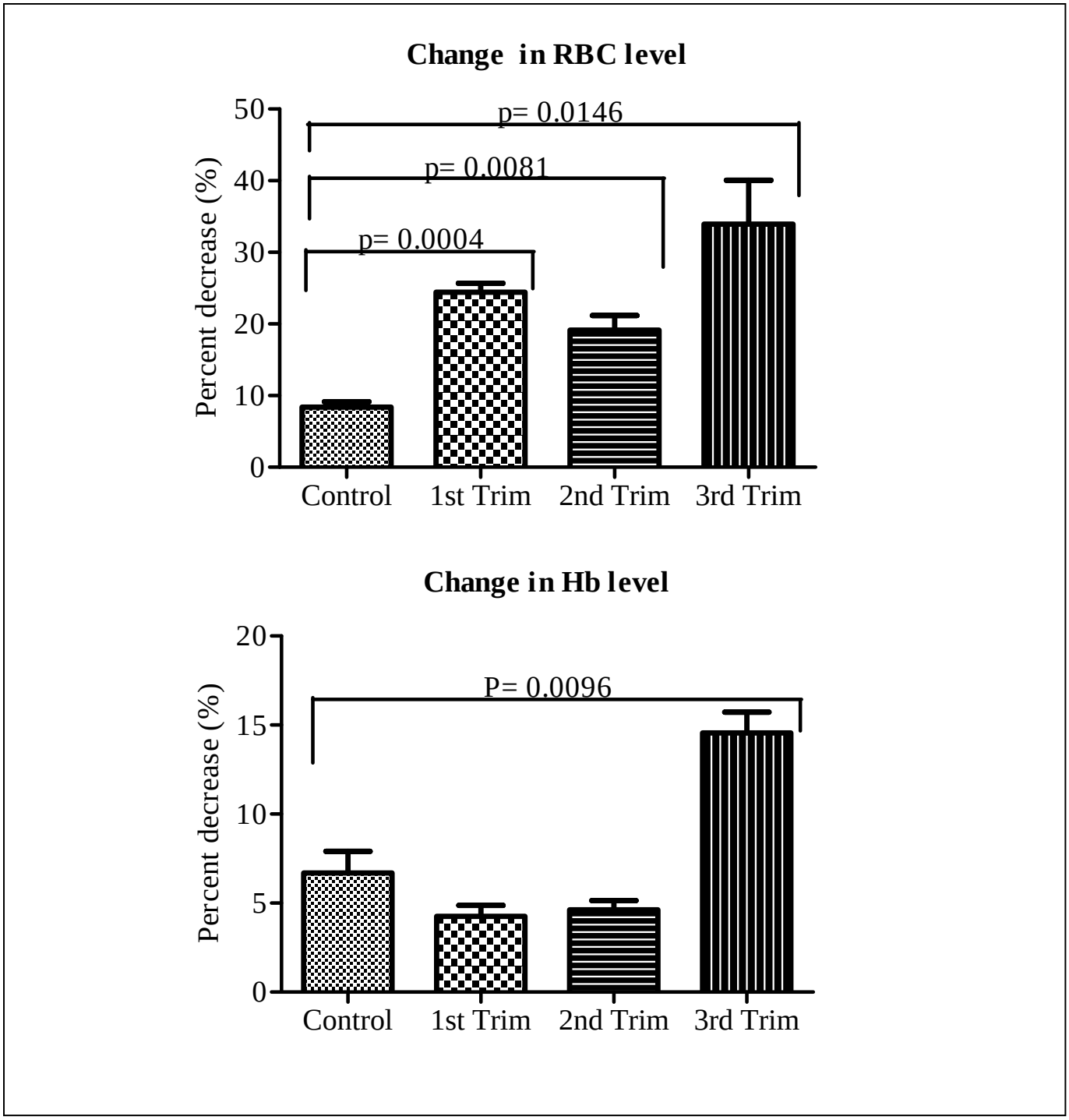


**Figure 10: Change in red blood cell (RBC) and haemoglobin (Hb) levels in *P. knowlesi* infected pregnant baboons and non-infected controls.** Trim means trimester.

There was an increase in the level of WBC in the infected baboons at first (19.72%) and third (33.26%) trimesters. Further analysis demonstrated a significantly different change in the level of WBC at third trimester (Student *t* test P= 0.00334) than non-infected controls (Figure 11).

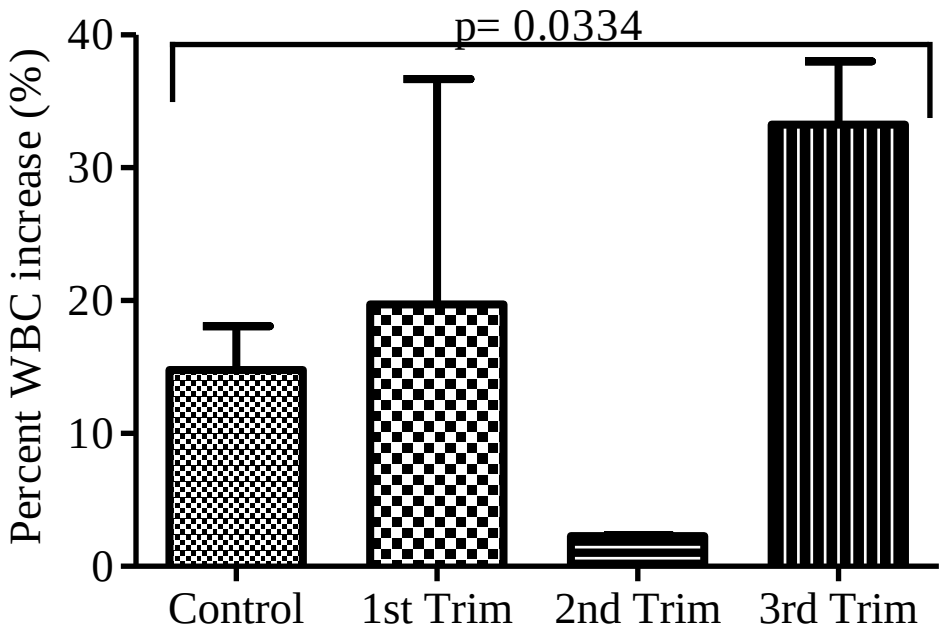


**Figure 11: Change in WBC levels of *P. knowlesi* infected pregnant baboons at different trimesters. Trim means trimester.**

### 4.3.3 Parasitaemia profile in *P. knowlesi* infected non-immune baboons

Pregnant baboons were infected with *P. knowlesi* malaria parasites ($2x10^5$) in the first, second and third trimesters. Patency was observed on day 2PI during early stage of pregnancy (first and second trimesters) and day 4PI in third trimester (Table 2). Peak parasitaemia in third trimester and second trimester was on average three fold and two fold higher than first trimester respectively (Figure 13).

This study observed 25% preterm deaths, two (Pan3579 and Pan3592) in first trimester and one (Pan3492) in second trimester animals. Parasitaemia detection in cord blood and the delivered foetuses by microscopy were negative for malaria suggesting that absence of congenital malaria in their foetuses.

**Table 2: Parasitaemia post infection levels in *P. knowlesi* infected baboons at different trimesters**

| Experimental Group | Patency | Peak parasitaemia/µl of blood | Peak parasitaemia reached |
|---|---|---|---|
| **1st Trimester** | Day 2 PI | $3.7x10^4$ | Day 7PI |
| **2nd Trimester** | Day 2PI | $8.0x10^4$ | Day 7PI |
| **3rd Trimester** | Day 4PI | $1.4x10^5$ | Day 8PI |

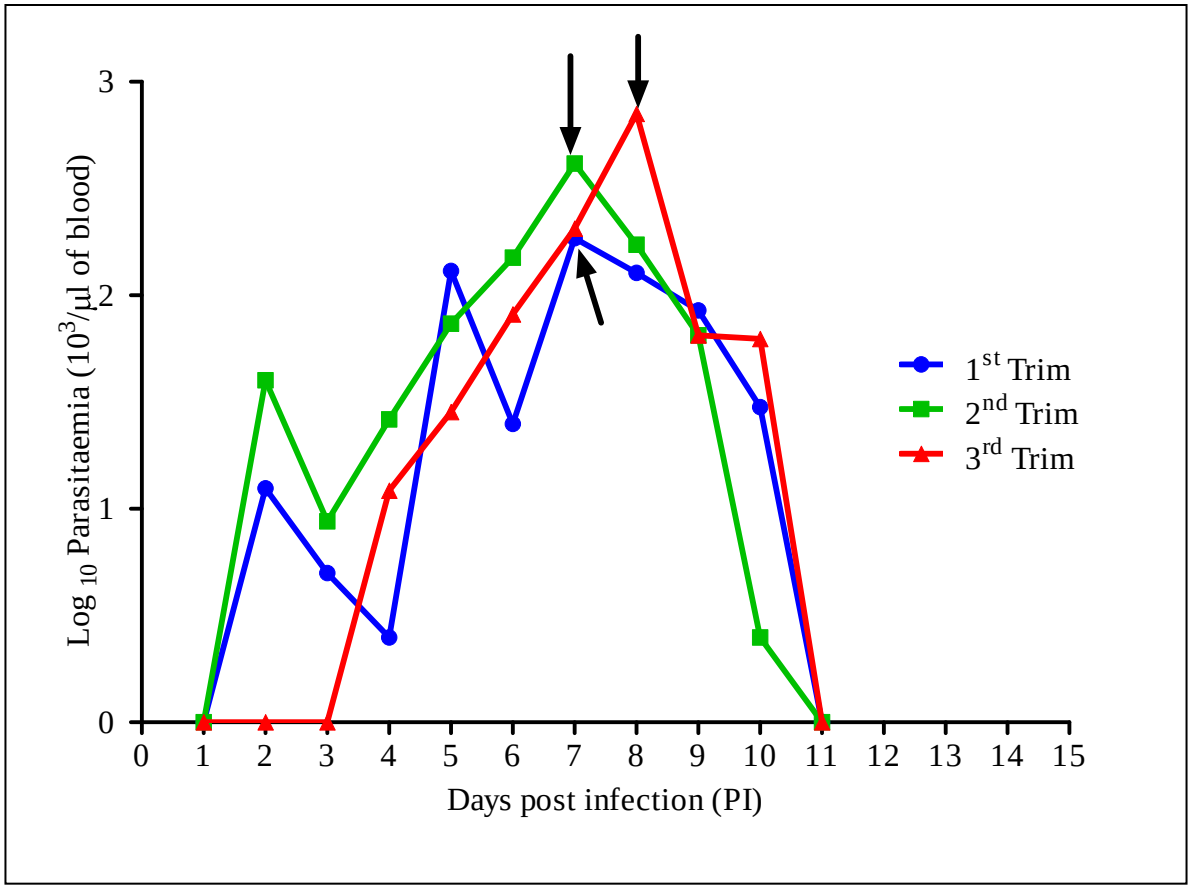


**Figure 12: Parasitaemia profile for *P. knowlesi* infected baboons at different trimesters (Trim). Arrows indicate time point of treatment with Pyrimethamine (10mg/Kg body weight).**

### 4.4 Discussion

Pregnant baboons were infected with *P. knowlesi* parasites in their first second and third trimesters and their clinical changes and haematological profiles observed and compared to non-infected pregnant controls. This study demonstrated that increased parasitaemia level in *P. knowlesi* infected pregnant baboons is associated with symptoms such as poor appetite, decreased body weight, increased body temperature, reduced RBC levels, reduced Hb levels and increased WBC levels. These findings were apparent during third trimester and correlate well with human studies where *falciparum* malaria infection in pregnant women is commonly

associated with increased body temperature and reduced RBC and Hb levels (Luxemburger *at al*. 2001).

This study demonstrated that pregnant baboons experience diarrhea (loose stool), poor appetite, reduced body weight and increased body temperature after infection with *P. knowlesi* malaria parasites. These are common malaria symptoms observed in humans (Choices 2014).

There was a significant decrease of RBC levels in pregnant baboons infected with *P. knowlesi* at all trimesters compared to controls in this study. Similarly, pregnant baboons infected at third trimester showed significant reduction in Hb levels. Low levels of RBC and Hb are characteristics of anaemia. Anaemia is one of the most common complications of malaria in pregnant woman living in high transmission areas (Menendez, Fleming, and Alonso 2000). Although the pathogenesis of malaria infection during pregnancy is still not clearly understood, it is believed that RBC destruction, accelerated removal of both parasitized and non-parasitized RBCs as well as parasitaemia levels are the main determinants (Kitua *at al*. 1997; Haldar and Mohandas 2009). As a result, patients may suffer from either severe anaemia due recrudescence of malaria infection (Phillips and Pasvol 1992), or may develop mild anaemia in immune or semi-immune populations(Newton *at al*. 1997). In fact, it is estimated that approximately 500,000 pregnant women develop severe anaemia due to *falciparum* malaria contributing up to 10,000 maternal anaemia-related deaths in sub-Saharan Africa (Uneke 2007; Steketee *at al*. 2001b; Guyatt and Snow 2004). In the past, there has not been a correlation in individuals between Hb levels and parasitaemia index during pregnancy, although women with malaria are often anaemic (B. J. Brabin 1983). In this study, reduced levels of RBC and Hb were apparent

during *P.knowlesi* infection in third trimester which also demonstrated higher parasitaemia levels compared to the first and second trimester infections. These findings correlate well with human studies which have demonstrated that high parasitaemia density contributes to reduced Hb levels (McElroy *at al*. 2000). According to Chritophers (Christophers 1924), Hb levels were lower in immune adults who had lower parasitaemia levels.

This study observed increased levels of WBC in pregnant baboons infected with *P. knowlesi* at first and third trimesters, while infection at second trimester demonstrated a reduction in WBC levels. These findings are similar to those observed in human studies which have demonstrated mixed trends in WBC levels during malaria infection. Generally, malaria infected patients tend to have significantly lower WBC levels in comparison to non-malaria infected patients (Erhart *at al*. 2004), although some studies have demonstrated higher WBC counts in infected patients compared to community controls (Ladhani *at al*. 2002). The increased levels of WBC in peripheral circulation observed in this study suggest that *P. knowlesi* malaria is associated with cell-mediated immunosuppression. Immunosuppression during pregnancy is important in maintaining the foetal allograft. Although some murine and human studies have demonstrated that suppression of cell-mediated immune responses plays a major role in the increased susceptibility to malaria during pregnancy, their findings have been based on histopathological tissues and not peripheral circulation (Ordi *at al*. 1998; Ismail *at al*. 2000; Leopardi *at al*. 1996; Walter, Garin, and Blot 1982). This study however, bases its findings on WBC levels in peripheral circulation of *P.knowlesi* infected pregnant baboons.

Although this study observed 25% preterm deaths during early gestation (first and second trimesters), peripheral parasitaemia was highest in third trimester. The processes involved in placental development are highly regulated and continues throughout pregnancy to ensure normal growth of the developing fetal tissues and the maintenance of a healthy pregnancy. The placenta also acts as an interface link between maternal and fetal tissues playing critical roles such as preventing allograft rejection of the fetus, ensuring fetal nutrient supply along with respiratory gas exchange and enabling the transfer of fetal toxic metabolic waste into the maternal blood circulation for elimination (Ahokas and McKinney 2009). This explains in part, the observed preterm deaths in *P. knowlesi* infected pregnant baboons as first and second trimester since the placenta at these pregnancy stages is still not fully developed.

As an immune privileged site, the placenta is ideal for the accumulation of cytoadherent *Plasmodium* malaria parasites, causing adverse pregnancy outcomes like preterm delivery (Brabin *at al*. 2004). Semi-immune women (i.e., those residing in areas of unstable malaria transmission) often have higher placental parasitaemia levels than peripheral parasitaemia levels (Uneke 2007). This phenomenon explains in part, the lower peripheral parasitaemia in first and second trimester in association with preterm deaths observed this study.

Since there are no predictive values of haematological and clinical indices associated with malaria and pregnancy (Maina *at al*. 2010), this study suggest that decreased body weight ≥ 4%, increased body temperature ≥1%, reduced RBC count ≥19%, reduced Hb count ≥ 4% and

increased WBC count ≥19% in combination with poor appetite, diarrhea and peripheral parasitaemia are predictive for placental malaria in *P. knowlesi* baboons.

# CHAPTER 5: PATHOGENESIS OF PLACENTAL MALARIA IN NON-IMMUNE OLIVE BABOONS

## 5.1 Introduction

The sequestration of malaria parasites in the placenta is one of the major manifestations of PM in humans. It is possible to detect placental infection even in the absence of peripheral blood parasitaemia (Uneke 2007). A number of studies aimed at assessing the prevalence and risk factors associated with PM in sub-Saharan Africa have been conducted. These studies have yielded inconsistent results due to differences in sample collection, analysis and definition of PM (Brown J Okoko *at al*. 2002; Tako *at al*. 2005). However, most of these studies relied on the results of the placental blood smear whose sensitivity is lower compared to placental histopathology (Shulman and Dorman 2003; van Geertruyden *at al*. 2004). Placental histology is therefore considered as the gold standard for malaria diagnosis during pregnancy since it is able to show signs of active, past and chronic infection (Moshi, Kaaya, and Kitinya 1995).

The pathogenesis of PM is only partially understood (Brabin *at al*. 2004). Therefore, evaluation of PM requires a good understanding of the questions and issues concerning both the foetus/infant and the mother. Studies on PM immunopathology have been conducted in rodents. Data obtained from these models cannot be correlated to humans because of their difference in reproductive system (Davison *at al*. 1998; King 1993). To bridge this gap, animal models such as NHPs whose reproductive system mimics the human situation are required to produce reliable data (Davison *at al*. 2000; Martin and Ramsey 1970). Previous work in the malaria laboratory at IPR has demonstrated that pregnant baboons experimentally infected by *P. knowlesi* are

susceptible to PM. This chapter therefore, focuses on the validation of this phenomenon by exploring the underlying mechanism(s) involved in this condition.

## 5.2 Materials and Methods

Ten pregnant baboons in their third trimester (day 150) were acquired and used to determine the pathophysiology of PM in non-immune baboons. They were randomly grouped as seven infected (Pan2724, Pan3305, Pan2809, Pan3314, Pan2859, Pan3392, Pan3233) and three non-infected (Pan2870, Pan3023, Pan3035). Malaria infection was initiated with *P. knowlesi* blood stage parasites through the femoral vein on 160th day of gestation (for pregnant baboons). Peripheral smear, placental smear and histological samples were collected during the study period. Placental samples were harvested through CS and baboons treated orally with Pyrimethamine (1 mg/kg of body weight for three days). A Spearman's rank correlation was run to determine the relationship between the different variables of severity scores. Probability values of $P < 0.05$ were considered significant.

### 5.2.1 Caesarean section and collection of placental tissue and blood samples

Caesarean sections were performed according to IPR Standard Operating Procedures (SOP). After surgery the delivered infants were weighed, tagged with animal numbers and given back to their mothers for nursing. Placental tissues were weighed and placed in sterile surgery pan. Umbilical cords and chorionic membranes were stripped off and placentas rinsed and submerged in sterile saline buffers containing 0.1% heparin and 2% penicillin-streptomycin (Moore *at al*. 1999). Intervillous whole blood samples were obtained after incision of placental (maternal side) cotyledon quadrants using sterile scalpel blades for separation of IVBMC and intervillous blood

serum as described previously (Moore *at al*. 1999; Othoro *at al*. 2006). Cord blood samples were also obtained from the umbilical vein (Brustoski *at al*. 2005).

### 5.2.2 Gross pathology and sample collection

Placental tissues were examined grossly by a trained veterinary pathologist. They were observed for any form of extensive tearing, damage, or gross abnormalities and recorded on a data sheet.

### 5.2.3 Processing of placental tissue samples for histopathology

Several placental biopsy specimen of approximately 2x2 cm were obtained for each placenta sample, and fixed in 10% neutral buffered formalin in separate processing jars at room temperature. These jars were transferred to the pathology lab for processing.

During processing, formalin fixed tissues were dehydrated in varying concentrations of ethanol. They were first put in 80% ethanol for 1hour then in two washes of 95% ethanol for 4 hours and 2 hours respectively. The next three washes were carried out in absolute ethanol for 2 hours, and the rest for 1 hour respectively. Tissues were then cleared in three washes of toluene for 1hour each followed by infiltration in paraffin bath for 2 hours. Tissue embedding process was next. This process provides the sectioning medium and means for correct orientation of the tissue. An appropriate size of embedding tray was selected to ensure that the tissue is as flat as possible at the bottom of the embedding tray. Plastic embedding moulds were correctly labeled with animal number, date and tissue type. Immediately after paraffin infiltration, warm forceps were used to transfer the tissue from infiltration tray to a warm embedding tray ensuring that the layer of paraffin did not solidify around the tissue.

After fixation, samples were embedded in paraffin wax and the sectioning done using a microtome, processed, and stained with haematoxylin-eosin (H&E) stain. This involved washing sections in double distilled water, placing them in haematoxylin solution for 5 minutes then washing further in tap water. The sections were placed in 1% lithium carbonate, rinsed in tap water, put in 1% acid alcohol for a few seconds and then rinsed again in tap water before placing them in eosin for 5 minutes. Next, sections were dehydrated in absolute alcohol and xylene before mounting on DPX mountant in a fume cupboard. After the mountant had hardened sufficiently, the slides were correctly labeled with the date, animal number and tissue type before placing the slides on a slide tray. Finally, the specimens were subjected to microscopic examination at a magnification of x40 and x100.

### 5.2.4 Placental parasitaemia

For placental blood and cord blood, (collected during CS) pricking of placenta cotyledon quadrants on the maternal side using a scalpel and collection of umbilical vein blood (for cord blood) was done to obtain blood for thin smears as described in section 4.2.2.

## 5.3 Results

### 5.3.1 Gross pathology in *P.knowlesi* infected baboons

The harvested dams were maroon-red in colour with intact membranes. The maternal surface of the placenta had cotyledons while the foetal surface was densely distributed with blood vessels. Baboon placentas weighed on average 167.3g and 165.9g for the infected and non-infected groups respectively. All animals had no fibrinoids except two (Pan3233 and Pan2724) which had slightly marked fibrinoids. Slight placental calcification was observed in all groups. Cord

insertion was intact either centrally or eccentrically placed and with three blood vessels regardless of the group. Placental praevia was complete and no haemorrhage, whether retro-placental or retro-membranous was observed in all the examined placentas. These observed features present similarity of baboon placenta to human placenta.

### 5.3.2 Parasitaemia patterns in *P. knowlesi* baboon placental tissue

Thin blood smears prepared from placental maternal surface, foetal surface, cord blood and maternal peripheral circulation were observed and compared for parasitaemia levels. Placental parasitaemia (from maternal region) was on average 19-fold higher than peripheral parasitaemia in the same animal (Table 3). This difference was significant (P= 0.00823). Parasitaemia differential count demonstrated the abundance of rings and late stage parasites (schizonts) in peripheral and placental (maternal side) blood samples respectively (Figure 13). In spite of high parasitaemia levels in blood smears from placental maternal section, the absence of parasitized red blood cells in smears from cord blood and placental foetal region was observed (Table 3). This was further confirmed by histology where H&E slides showed absence of parasitized erythrocytes on the foetal vessel and placental chorionic plate (Figure 14 and 15). Infiltration of parasitized erythrocytes and inflammatory cells like macrophages, neutrophils and monocytes in the IVS was observed in the baboon placenta (Figure 14).

**Table 3: Parasitaemia levels in *P. knowlesi* infected baboons at caesarean delivery at day 160 of gestation.**

| Baboon number | Group | Peripheral parasitaemia (%) | Placental parasitaemia (cotyledon) (%) | Placental parasitaemia (chorion) (%) | Cord parasitaemia (%) | *Fold increase in placental parasitaemia |
|---|---|---|---|---|---|---|
| **2724** | PI | 0.80 | 18.31 | - | - | 22.89 |
| **3305** | PI | 0.69 | 4.46 | - | - | 6.5 |
| **2809** | PI | 0.03 | 0.74 | - | - | 24.67 |
| **3314** | PI | 0.92 | 4.8 | - | - | 5.2 |
| **2859** | PI | 0.39 | 14.25 | - | - | 36.54 |
| **3392** | PI | 0.6 | 4.3 | - | - | 7.2 |
| **3233** | PI | 0.58 | 18.23 | - | - | 31.43 |
| **2870** | PNI | - | - | - | - | - |
| **3023** | PNI | - | - | - | - | - |
| **3035** | PNI | - | - | - | - | - |

*Fold increase was calculated as placental parasitaemia (from maternal side) over peripheral parasitaemia. PI is pregnant infected group and PNI is pregnant not-infected group (parasitaemia was taken at corresponding time to PI group).

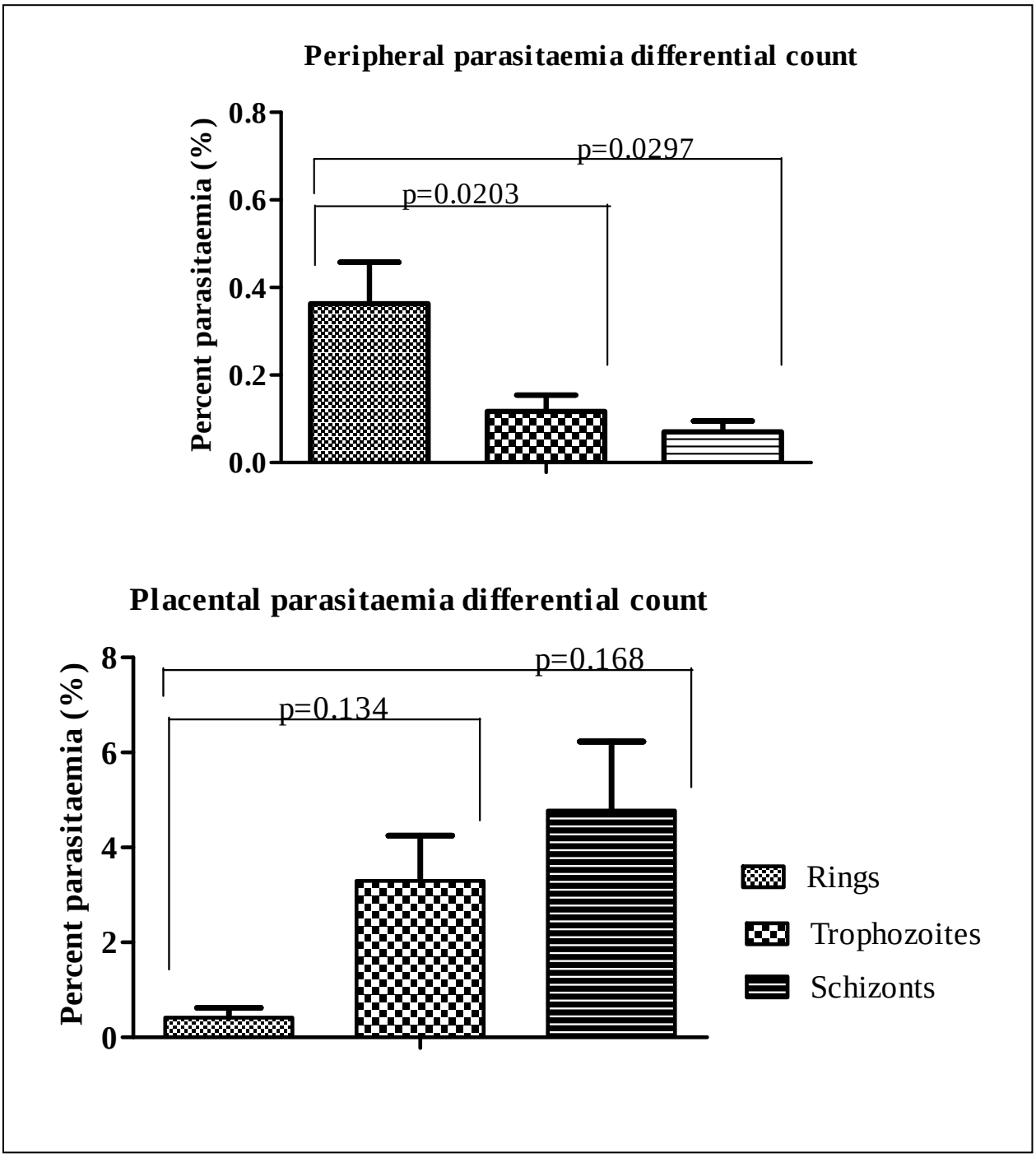


**Figure 13: Parasitaemia differential counts in peripheral and placental blood samples.** Ring stage was significantly dominant in peripheral circulation while mature parasite forms (trophozoites and schizonts) were significantly dominant in placental circulation. Bars show the mean ± SD.

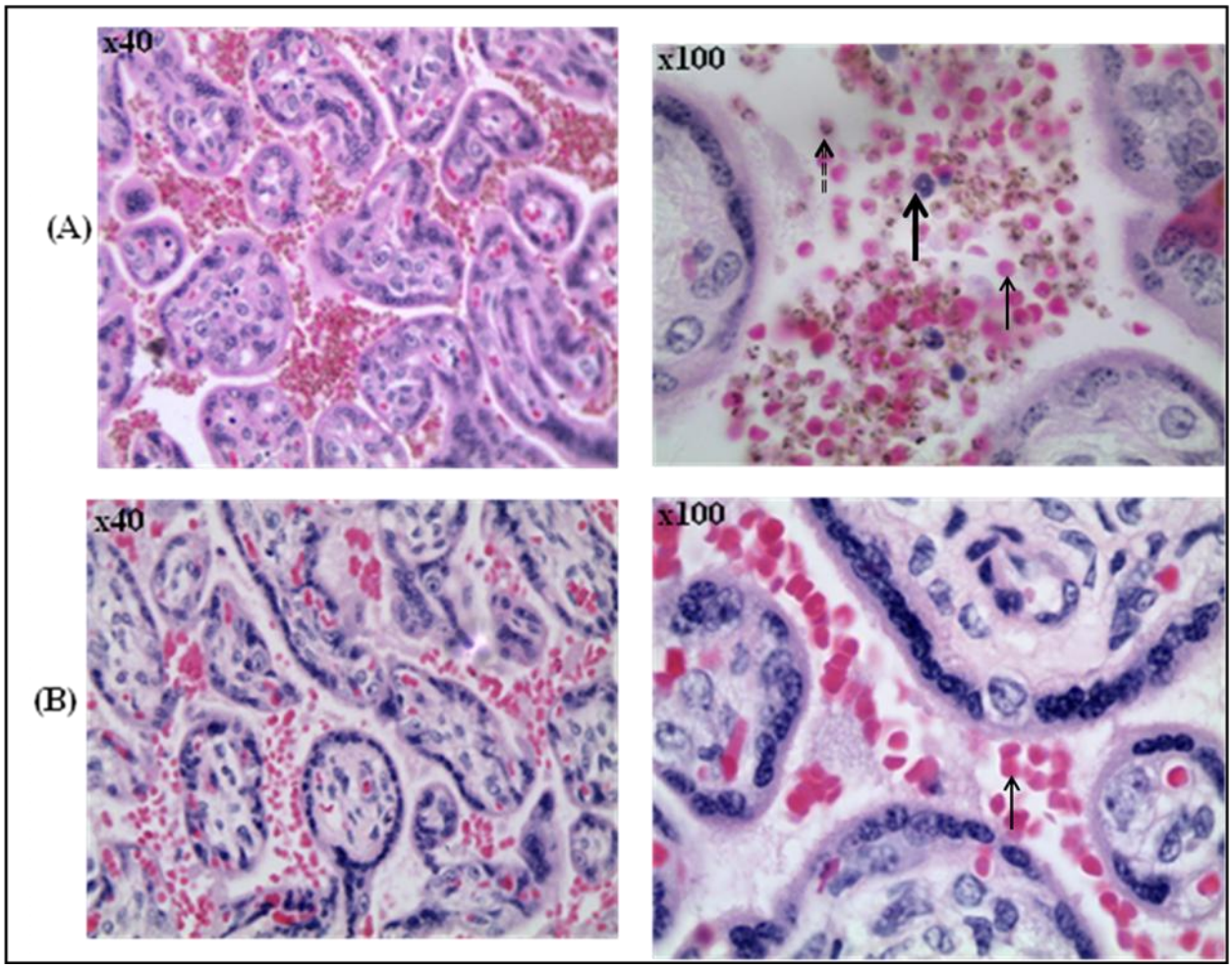


**Figure 14: Photomicrograph showing placental tissue of H&E-stained placental biopsies**. Placental tissue from *P. knowlesi*-infected baboon(A) is described by infiltration of parasitized red blood cells (broken arrow), inflammatory cells (thick arrow) and non-parasitized erythrocytes (thin arrow) compared to tissue from non-infected baboon (B). The volume of in filtered red blood cell increases in the baboon placenta following *P. knowlesi* infection.

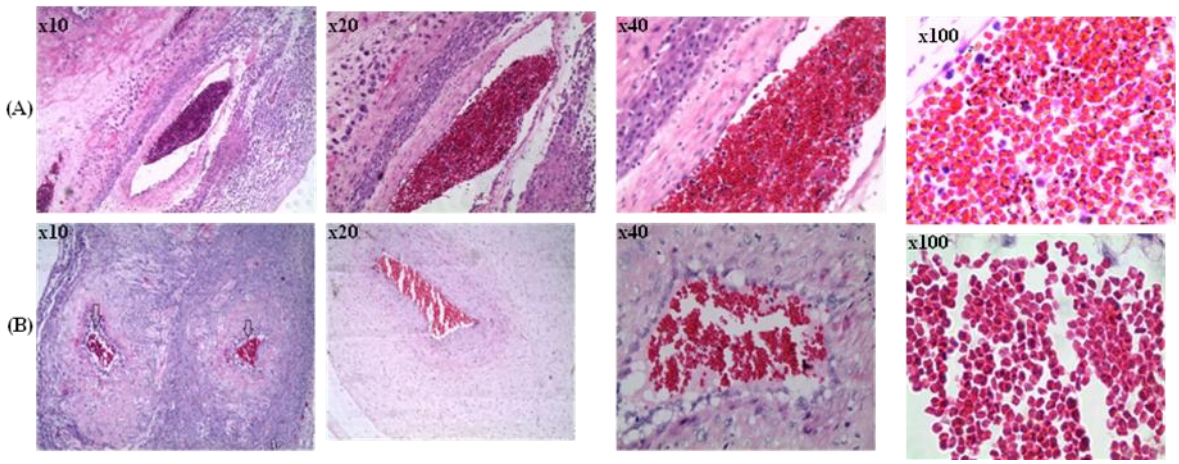


**Figure 15: Photomicrograph representing (A) placental maternal region (basal plate) and (B) placental foetal region (chorionic plate) of H&E stained placenta of *P. knowlesi* infected baboon (Pan 3233)**. Parasitized erythrocytes and immune cells are observed in (A) the basal plate (maternal side) and absence of parasitized cell in the (B) foetal red blood cells on the chorionic plate (foetal side) in slides.

### 5.3.3 Placental damage in *P. knowlesi* infected baboons

Haematoxylin and eosin (H&E) stained placental biopsies were analyzed by severity score ranging from 0 to 4, with 0 representing "none", 1 representing minimal and 4 representing the most severe according to Davison *at al.*, (Davison *at al*. 2000) with a few modifications. The total score for each parameter was recorded on all layers of the placenta and the average obtained. Infected group had significantly higher scores for damage and inflammation compared to the control group (Table 4). Total placental damage score consisted of fibrin necrosis of the villi (Figure 16), chorionic plate thrombosis, syncytiotrophoblast disruption and chorionic plate syncytiotrophoblast disruption. Malaria parasite score increased with increase in total placental damage score ($r_s$ = 0.7650, P< 0.05) and inflammatory score ($r_s$ = 0.8590, P< 0.05). This study demonstrated that placental damage and infiltration of immune cells is directly associated with *P. knowlesi* infection in baboons and subsequent sequestration in the placenta.

**Table 4: Histopathological scores from H&E stained baboon placental tissues**

| Group | Number of animals (N) | Mean total placental damage score (TPDC) | Mean malaria pigment score (MPS) | Mean inflammatory score (IS) | Mean parasitaemia score (PS) |
|---|---|---|---|---|---|
| **PI** | 7 | 12 | 5.79 | 4.71 | 7.29 |
| **PNI** | 3 | 6.67 | 0.00 | 1.5 | 0.00 |

PI means pregnant infected and PNI means pregnant not infected

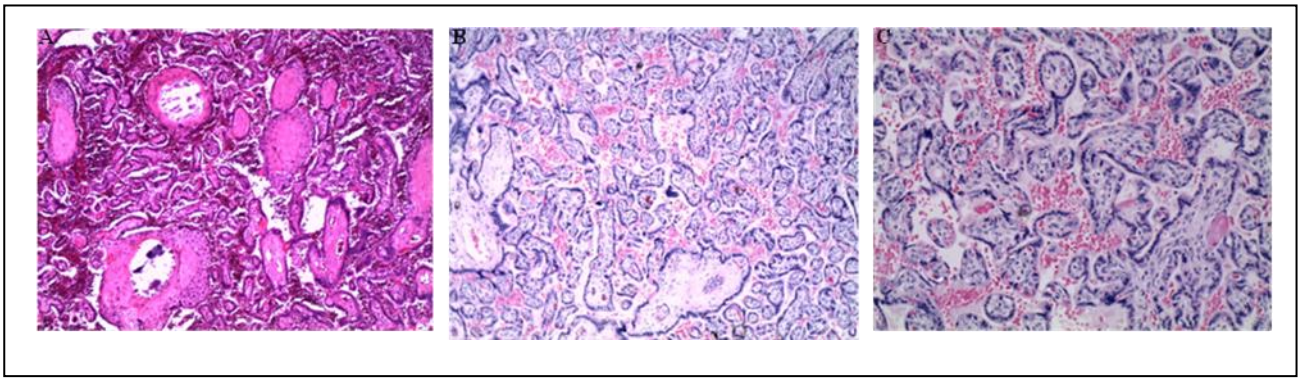


**Figure 16: Photomicrograph of villi showing fibrin necrosis in H&E placental biopsies of (A) infected and (B and C) non-infected baboon at ×10 and ×20 magnifications, respectively.** Necrosis was more severe in malaria-infected placental tissue.

### 5.3.4 Adverse pregnancy outcome

Of the ten *P. knowlesi* infected baboons, five (50%) had abortion and one (10%) had still birth. Although infants born from malaria infected baboons had lower birth weights compared to infants born from non-infected baboons, the difference was not significant (P= 0.2500).

## 5.4 Discussion

The infiltration of parasitized RBC and inflammatory cells in the placenta of non-immune baboons have for the first time been demonstrated in this study. During *P. knowlesi* infection in pregnancy, symptomatic disease is manifested in combination with increased placental parasitaemia. Consequently, accumulation of parasites in the placental IVS combined with infiltration of inflammatory cells leads to placental damage.

In this study, higher levels of placental parasitaemia compared to peripheral parasitaemia was observed in the same animal. The distribution of parasites in an infected pregnant woman varies with endemicity. The proportion of parasitized erythrocytes is often higher in the placenta than in the peripheral blood in endemic areas (Bulmer *at al*. 1993; Walter, Garin, and Blot 1982) because infected erythrocytes are preferentially retained in the placenta (Mustafa 2011; Watkinson and Rushton 1983). Haemozoin has also been frequently observed in the placentas of malaria-infected women whether in the presence or absence of peripheral parasitaemia (Bulmer *at al*. 1993; Galbraith *at al*. 1980). Findings in this study correlate well with the those observations in human studies (Uneke 2007; Uneke 2008; Adebami *et al.* 2007)

Observation of inflammatory cells (monocytes, macrophages and neutrophils) in *P. knowlesi*-infected baboon placental tissues is similar to human findings. A study conducted in a *P.falciparum* hyperendemic area in Tanzania revealed a marked increase in the levels of monocytes and macrophages and cytotoxic T cells in the IVS of placentas with active malaria infection (Ordi *at al*. 2001) .

This study has also demonstrated accumulation of parasitized erythrocytes in the IVS of the baboon placenta, in addition to high levels of mature forms of the parasite in the placenta. This suggests possible cytoadherance property of *P. knowlesi* parasites in the baboon placenta. It is documented that only mature *falciparum* malaria parasites show cytoadherance properties (Berendt, Ferguson, and Newbold 1990). These parasitized erythrocytes have the capacity to adhere in the endothelium via parasite derived proteins expressed on their surface (Hviid *at al*. 2010). In fact, syncytiotrophoblast cells of the human placenta expresses different and variable amounts of host cell receptors onto which the parasites can bind. The principal molecule that mediates adhesion of infected erythrocytes is *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1), a large, highly variant parasite antigen protein, encoded by the var multigene family (Baruch 1999). These adhesion phenotypes are not homologous and as a result different parasites can bind to various numbers and combinations of host molecules, such as chondroitin sulphate A (CSA), hyaluronic acid (HA) and Fc receptors (Beeson *at al*. 2002; Duffy 2007; Muthusamy *at al*. 2004). In seeking to identify the ligand and receptor molecules associated with accumulation of *P. knowlesi* infected erythrocytes in the baboon placenta,

chondroitin sulphate proteoglycan (CSPG) 4 and, hayaluronan and proteoglycan link protein 1 (HAPLN1) were predicted as putative receptor molecules in the baboon with high similarity to human CSA and HA respectively. In addition, *P. knowlesi* erythrocyte binding proteins (EBP-alpha, EBP-beta and EBP-gamma) matched closely to the placental *P. falciparum* ligand Var2csa (Nyamagiri *at al*. 2014).

Histopathological findings also demonstrated that PM in non-immune baboons is characterized by placental damage due to fibrinous necrosis of the villi, chorionic plate thrombosis, syncytiotrophoblast disruption, chorionic plate syncytiotrophoblast disruption, and thickening of trophoblastic basement membrane. The process was also accompanied with infiltration of inflammatory cells in the placental tissue. These findings correlate well with human studies where placental *falciparum* malaria pathology is characterized by excess perivillous fibrinoid deposits, excessive syncytial knotting, trophoblastic membrane thickening, which have been associated with destruction/damage of placental tissues, and proliferation of cytotrophoblastic cells (Davison *at al*. 1998; Martin and Ramsey 1970; Walter, Garin, and Blot 1982; Hviid *at al*. 2010; Yamada *at al*. 1989). It is hypothesized that placental damage, especially the thickening of the trophoblastic basement membrane, alters the maternal foetal exchange, leading to malaria-associated placental lesions and poor foetal outcomes (Baruch 1999). The same mechanism is likely to take place in *P. knowlesi* associated PM in non immune baboons.

Therefore this study demonstrates that during *P. knowlesi* infection, symptomatic disease is manifested in combination with increased placental parasitaemia. The accumulated parasites in the placental IVS together with infiltered inflammatory cells lead to placental damage.

Consequently, poor pregnancy outcome like maternal anaemia, abortion and still birth are observed.

# CHAPTER 6: ACQUIRED PASSIVE IMMUNITY IN BABOONS INFECTED WITH *PLASMODIUM KNOWLESI* MALARIA PARASITES

## 6.1 Introduction

Malaria being one of the most important public health problems worldwide is responsible for 8% of mortality among children below the age of five (Piñeros-Jiménez *at al*. 2011b). In spite of this, data on burden of congenital and neonatal malaria is scarce and contradictory, with some recent studies reporting a high burden. In fact, studies done in areas of high malaria endemicity demonstrated that congenital and neonatal malaria are rare in occurrences (Mwaniki *at al*. 2010).

Congenital malaria occurs when infants are infected with malaria parasites from their mother prior to or during birth. It is recognized as a serious problem in *P. falciparum*-endemic sub-Saharan Africa with prevalence ranging from 3% to 54.2%. In these regions, neonates may be protected from the disease through the transfer of maternal antibodies against the parasite. Further studies have demonstrated an association between congenital malaria with the diagnosis of malaria in the mother during the last trimester of pregnancy or during delivery, and the presence of placental infection (Piñeros-Jiménez *at al*. 2011a). These emerging issues have made PM associated with foetal outcome an important area of study.

Currently, it is evident that children born to mothers with PM are at a higher risk of acquiring malaria infections in the first 2 years of life compared to those born to women without PM (Bonner *at al*. 2005). According to immunological theories, early exposure to an antigen *in utero* can lead to immunological tolerance causing the host to be more vulnerable to the infectious

agent (McGregor 1984) . *In utero* exposure to malaria antigen has been demonstrated in humans infected with *falciparum* malaria during pregnancy (Desowitz 1988; Xi *at al*. 2003). However, the impact of PM on the development of the immune responses especially in infants is not clearly defined (Bonner *at al*. 2005).

Naturally acquired immunity (NAI) to *falciparum* malaria protects millions of people routinely exposed to this infection from severe disease and death. Protection against *P. falciparum* is acquired at a slower rate compared to *P. vivax* and *P. ovale*. On the other hand, protective immunity against *P. vivax* does not persist for long periods of time in the absence of multiple exposures. Slow onset of NAI to *P. knowlesi* malaria has been demonstrated in rhesus macaques, because of antigenic variation by the parasite (Doolan, Dobaño, and Baird 2009; Hviid *at al*. 2010). This kind of immunity develops when one is exposed to a live pathogen like malaria parasites, mounting a primary immune response that leads to immunological memory ("Immunity: Active and Passive Immunity | Infoplease.com" 2014). It can either be active or passive.

Naturally acquired passive immunity is the transfer of active immunity, in the form of readymade antibodies, from one individual to another for example, when maternal antibodies are transferred to the foetus via the placenta by FcRn receptor on placental cells. Immunoglobulin G (IgG) is the only antibody isotype that can pass through the placenta (Coico and Sunshine 2009).

This chapter focuses on how *in utero* exposure of baboon infants to maternal *P.knowlesi* infection protects them from progression of the disease by detecting IgG antibody in serum

samples isolated from infants born to mothers with and without PM, their mothers, cord blood and placental blood.

### 6.2 Materials and methods

Blood obtained from pregnant baboons infected at first, second and third trimesters as described in section 4.2 together with pregnant non infected control (Pan3174, Pan3366 and Pan3415) were isolated for serum. Pregnant baboons experimentally infected with *P. knowlesi* at third trimester together with their control counterparts were left to deliver at term. Infants from PM positive mothers (Pan 3760, Pan3775 and Pan3777) and those from PM negative mothers (Pan3772, Pan3770 and Pan3767) were challenged by *P. knowlesi* H strain infection as demonstrated in the study design in figure 6. Parasitaemia profile was observed as described in section 4.2.1. Baboon infants were treated on day 5 PI while their mothers were treated soon after delivery by Pyrimethamine (1mg/Kg body weight). Blood samples were collected once every week beginning with baseline sampling and IgG antibody levels detected by direct ELISA.

Whole blood samples drawn from the femoral vein of both mothers and infants were allowed to clot overnight at 4$^{o}$C then centrifuged (at 18-20$^{o}$C 500x *g* for 10 minutes) to separate the sera. High binding ELISA plates (Dynatech laboratories, Sussex) were coated overnight with 100µl of crude *P. knowlesi* antigen (1x10$^{6}$) diluted in bicarbonate buffer, pH 9.6. The remaining coating buffer was washed off and non specific binding sites blocked with 3% BSA in PBS for 1 hour at 37$^{o}$C. Unbound BSA was washed off six times with 0.05% Tween 20 in PBS. One hundred microliters of serum samples were dispensed into the wells and incubated for 1 hour at 37°C. Unbound serum was washed as above and 100µl of 1/2000- diluted peroxidase conjugated goat

anti-human IgG added before incubating for 1 hour at 37°C. Unbound conjugate was washed off as before followed by addition of 100µl of TMB (Sigma). The plates were incubated at 37$^{o}$C in the dark to allow for colour development. Optical densities (OD) were read at 630nm after 30 minutes in a Dynex microplate reader. Immunoglobulin G (IgG) antibody detection was also assessed in sera isolated from placental and cord blood samples.

## 6.3 Results

### 6.3.1 Maternal IgG antibody levels in baboon sera

Comparison of IgG levels in pregnant *P.knowlesi* infected baboons (PM positive) and pregnant controls (PM negative) revealed that PM positive mothers had significantly higher IgG OD levels compared to PM negative mothers (2way ANOVA P= 0.0030) as illustrated in figure 17. Further, IgG antibodies levels were detected in pregnant baboons at different trimesters in their gestation period and their OD levels compared. In the first and third trimesters IgG titers increased from baseline levels by week 1 PI then reduced by week 2 PI following treatment ( after sampling in week 1 PI). In second trimester, IgG levels were similar at baseline, week 1 PI and at week 2 PI (Figure 18). At week 1PI, IgG antibody levels were highest in first and third trimesters (OD levels 0.142 and 0.144 respectively). The observed changes were not significant.

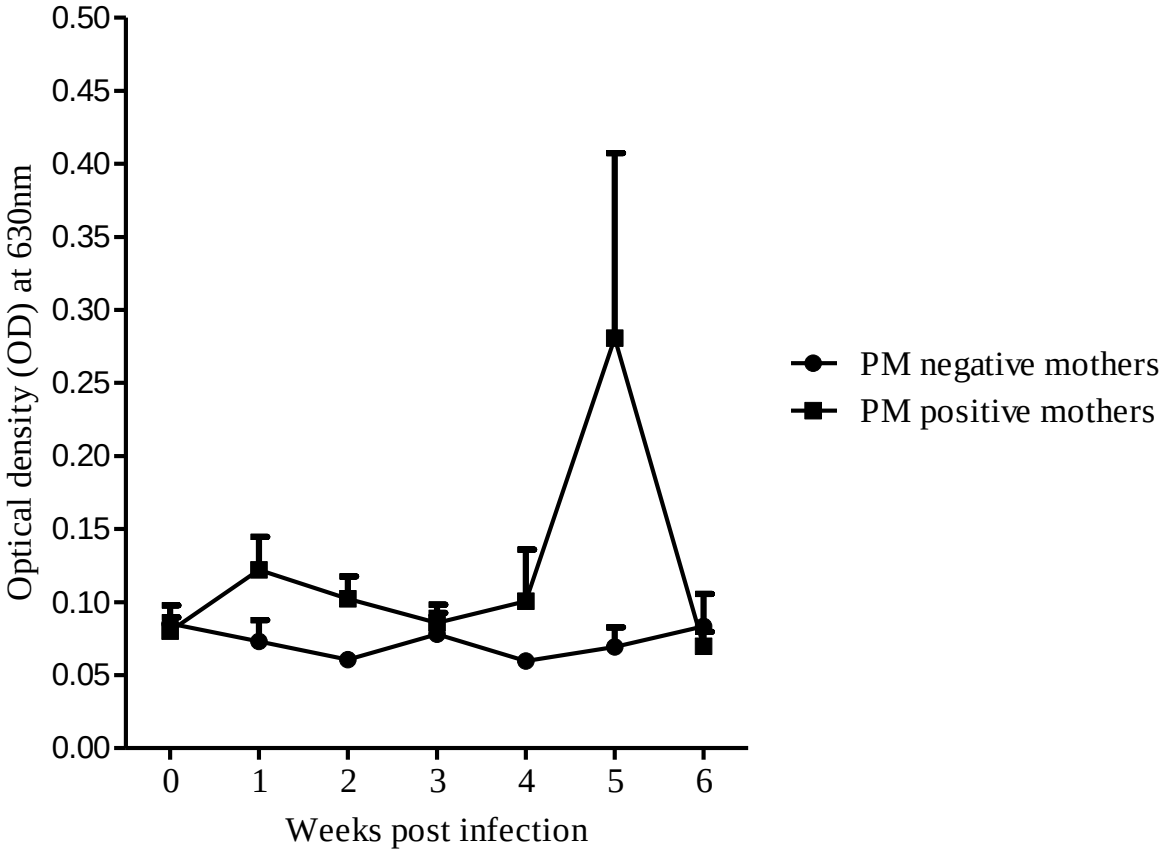


**Figure 17: Changes in IgG levels in PM positive and PM negative baboons.** Placental malaria positive mothers had higher IgG titers compared to PM negative mothers. The difference was statistically significant (2way ANOVA P= 0.0030)

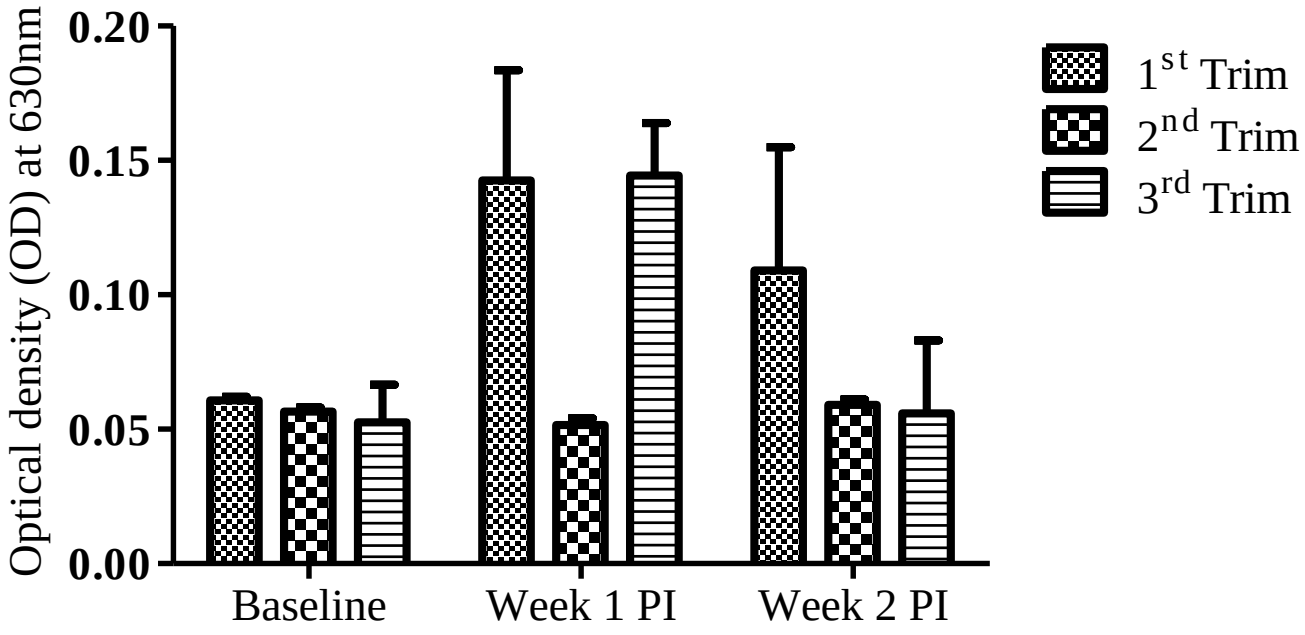


**Figure 18: Comparison of IgG levels in pregnant *P. knowlesi* infected baboons at different trimesters. Serum samples were analyzed at baseline and at 1 week and 2 weeks post infection (PI).**

### 6.3.2 Parasitaemia profile and maternal IgG levels in baboon infants

Baboon infants were infected with *P. knowlesi* blood stage parasites ($2x10^5$) one month post partum and their IgG levels detected. Patency was observed on day 3PI in both groups (group A consisting of infants born to PM positive mothers and group B consisting of infants born to PM negative mothers). Peak parasitaemia was detected on day 5PI, with group B infants having parasitaemia levels 4 fold higher (on average) than group A infants (Figure 19). This change was significant (Student *t* test p= 0.0287), suggesting the suppression of parasite propagation due to in utero sensitization by *P. knowlesi* malaria.

Differences in IgG antibody levels in infants born to PM negative baboons (group A infants) and PM positive baboons (group B infants) was also compared at baseline, one week PI and at two weeks PI. The calculated means were not significantly different (Figure 20). This implies that suppressed *P*. knowlesi parasitaemia in baboon infants was due to increased levels of maternal IgG.

Further analysis of IgG antibody levels was assessed and compared for maternal peripheral, placental, cord and infant sera samples. The IgG levels detected in peripheral maternal samples correlated with foetal blood samples (OD levels 0.085 and 0.090 respectively) which suggest that the transfer of IgG from mother to child was at equilibrium. Maternal IgG levels in sera isolated from placental foetal region, placental maternal region and cord blood had similar OD levels (0.068, 0.07 and 0.066 respectively) as illustrated in figure 21, demonstrating the sensitization of baboon infants is *in utero*.

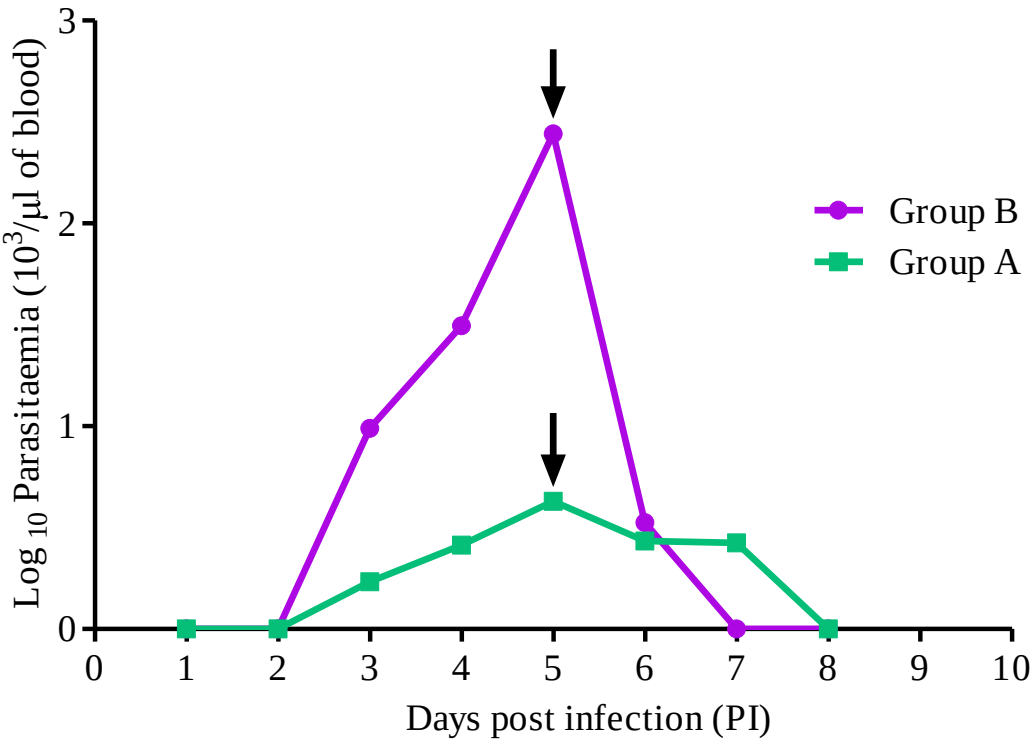


**Figure 19: Parasitaemia profile of baboon infants born to placental malaria positive mothers (A) and placental malaria negative mothers (B).** Infants were infected a few weeks after delivery and their parasitaemia levels monitored. At peak parasitaemia (Day 5PI), parasitaemia levels in group B were significantly higher than group A (P= 0.0287).

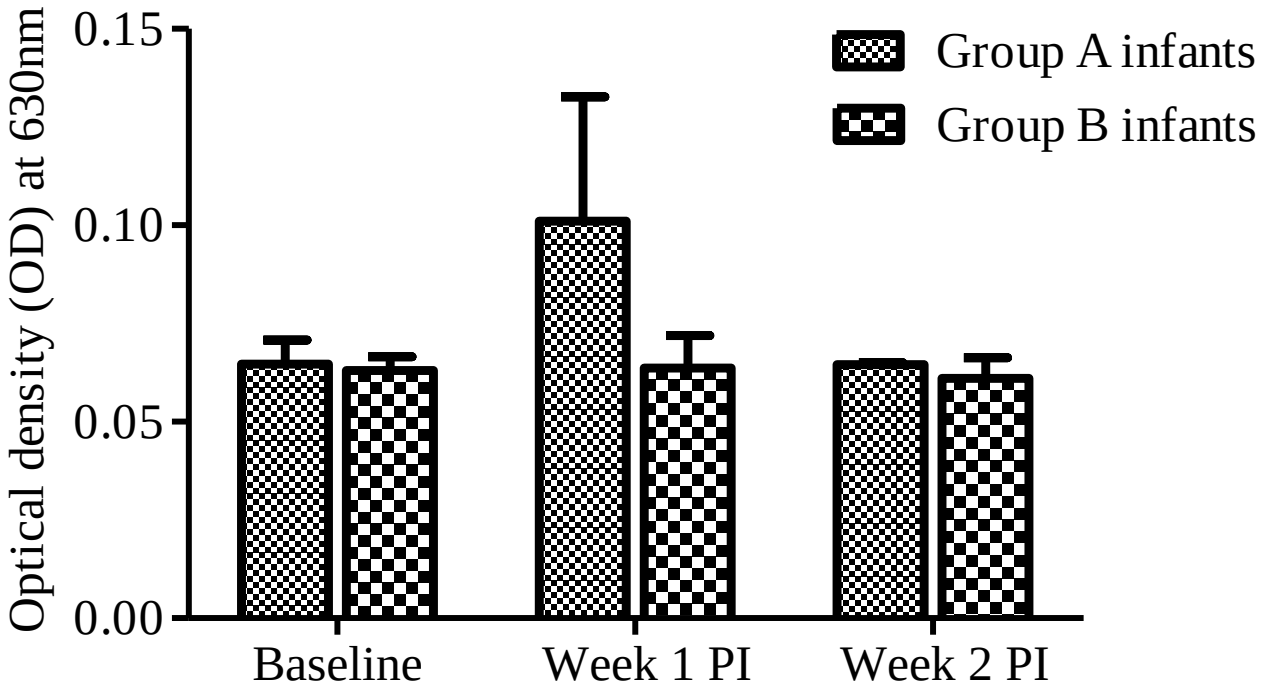


**Figure 20: Comparison of IgG levels in infants born to PM positive mothers (group A infants) and PM negative mothers (group B infants).** Serum samples were analyzed at baseline and at 1 week post infection (PI) and at 2 weeks PI. Treatment was done at week 1 PI. The difference in means was not significantly different.

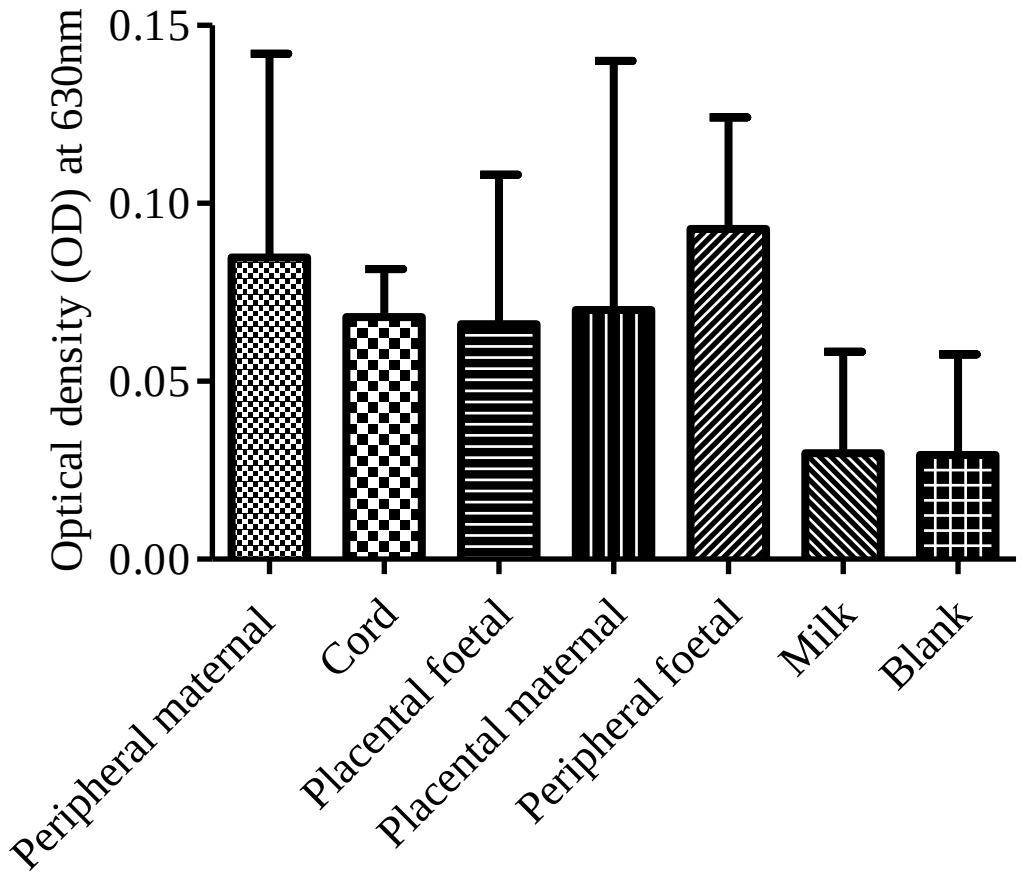


**Figure 21: Comparison of IgG levels in various sera samples isolated from pregnant *P. knowlesi* infected baboons.**

To mimic a semi-immune condition, the three pregnant baboons that were previously infected at third trimester (Pan3414, Pan3443 and Pan3592) were re-infected and their IgG levels detected and compared to their previous OD levels. Levels of IgG antibodies increased progressively after the second infection (Figure 22). Although the differences in IgG levels between first initial infection and second infection were not significantly different, the results imply that IgG levels increases with increase in exposure to *P. knowlesi* malaria parasite.

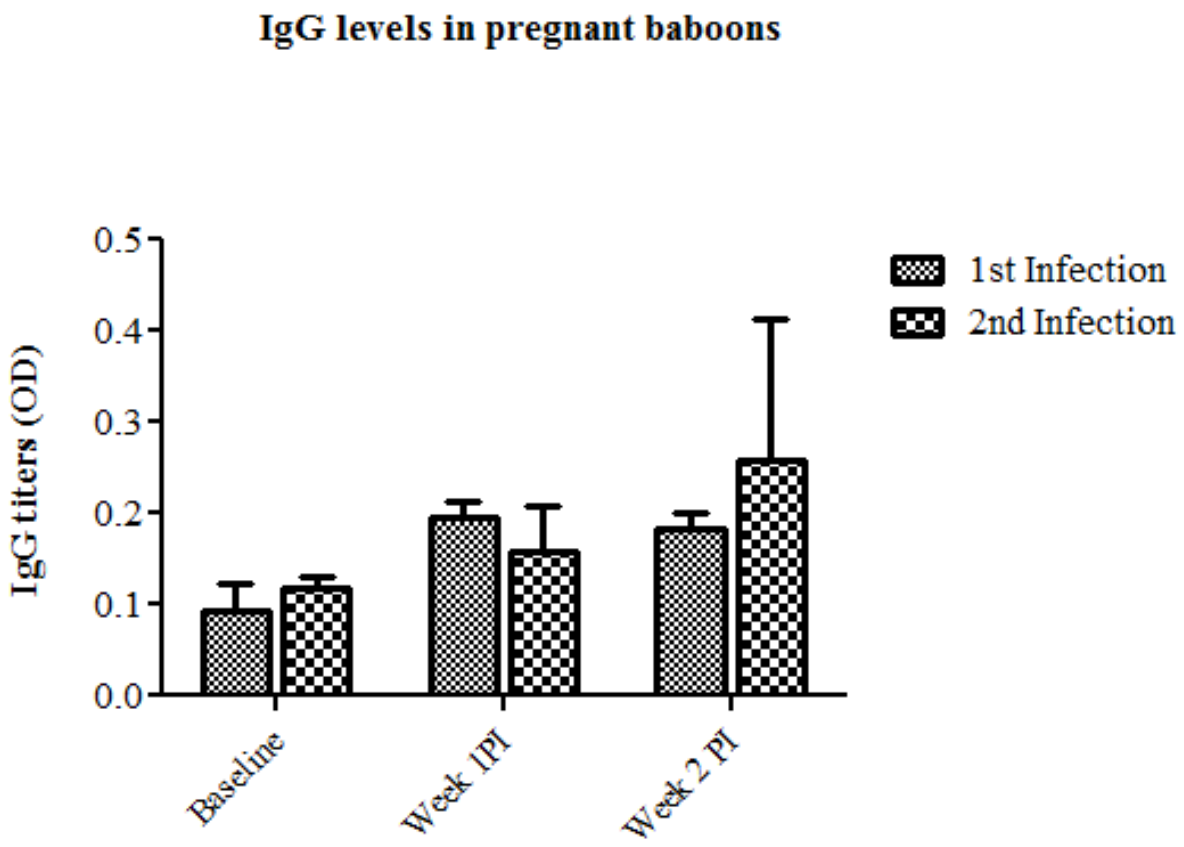


**Figure 22: Comparison of IgG levels in pregnant baboons during first and second infections with *P. knowlesi*.** Serum samples were analyzed at baseline and at 1 week and 2 weeks post infection (PI). Treatment was done at week 1 PI.

### 6.4 Discussion

To determine the importance of PM infection in protecting baboon infants against progression of malaria, IgG antibody levels were detected in serum samples isolated from infants born to mothers with and without PM, their mothers, cord blood, placental maternal section and placental foetal section.

Findings in this study indicate that IgG levels were higher in non-immune PM positive mothers than non-immune PM negative mothers. Further analysis demonstrated that IgG antibody levels in pregnant baboons was highest in first and third trimesters (OD levels 0.142 and 0.144 respectively) following infection with *P. knowlesi* malaria parasites. In humans, it is documented that the transfer of IgG antibodies depends on gestational age (Palmeira *at al*. 2011). This transfer of IgG antibodies from mother to foetus increases as the pregnancy progresses with the largest amount of transfer occurring at the third trimester (Saji *at al*. 1999). At term, depending on the immunological experience of the mother, placental transfer allows the newborn to acquire different specificities of IgG antibodies resulting in identical recognition patterns of antigens between the mother and her offspring (A. Malek *at al*. 1996). This explains the higher levels of IgG observed in pregnant baboons infected with *P. knowlesi* at third trimester.

Peripheral parasitaemia levels were lower in baboon infants born to non-immune PM positive mothers compared to those born to non-immune PM negative mothers. In addition, IgG antibody levels were higher in infants born to PM positive mothers compared to those born to PM negative mothers. The difference was not statistically significant probably due to the small sample size used. Earlier studies showed that neonatal IgG levels were not influenced by PM and found no evidence to indicate that malaria infection of the placenta induced an immune response in the foetus (Logie *at al*. 1973). Recent studies demonstrate the contrary. It is documented that antibody transfer during pregnancy can be affected by several factors and clinical conditions such as PM (Bulmer *at al*. 1993; Fried *at al*. 1998). Indeed, some studies have also demonstrated that PM in humans results in the exposure of the foetus to malaria antigens which leads to prenatal sensitization to malaria specific antibodies (Desowitz 1988). Maternal malaria and

immunity indirectly benefit young infants by enhancing resistance against blood-stage infection and morbidity in the first few months after birth. The mechanism underlying this phenomenon involves IgG antibodies raised against blood-stage *P. falciparum*. These antibodies are acquired from the maternal circulation during gestation, target merozoite invasion ligands and parasite antigens transported to the surface of the infected erythrocyte, and presumably protect the young infant from severe malaria and high-density parasitaemia by slowing the growth rate of intraerythrocytic parasites (Malhotra *at al*. 2005). However, IgG antibodies of maternal origin decrease progressively in the infant's circulation and are completely lost by 6 to 9 months after birth (Desowitz, Elm, and Alpers 1993). This suggest that the reduced parasitaemia observed in baboon infants born to non-immune PM positive mothers is due to prenatal sensitization to *P. knowlesi* blood-stage malaria parasites which inhibit parasite propagation hence the low parasitaemia observed.

Further, this study also demonstrated that baboon sera isolated from maternal peripheral circulation and foetal peripheral circulation had similar OD levels of IgG antibodies while those isolated from placental blood and cord blood had similar OD levels of IgG. These findings suggest that maternal IgG antibodies against *P. knowlesi* are acquired *in utero*. Placental transfer of maternal IgG to the foetus is an important mechanism that provides protection to the infant while his/her humoral response is inefficient. Immunoglobulin G (IgG) antibody can successfully cross the human placenta, a mechanism mediated by FcRn expressed on syncytiotrophoblast cells. However, IgG transfer is dependent on factors such as maternal levels of IgG, IgG subclass, placental integrity among others (Palmeira *at al*. 2011). Although, earlier studies demonstrated that neonatal IgG levels exceeded maternal ones (Logie *at al*. 1973), many other

studies done in Africa have demonstrated that foetal IgG antibodies' level correlate with maternal ones and that total IgG concentrations in cord sera tends to be lower than in their mothers (Okoko *at al*. 2001). Therefore, findings obtained in this study demonstrate that maternal IgG is acquired in utero and their levels exist in equilibrium in both maternal and foetal circulation in non-immune baboons infected with *P. knowlesi* malaria parasites.

In attempting to mimic the semi-immune condition of individuals living in malaria holoendemic areas, pregnant baboons were re-infected with *P. knowlesi* malaria parasites and their IgG levels compared to IgG levels of initial infection. Results indicate that IgG levels were generally higher during the second infection compared to levels observed during initial infection. There is growing evidence suggestive of the protective role of IgG in *P. falciparum* infection (Aucan *at al*. 2000) and that effective immunity can be induced in humans by repeated infections (Doolan, Dobaño, and Baird 2009). Natural (innate) immunity to malaria is believed to be an inherent property of the host, a refractory state or immediate inhibitory response to the introduction of parasite independent of previous infection (Hawking 1980; Doolan, Dobaño, and Baird 2009). This explains the higher IgG levels observed by week 1PI during initial *P. knowlesi* infection in pregnant baboons. On the other hand, active (acquired) immunity is believed to results due to previous encounter with the same pathogen (Hawking 1980). This explains the higher OD levels of IgG observed in pregnant baboons during the second infection compared to the initial infection. Therefore, this study presents the importance of experimentally induced immunity against *P. knowlesi* malaria and its potential as a good model for vaccine development.

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# CHAPTER 7: CELL MEDIATED IMMUNITY IN NON-IMMUNE AND SEMI-IMMUNE OLIVE BABOONS

## 7.1 Introduction

Cell mediated immunity is an immune response that involves the activation of phagocytes, antigen specific cytotoxic lymphocytes and the release of various cytokines in response to an infectious antigen (Jr *at al*. 2001). Lymphocytes play a central role in the regulation of both cell-mediated and antibody mediated immune responses against various antigens. There are two main classes of these lymphocytes which include the B and the T lymphocytes (Glenny and Südmersen 1921).

B lymphocytes arise from pluripotent stem cells and differentiate to fully mature antibody secreting plasma cells (Calvert *at al*. 1984). They express CD 20 on their surfaces which is a cell surface non-glycosilated hydrophobic phosphoprotein that is expressed during early pre-B-cell development (Reff *at al*. 1994). T lymphocytes on the other hand play a central role in cell mediated immunity. They can be distinguished from other lymphocytes by the presence of T-cell receptor on their surfaces which commonly interact with CD3 expressed on mature T cells (Siegel and Gleicher 1981).

There are several types of T cells which include Memory T cells, cytotoxic T ($T_c$) cells, helper T ($T_h$) cells, just to mention but a few. Cytotoxic T cells destroy virus-infected cells, tumour cells and are also involved in transplant rejection. They are commonly known as $CD8^+$ T cells as they express CD8 glycoprotein on their surfaces. Helper T cells which assist other lymphocytes

(particularly B cells) in immunologic processes are also known as $CD4^+$ T cells because they express CD4 glycoprotein on their surfaces. When T helper cells are activated, they produce cytokines that regulate and assist in active immune responses (Gutcher and Becher 2007).

Cytokines are soluble proteins that play an important role in immunity, inflammation, and hematopoiesis. They are rapidly produced by a variety of cell types and secreted in response to specific and non-specific stimuli. T lymphocytes play an important role in the regulation of the immune system by secreting key cytokines that can drive T-cell differentiation into several characteristic subsets based on the stimuli. They can further be differentiated into Th1 and Th2 which produce pro-inflammatory and anti-inflammatory cytokines respectively (Korn *at al*. 2009).

Memory T cells are a subset of antigen specific T cells that persist long after the infection has been resolved. They have the capacity to expand into large numbers of effector T cells upon re-exposure to a previously encountered antigen thereby providing the immune system with "memory" against previous infection. Memory T cells can either be CD4+ or CD8+ and they express cell surface protein CD45RO (Willinger *at al*. 2005).

This chapter focuses on the assessment of the maternal immunological competence and baboon responses to *P. knowlesi* blood stage malaria antigens by quantification of T lymphocyte population and cytokine expression in pregnant baboons.

## 7.2 Materials and Methods

### 7.2.1 Blood mononuclear cells preparation

Whole blood samples were obtained from the femoral vein and umbilical vein and diluted in an equal volume of Alsever's solution (20.5g dextrose, 4.2g NaCl, and 8.0g Sodium citrate in one liter of water) in a 20ml syringe. Placental blood was obtained by placental perfusion using a catheter and collected in 20 mM EDTA/PBS according to Moore *at al.,* (1997). The whole blood-Alsever's mixture (10ml whole blood and 10ml Alsever's solution) was overlaid directly from the syringe onto 10ml of lymphocyte separating medium (LSM®) layer already placed on a 50ml centrifuge tube. This was followed by centrifugation for 30minutes in Sorvall RT 6000 D at 20°C, at a speed of 800x *g* with no breaks. After, the upper plasma layer was transferred into labeled tubes in 1ml aliquots and stored at -20°C until used. The mononuclear cell layer (buffy coat layer) was transferred into another 50ml tube. The entire interface was removed with a minimum amount of LSM and supernatant. Alsever's solution was added (approximately 30ml) to the isolated PBMC solution and the cells resuspended evenly using a serological pipette by gently drawing in and out. Washing was done by spinning at 400x *g* at 18°C to 20°C. The supernatant was discarded and cells resuspended in Alsever's solution, washed again and most of the pellet removed. Each centrifugation step was preceded by loosening the pellet and resuspending in 10ml complete RPMI1640. Finally, approximately 200µl of the resuspended cells were transferred into a sterile eppendorf microcentrifuge tube and spun for 10min in Sorveall RT 6000 D rotor at 500x *g* at room temperature. The supernatant was discarded and the pellet saved. The mononuclear cells were processes for FACS analysis and cryopreservation (Gicheru *at al*. 2001).

### 7.2.2 Cell enumeration

Mononuclear cells were enumerated in order to perform accurate dilution for FACS analysis and have $2x10^5$ cells/well distribution. Trypan blue solution (90µl, Sigma) was added to a 96 well microtitre plate well. Ten µl of each of the resuspended cells for enumeration was transferred from an eppendorf tube to the respective 96 well plate. Gentle mixing was done in order to mix the cells evenly with trypan blue. The dilution factor of 10 was noted for use in calculating the number of cells per ml. Cells were allowed to stain for 3-5 minutes. Trypan blue stains non-viable cells blue and leave viable cells unstained. With the cover slip in place, a P20 pipette man was used to transfer a small amount of trypan blue-cell suspension mixture to both chamber of the haemocytometer. Each chamber was allowed to fill by capillary action. Starting with chamber 1 of the haemocytometer, all the cells in the four 1mm corner square were counted and average number per 1mm square calculated by dividing by 4. A manual tally counter was used. Cells touching the middle line at the bottom and right side were not counted. The procedure was repeated for chamber 2. Calculations were done as follows:

Cells per ml = Average count per square x dilution factor x $10^4$ (count 10 squares)

Total cells = cells per ml x original volume of fluid from which cells were picked (10ml).

Enumerated cells were resuspended in the right concentration for FACS analysis. The coverslip and haemocytometer were then decontaminated by first rinsing with 70% ethanol then in deionized water before drying and storing for future use (Gicheru *at al*. 2001).

### 7.2.3 Fluorescence activated cell sorting (FACS) analysis of baboon PBMCs

Flow cytometry was done using peripheral blood mononuclear cells (PBMCs) of pregnant baboons infected with *P. knowlesi* malaria parasite at first, second and third trimesters. All reagents and requirements were assembled. Cells (PBMCs) were washed in incomplete culture media three times at 400x *g* for 10 minutes at 4ºC, each time discarding the supernatant, dislodging the pellet and resuspending it in 10ml of the media. The samples were then reconstituted in 5ml complete media before enumerating the cells in a neuber chamber. Once the enumerated cells were obtained to be at least $2x10^5$ cells, they were reconstituted in 1ml complete media.

A template of a 96 well plate was prepared to indicate how the samples will be loaded. The 96well plate was labeled according to the template and 2µl of CD3, CD20, CD45 and CD3/CD20/CD45 added on the respective wells. Next, 100µl of the respective samples were added as per the template, the plate covered with aluminium foil and incubated on a rocking platform on ice for 30 minutes. Spinning was then done at 400x *g* for 5 minutes at 4ºC, the supernatant discarded and 100µl of 1X phosphate buffered saline (PBS) with 2% bovine serum albumin (BSA) added. Spinning was repeated as before and washing done twice and finally resuspending the samples in 100µl of 1X PBS in 2% BSA. The samples were taken to the FACS room where each sample was transferred into its respective labeled tube. In each tube, 100µl of FACS fixing solution was added and mixed well in order to fix the cells, before acquisition.

### 7.2.4 Preparation of human Th1/Th2 cytokine standards for cytometric bead arrays (CBA)

Lyophilized human Th1/Th2 standards was transferred into a 15ml polypropylene tube (BD Falcon) and labeled as "top standard". The standard was then reconstituted with 2.0 ml of assay diluent. The reconstituted standard was left to equilibrate for at least 15 minutes before making dilutions. The reconstituted protein was mixed by pipette only and not vortexing.

Next, 12 × 75 mm tubes (BD, Falcon) were labeled and arranged in the following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 and 300 µl of assay diluents pipette in each tube. Serial dilution was performed by transferring 300 µl from the “top standard” to the 1:2 dilution tube and mixing thoroughly. This was continued by transferring 300 µl from the 1:2 tube to the 1:4 tube and so on to the 1:256 tube and mixed thoroughly by pipetting. One other tube containing assay diluents was also prepared to serve as 0 pg/ml negative controls.

### 7.2.5 Preparation of mixed human Th1/Th2 cytokine capture beads

The number of assay tubes required including standards and controls was determined. Each capture bead (IL-2, IL-4, IL-5, IL-6, TNF and IFN-γ) suspension was vortexed vigorously before mixing to form a cocktail containing 10 µl aliquot of each bead per sample. The mixed capture beads were centrifuged at 400*g* for 5 minutes. The supernatant was aspirated carefully and discarded. The pellet was resuspended in serum enhancement buffer and vortexed thoroughly. The mixture was covered with aluminum foil and incubated for 30 minutes at room temperature.

### 7.2.6 Serum assay procedure

Mixed capture beads (50 µl) was added to the appropriate well labeled assay tubes. Next, 50 µl of NHP Th1/Th2-PE detection reagent was added to the assay tubes followed by addition of 50 µl of each serum sample and 50 µl of the Human Th1/Th2 cytokine standard dilutions to the control assay tubes. The assays were covered with aluminium foil and incubated for 3 hours at room temperature. After, 1 ml of wash buffer was added to each assay tube, centrifuged at 400*g* for 5 minutes and the supernatant discarded. The pellet from each tube was resuspended in 300 µl wash buffer before analyzing the samples on the flow cytometer.

## 7.3 Results

### 7.3.1 Lymphocyte population at different trimesters

Lymphocyte cell surface markers for B cells and T cells in *P. knowlesi* infected baboons were quantified at different trimesters using FACS. Generally, the proportion of CD3 cell surface markers for T lymphocytes was more compared to CD20 surface markers for B lymphocytes. There was a 0.8 fold decrease in the proportion of CD3 surface markers observed in first and second trimester infected pregnant baboons, and an 8.4 fold increase in thirds trimester infected pregnant baboons from baseline values (Figure 23 and 24). Proportion of CD20 surface markers also decreased by 0.6, 0.3 and 2.3 fold in first, second and third trimester infected pregnant baboons respectively, while an 8 fold increase was observed in third trimester infected pregnant (Figure 23 and 25).

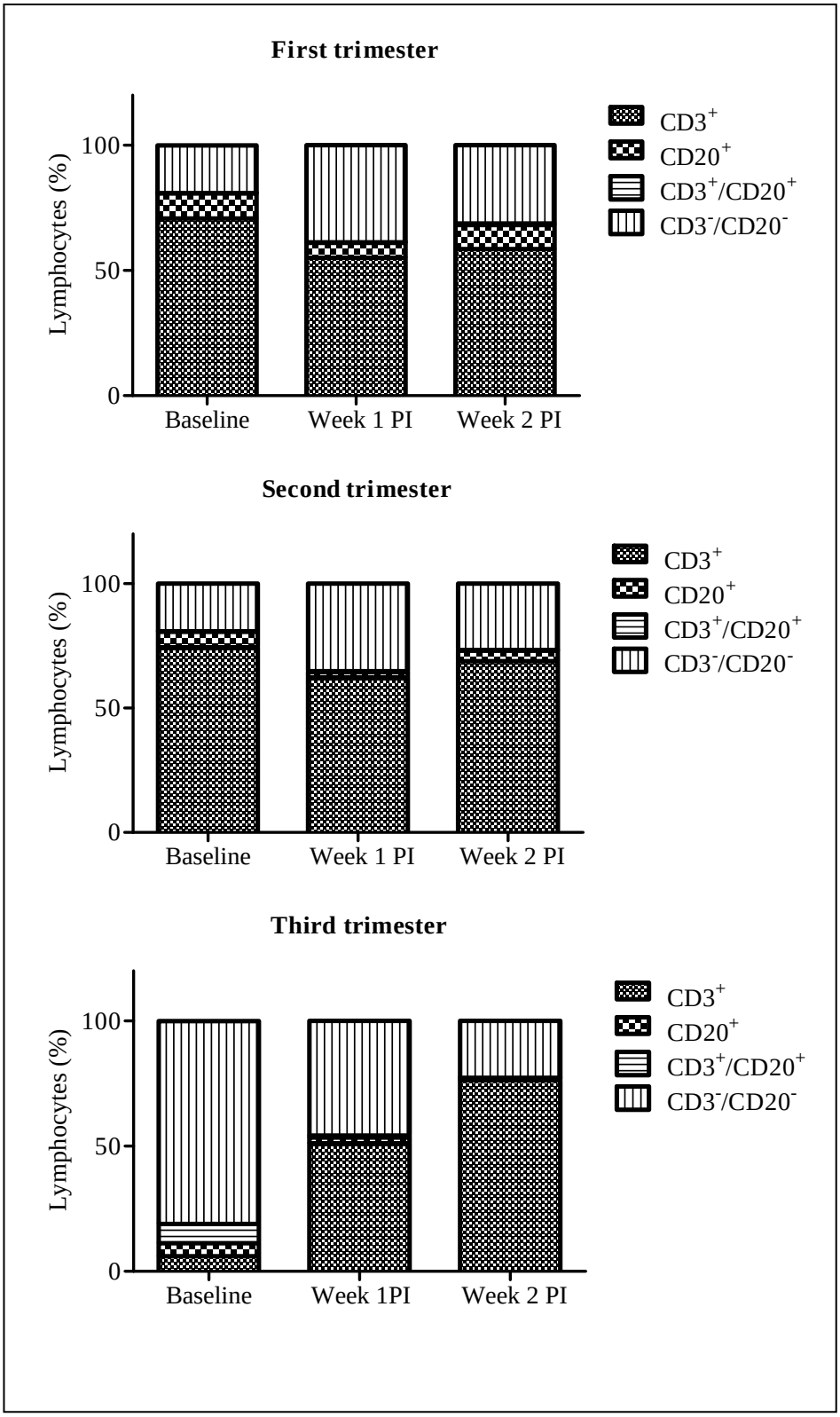



**Figure 23: Relative contribution of each lymphocytes cell by percentage in PBMCs isolated from pregnant baboons infected with *P. knowlesi* at different trimesters**

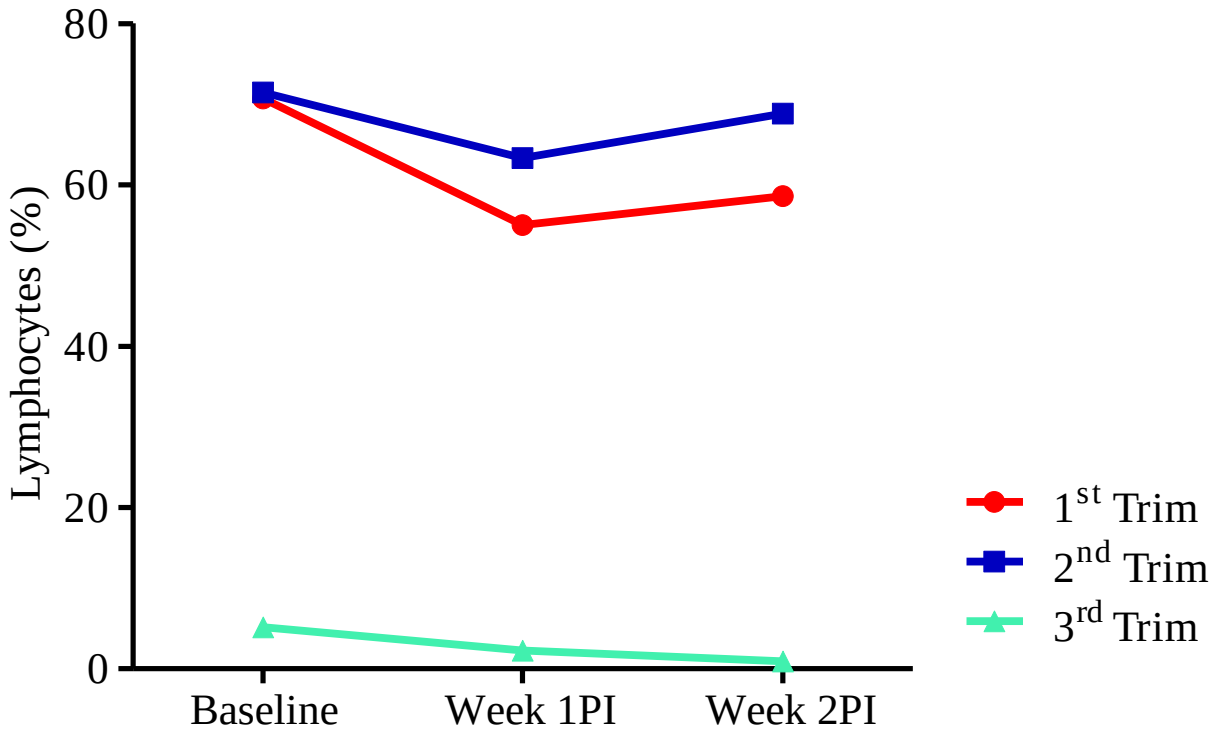



**Figure 24: Proportion of CD20 lymphocytes in pregnant baboons infected with *P. knowlesi* malaria parasite at first, second and third trimesters. Trim is trimester and PI is post infection.**

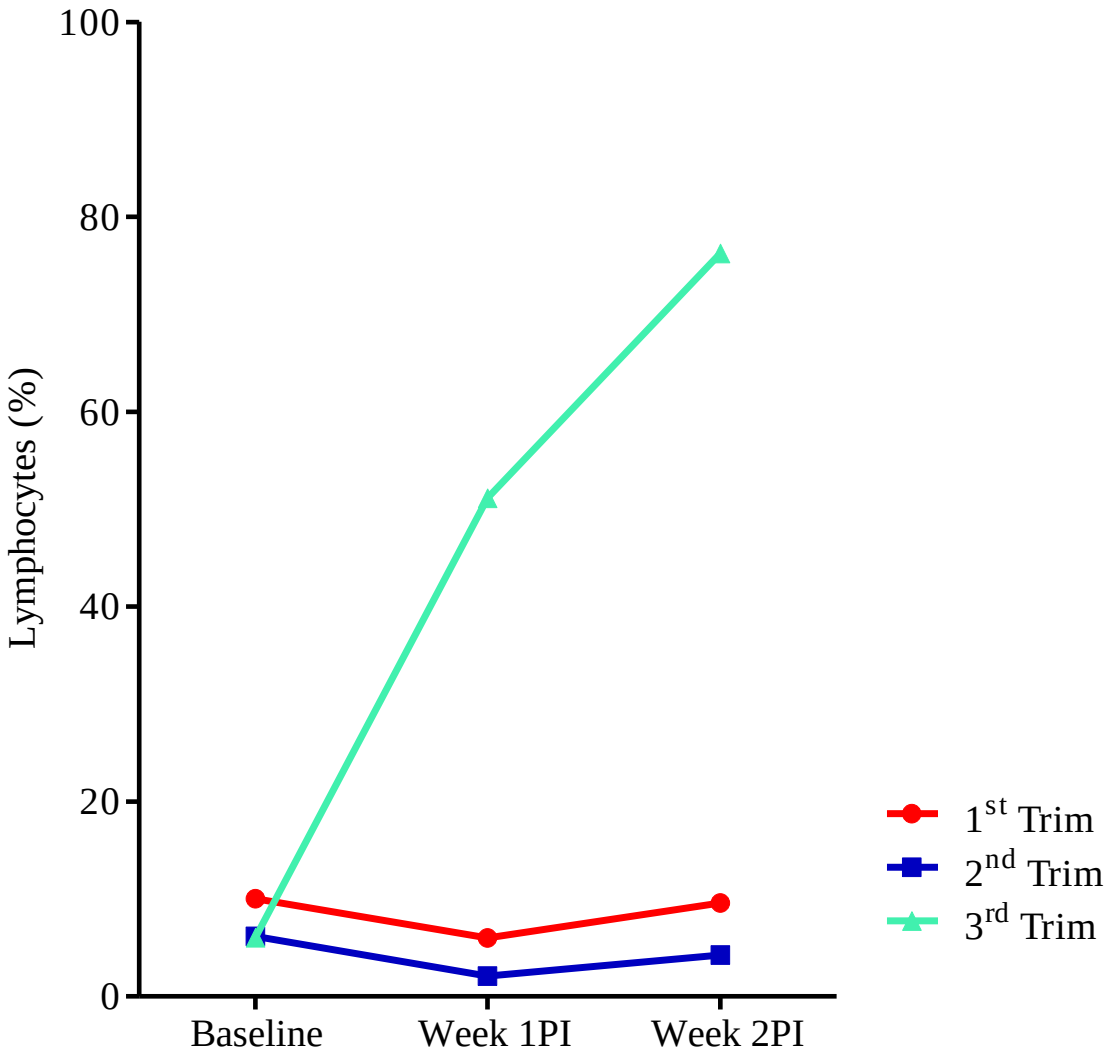



**Figure 25: Proportion of CD3 lymphocytes in pregnant baboons infected with *P. knowlesi* malaria parasite at first, second and third trimesters. Trim is trimester and PI is post infection.**

After infection of pregnant baboons with *P. knowlesi* malaria parasites (week 1 PI), the proportion of T lymphocytes represented by CD3 cell surface markers predominated (55%, 22% and 51% in first, second and third trimesters respectively) compared to non-infected pregnant controls. The proportion of B lymphocyte represented by CD20 cell surface markers was more in

non-infected pregnant controls (9%) compared to *P. knowlesi* infected pregnant baboons (6%, 2% and 3% in first, second and third trimesters respectively) (Figure 26). This implies that *P. knowlesi* infection in pregnant baboons is associated with an increased proportion of T cells that play an important immunological role in pregnancy associated malaria. B lymphocytes also play a role.

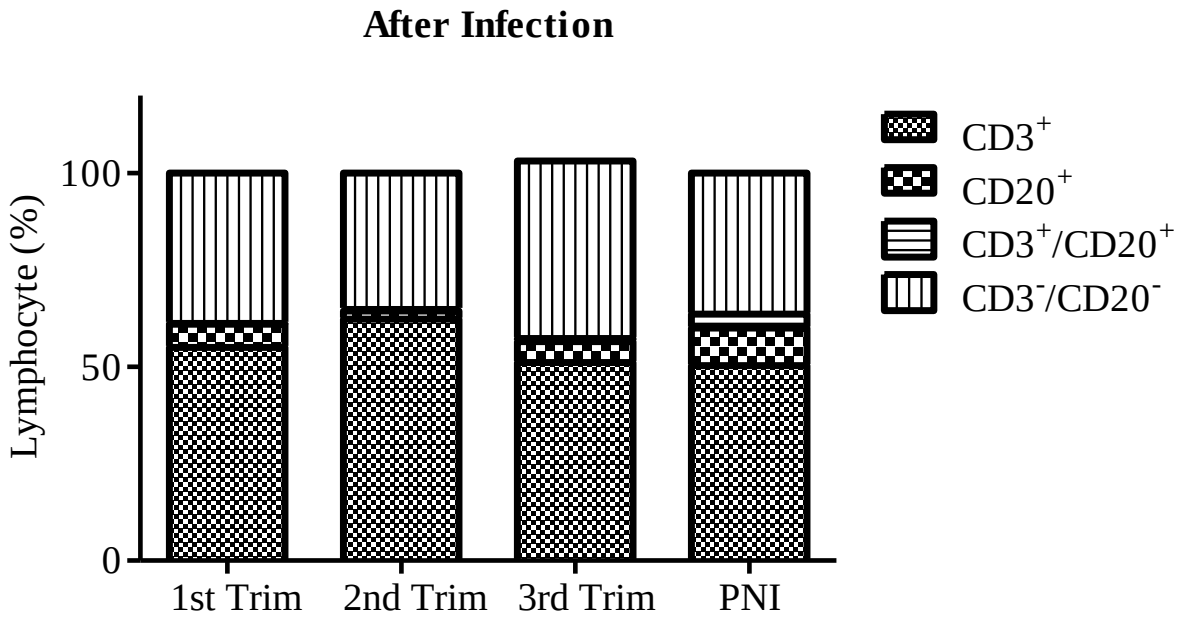



**Figure 26: Relative contribution of each lymphocyte cell by percentage in PBMCs isolated from pregnant baboons following infection with *P. knowlesi* (week 1 PI).** Trim means trimester while PNI is pregnant non-infected baboons.

### 7.3.2 Cytokine profiles in *P. knowlesi* in infected Olive baboons

Baboon serum isolated from peripheral blood, cord blood, blood from placental foetal region and blood from placental maternal region were analyzed for pro-inflammatory (Th1) and anti-

inflammatory (Th2) cytokine profiles using FACS caliber. Results were analyzed using FCAP Array 3v software.

Generally, peripheral blood had higher levels of anti-inflammatory cytokines IL-5 and IL-6 (109.91pg/ml and 749.64pg/ml respectively) while pro-inflammatory cytokines (IFNγ and TNF-α) were below detectable levels of 20pg/ml (Figure 27) by week 2PI.

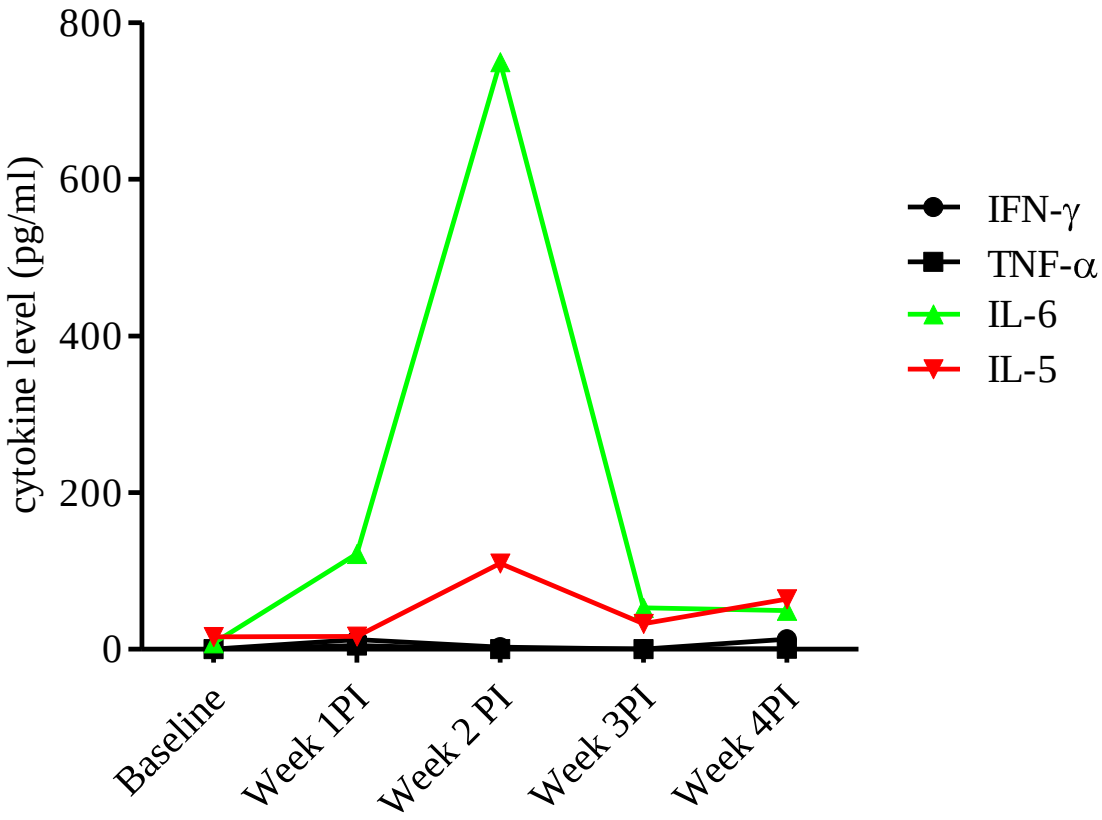



**Figure 27: Cytokine levels in peripheral blood samples of *P.knowlesi* infected pregnant baboons. PI means post infection.**

Analysis of the different samples at week 1PI revealed TNF-α was expressed in serum sample issolated from placental maternal layer (41.13pg/ml), while IFN-γ levels were below detectable levels of 20pg/ml. Interleukin-5 was detected in cord blood (20.31pg/ml) while IL-6 in peripheral, cord and placental foetal samples with 121.96pg/ml, 102.5pg/ml and 221.3pg/ml respectively (Figure 28).

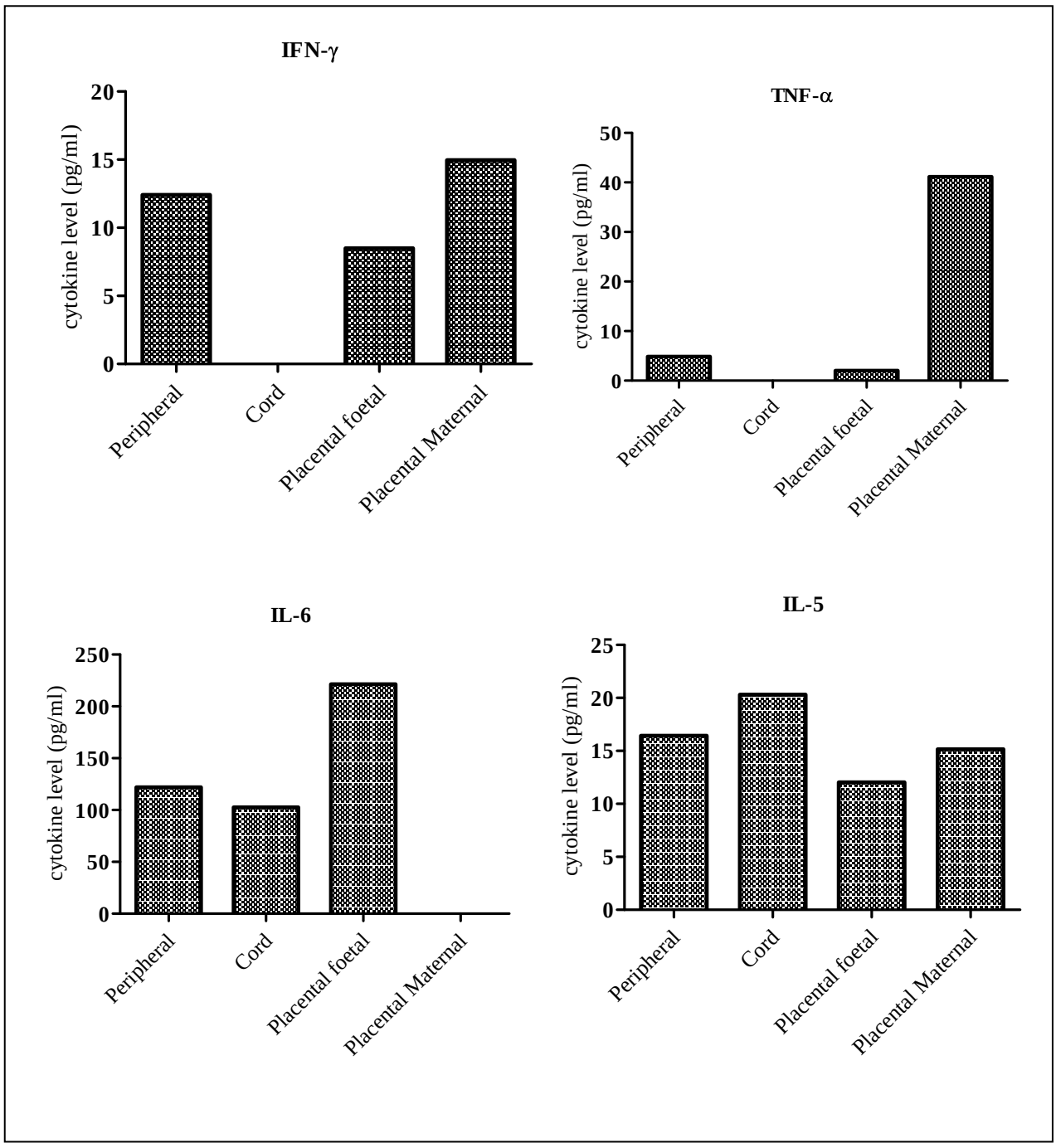



**Figure 28: Pro-inflammatory (IFN-γ and TNF-α) and anti-inflammatory (IL-5 and IL-6) cytokines in peripheral, cord, placental foetal and placental maternal blood samples by week 1 post infection.**

## 7.4 Discussion

Although malaria is a major cause of morbidity and mortality in tropical countries, individuals living in these areas develop some level of immunity against the disease (Shulman and Dorman 2003). This partial immunity is mainly due to antiparasitic antibodies (Sabchareon *at al*. 1991). Monocytes, T cells and cytokines also play a role in determining the level of malaria parasitaemia as demonstrated in human studies and murine models (Taylor-Robinson 1995; Winkler *at al*. 1998). Cytokines particularly play a major role in the determination of malaria severity and outcome. Some studies suggest that the balance between pro- and anti-inflammatory cytokines determines the degree of parasitaemia, level of anaemia and clinical severity while others suggest that the absolute levels of cytokines determines malaria outcome (Winkler *at al*. 1998; Day *at al*. 1999).

In this study pregnant baboons infected with *P. knowlesi* at first and second trimesters had decreased proportion of lymphocytes expressing CD3 and CD 20 cell surface markers following experimental infection with *P. knowlesi* blood stage malaria parsites. At third trimester however, population of lymphocytes expressing CD3 cell surface markers increased while cells expressing CD20 cell surface markers decreased. Indeed, lymphocytes that express CD20 represent B lymphocytes (B cells) while those that express CD3 are T lymphocytes (T cells). Although acquired immune responses to all forms of malaria parasites are thymus dependent, antibodies have the capacity to facilitate recovery from primary malaria infections and increase the effectiveness of cell-mediated immune responses in these infections (Allison and Eugui 1983). In fact both antibodies and T cells can control parasite growth in human and animal model systems,

but such responses are regulated by parasite load. Because high parasite load is associated with pathology, and cell-mediated responses may also harm the host through production of inflammatory cytokines. Immunity without pathology requires rapid parasite clearance, effective regulation of the inflammatory anti-parasite effects of cellular responses, and the eventual development of a repertoire of antibodies effective against multiple strains. This phenomenon can be achieved by exposure to malaria antigens in low dose, leading to augmented cellular immunity and rapid parasite clearance (Good *at al*. 2005). Evidence from various studies conducted in humans show that antibodies and $CD4^+$ (a subset of CD3) protect against blood stage *Plasmodium* infections. Investigations into the mechanism of protection in mice have also emphasized the importance of antibodies in controlling chronic malaria (von der Weid, Honarvar, and Langhorne 1996). However, in malaria endemic settings, memory antibody responses against *Plasmodium* parasites are short-lived and require constant parasite exposure (Crompton *at al*. 2010). Although this study observed higher levels of T lymphocyte population compared to B lymphocyte, it is emphasize the importance of both B and T lymphocytes in *P. knowlesi* infection during pregnancy.

A large body of evidence indicates that CD4+ T-cell responses can be categorized based on their profile of cytokine secretion. Type 1 T helper (Th1) cells produce pro-inflammatory cytokines IFN-γ, IL-2, and TNF-α, which activate macrophages and are responsible for cell-mediated immunity and phagocyte-dependent protective responses. By contrast, type 2 Th (Th2) cells produce anti-inflammatory cytokines IL-4, IL-5,IL-6, IL-10, and IL-13, which are responsible for strong antibody production, eosinophil activation, and inhibition of several macrophage functions (Romagnani 1999). Analysis of cytokine profile in pregnant baboons demonstrated

increased levels of anti-inflammatory cytokines (IL-5 and IL-6) following infection with *P. knowlesi* malaria parasites. Pro-inflammatory cytokines (TNF-α and IFN-γ) remained at non-detectable levels throughout the experimental period. Although high levels of IFN-γ as part of Th1 immune response has been associated with a better outcome of malaria in many malaria models (Jacobs, Radzioch, and Stevenson 1996), human studies have shown a more pronounced Th2 driven immune response during acute and uncomplicated malaria caused by *P. falciparum* , with a shift toward Th1 responsiveness induced by parasite clearance (Winkler *at al*. 1998). Indeed, immunological studies in rats suggest that a successful pregnancy occurs in a Th2 dominant situation while Th1 response is associated with pregnancy failure. This altered balance is not clear in humans (Guilbert 1996) although malaria has been reported to be more severe during human pregnancy (Ordi *at al*. 2001). It is evident that PM stimulates the production of inflammatory mediators, shifting immune responses away from the Th2 cytokines associated with healthy pregnancy towards Th1 type cytokines (Fried *at al*. 1998).

According to this study, PM in *P.knowlesi* infected baboons demonstrates a balanced Th1 and Th2 immune response, but their localization varies. Th1 immune response as demonstrated by TNF-α is pronounced within the placental maternal region where it functions to regulate immune cells(Benedict *at al*. 2009). On the other hand, Th2 immune response as demonstrated by elevated levels of IL-6 in maternal circulation, cord blood and placental foetal region acts to stimulate immune cells (Benedict *at al*. 2009). These findings correlate with those observed in human and in NHPs (Mustafa *at al*. 2012; Moormann *at al*. 1999; Fried *at al*. 1998).

## CHAPTER 8: GENERAL DISCUSSION AND CONCLUSSION

This study has demonstrated that *P. knowlesi* infection in pregnant baboons is associated with symptoms similar to those observed in humans such as poor appetite, diarrhea (loose stool), decreased body weight and increased body temperature (fever). Clinical symptoms suggestive of anaemia (reduced RBC and Hb levels) were also observed. These could be used as indicators of placental malaria. Since there are no predictive values of haematological and clinical indices associated with malaria and pregnancy (Maina *at al*. 2010) this study suggest predictive values of > 4.5% weight decrease, >0.5% temperature increase and > 9% RBC decrease to be associated with malaria during pregnancy regardless of the trimester.

The baboon model of PM has for the first time demonstrated the infiltration of *P. knowlesi* parasitized RBC and inflammatory cells in the placenta of non-immune baboons. During *P. knowlesi* infection in pregnancy, symptomatic disease is manifested in combination with increased placental parasitaemia. Consequently, accumulation of parasites in the placental IVS combined with infiltration of inflammatory cells leads to placental damage. In seeking to identify the ligand and receptor molecules associated with accumulation of *P. knowlesi* infected erythrocytes in the baboon placenta, chondroitin sulphate proteoglycan 4 (CSPG4) and, Hyaluronan and proteoglycan link protein 1 (HAPLN1) have been predicted as putative receptor molecules in the baboon with high similarity to human CSA and HA respectively. In addition, *P. knowlesi* erythrocyte binding proteins (EBP-alpha, EBP-beta and EBP-gamma) matched closely to the placental *P. falciparum* ligand Var2csa (Nyamagiri *at al*. 2014).

The study also demonstrated that IgG antibodies and cytokines are important during *P. knowlesi* infection in pregnant baboons. The pathophysiology of malaria during pregnancy is greatly due to the altered immunity and availability of the placental tissue because the placenta is an immune privileged site. There are several theories that have been postulated to explain the pathophysiology of PM. Some researchers postulated that loss of antimalarial immunity is consistent with general immunosupression of host's immune response in the attempt to prevent foetal rejection responsible causing the pregnant woman to be more susceptible to malaria. Others believe that cell mediated immune response is lost during pregnancy while passive antibody mediated immune response is transferred hence the mother becoming susceptible to malaria. Another hypothesis is that the placental tissue as a new organ in primigravidae allows the parasites to by-pass the existing host immunity or allows placental specific phenotypes of *P.falciparum* to multiply. The development of placental specific immunity may therefore explain the decreased susceptibility of malaria infection in multigravidae. It is also postulated that pregnant women display a bias towards type-2 cytokines and are therefore susceptible to diseases like malaria which require a type -1 response.

This study demonstrates that protective immunity against PM associated with *P. knowlesi* infection involves both antibody dependent and cell mediated immune responses. Pregnant baboons infected with *P. knowlesi* malaria parasites displayed a balanced type-1 and type-2 cytokine response. The baboon placenta however displays inflammatory response as demonstrated by the presence of inflammatory cells in their placental tissue and increased levels of TNF-α expressed in serum isolated from placental maternal region. This finding questions the

commonly held view that increased risk of pregnant women to malaria may be due to an underlying immune suppression.

This study also emphasizes the importance of maternal antibodies in protecting baboon infants born to PM positive mothers against progression of the disease. No congenital malaria was observed in this study. Therefore, it is likely that both *P. knowlesi* parasites and *P. knowlesi*-infected red blood cells activate dendritic cells through pattern recognition receptors (PRRs), are phagocytosed and their antigens presented to T cells. The PRR signaling leads to the secretion of cytokines that initiate the inflammation that underlies malaria pathogenesis and direct Th1 cell differentiation which provide help for B cell differentiation, antibody secretion and also secretion of IFN-γ which activates macrophages. IFN-γ-activated macrophages phagocytose opsonized parasites and infected red blood cells and subsequently kill them by nitric oxide (NO-) and Oxygen ($O_2$)-dependent pathways. Inflammation induces expression of endothelial adhesion molecules to which infected RBCs bind. Inflammation is curtailed by the secretion of anti-inflammatory cytokines from macrophages and regulatory populations of T cells (Riley and Stewart 2013).

Hence, the baboon model of PM is an ideal model for studies in malaria and pregnancy since it mimics the pathophysiology and immune responses associated with the disease in humans. As a result further exploration in the field of reproductive immunology will help in the understanding of immune regulation in PM.

# CHAPTER 9: RECOMMENDATIONS OF THE STUDY

This study recommends the following:

i. Developing a semi/partial immune state in the baboon model.

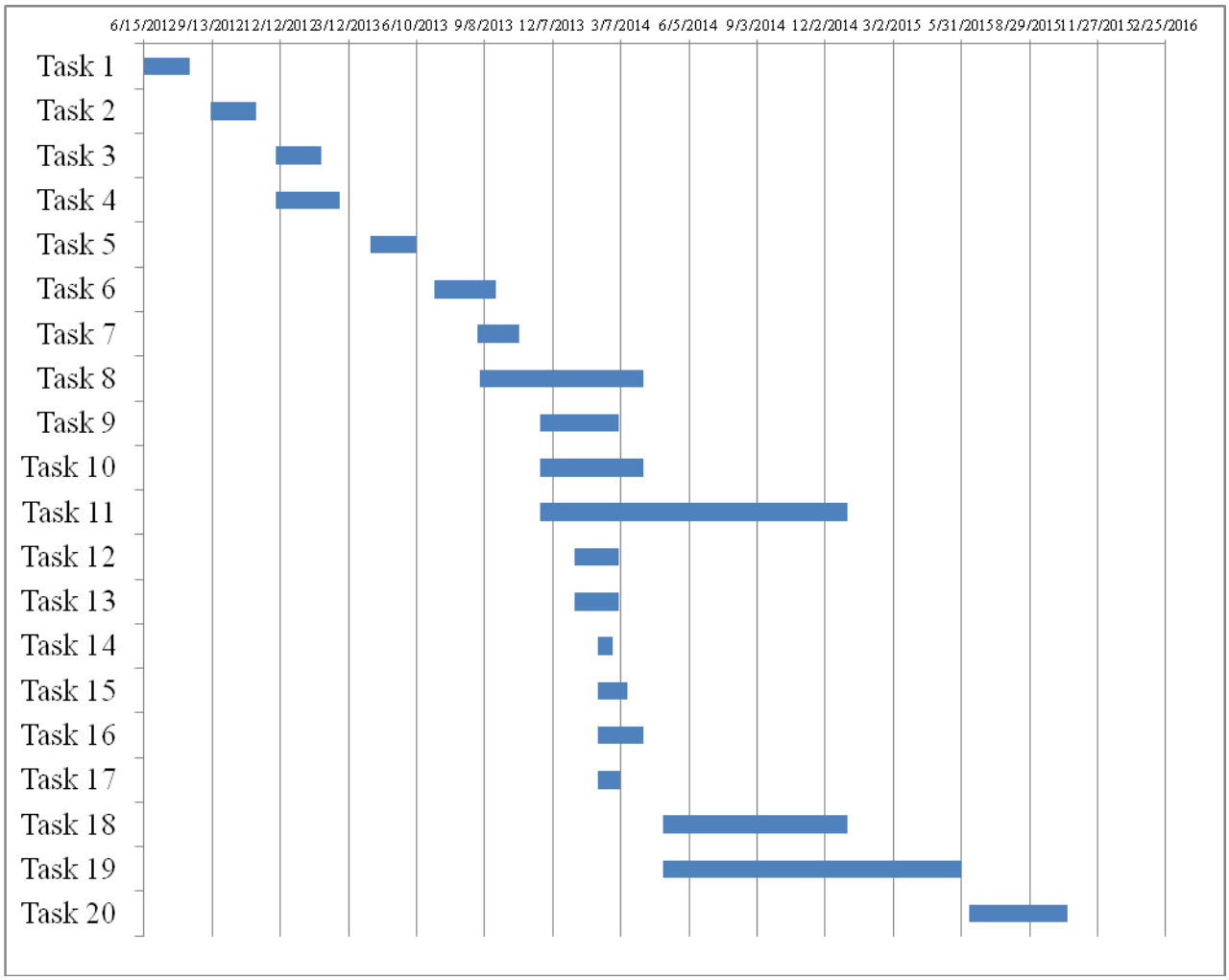
ii. Conducting further studies to assess the importance of IL10 during PM in baboons.

iii. Plan a larger collaborative study to validate findings on infant protection.

iv. Quantify the levels of IgG subclasses in sera isolated from pregnant baboons.

v. Explore and quantify specific cell surface markers expressed in baboon placentas following PM.

**TIME LINE**




KEY:

| | |
|---|---|
| **Task 1-** Ethical approval | **Task 11-** Data collection |
| **Task 2-** Proposal approval | **Task 12-** Caesarian and term deliveries of Pregnant Baboons |
| **Task 3-** Purchase of reagents | **Task 13-** Pathology (Gross and Histopathology) |
| **Task 4-** Acquisition of adult baboons | **Task 14-** Acquisition of baboon infants |
| **Task 5-** Time mating | **Task 15-** Maintenance of baboon infants |
| **Task 6-** Maintenance of adult baboons | **Task 16-** Sampling baboon infants |
| **Task 7-** Infection of adult baboons | **Task 17-** Infection of baboon infants |
| **Task 8-** Parasitology | **Task 18-** Immunological assays (ELISA, FACS and CBA) |
| **Task 9-** Sampling of adult baboons | **Task 19-** Data analysis |
| **Task 10-** Haematology and clinical observation | **Task 20-** Thesis preparation and submission |

**BUDGET**

| | **Item Description** | | **Cost (USD)** |
|---|---|---|---|
| 1 | Animal acquisition | 29animals x 500USD | 14,500.00 |
| 2 | Baboon maintenance | 3USD for 70days= 210USD<br>(29animals+15infants)=44<br>44animals x 210USD | 9,240.00 |
| 3 | Chemicals and reagents | Parasitological assay<br>Immunological assay<br>Pathology<br>Molecular biology | 5,000.00<br>7,000.00<br>5,000.00<br>7,000.00 |
| 4 | Stationary and library expenses | | 300.00 |
| 5 | Miscellaneous | | 200.00 |
| | TOTAL | | **48,240.00** |

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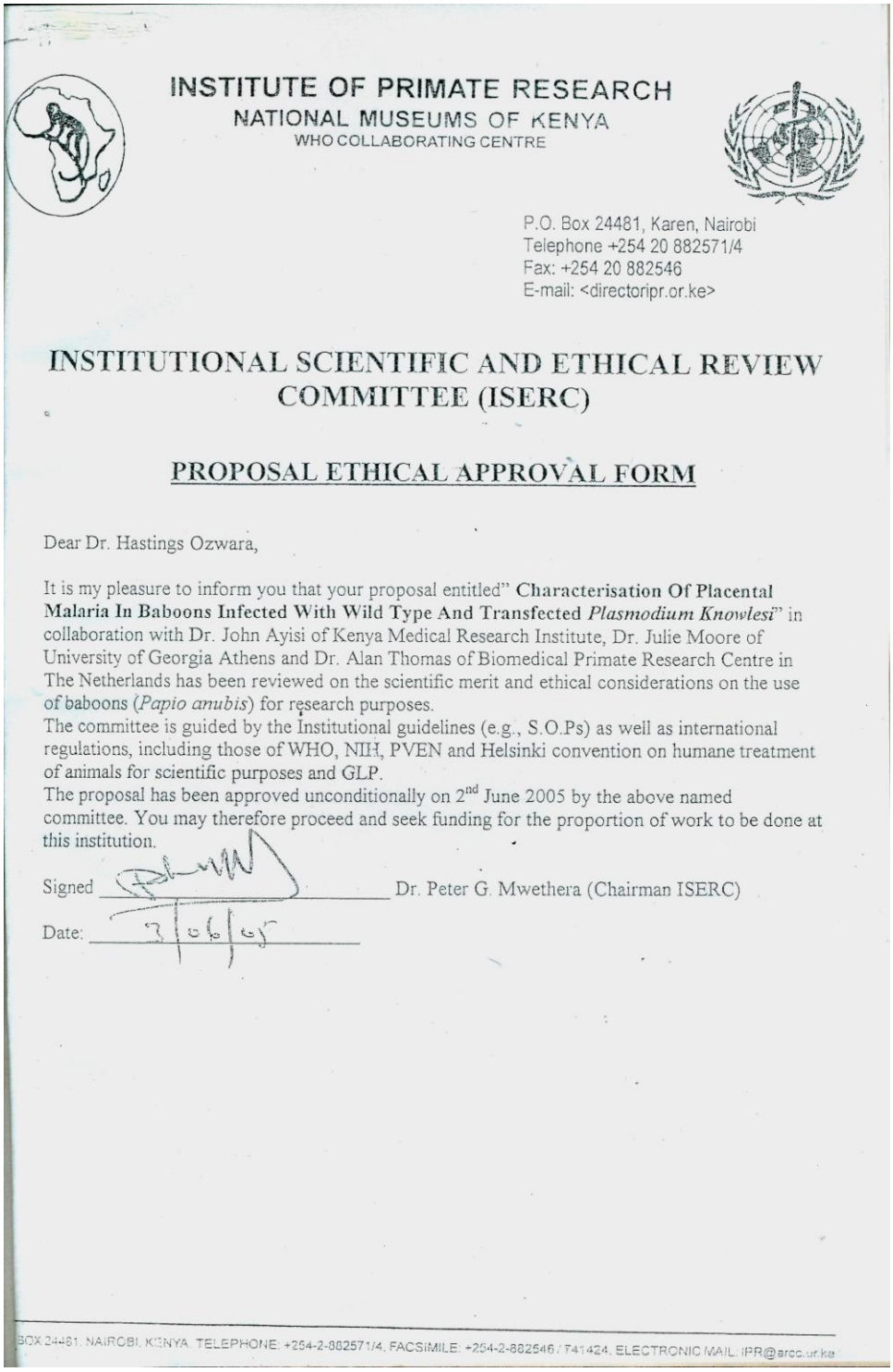
## APPENDICES

## Appendix I: Ethical approval



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INSTITUTIONAL SCIENTIFIC AND ETHICAL REVIEW COMMITTEE (ISERC)

PROPOSAL ETHICAL APPROVAL FORM

Dear Dr. Hastings Ozwara,

It is my pleasure to inform you that your proposal entitled" **Characterisation Of Placental Malaria In Baboons Infected With Wild Type And Transfected *Plasmodium Knowlesi*** in collaboration with Dr. John Ayisi of Kenya Medical Research Institute, Dr. Julie Moore of University of Georgia Athens and Dr. Alan Thomas of Biomedical Primate Research Centre in The Netherlands has been reviewed on the scientific merit and ethical considerations on the use of baboons (*Papio anubis*) for research purposes.
The committee is guided by the Institutional guidelines (e.g., S.O.Ps) as well as international regulations, including those of WHO, NIH, PVEN and Helsinki convention on humane treatment of animals for scientific purposes and GLP.
The proposal has been approved unconditionally on 2nd June 2005 by the above named committee. You may therefore proceed and seek funding for the proportion of work to be done at this institution.

Signed _____ Dr. Peter G. Mwethera (Chairman ISERC)

Date: 3/06/05

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## Appendix II: Reagents

**Alsever's solution**

| | |
|---|---|
| Dextrose | 10.25g |
| NaCl | 2.1g |
| Disodium citrate | 4.0g |
| Double distilled water | 500ml |

Sterilize by filtering through 0.2µm pore size filter

Store at 4°C

**Complete RPMI 1640 (pH 7.2)**

| | |
|---|---|
| RPMI 1640 | 445ml |
| FBS | 50ml |
| L-glutamine | 5ml |
| Gentamycin | 1ml (0.5mg/ml) |

Filter using 0.45µl pore size filter

**Appendix III: Daily Health Record Sheet**

**DAILY HEALTH REPORT SHEET**

**PLACENTAL MALARIA PROJECT, BIOCONTAINMENT FACILITY**

**ANIMAL NUMBER:**

| DATE | DEMEANOUR | APPETITE | STOOL | EYES | VET |
|---|---|---|---|---|---|
| | | | | | |
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**KEY: APPETITE CODE**: G- good, F3/4, ½ and P-poor. F= proportion of full appetite

**STOOL CODE**: N-normal, L-liquid, B-bloody, H-hard, A-absent

**EYES CODE**: N-normal, C-cloudy, O-opaque

**Appendix IV: Parasitaemia Data Sheet**

| **Animal Number: PAN** |
|---|

| **Sample Origin:** ☐ Peripheral ☐ Cord ☐ Placental ☐ Parasite Culture<br><br>☐ Retrieval Vial |
|---|
| **Type of smear:**<br><br>☐ Thick ☐ Thin |

| **Date** | **Time (am/pm)** | **DAI** | **R** | **T** | **S** | **G** | **E ($10^3$)** | **%** | **Remarks** |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |

**Key**: **DAI**- Day After Infection, **R**- Ring stage, **T**- Trophozoite stage, **S**- Schizont stage, **G**- Gametocyte stage, **E**- Total Erythrocytes, **%**- Percentage

**Appendix V: Gross Pathology Data Collection Forms**

Animal ID:_____ Date of Data Collection: _____

Time:_____

Number of previous births (gravidity) if known: _____

Composition of anaesthetic injection solution _____

1. Clinical Information:
   a) Auxiliary body temperature: _____ °C

   b) Body weight: _____ Kg

   c) Heart rate: _____

   d) Feotal Heart rate: _____

2. Blood Samples

   a) Peripheral Blood Collected
   10 ml Blood in Alsever's ☐

   6 ml Blood in 15 ml tubes ☐

   4 ml Blood in EDTA ☐

   Blood Smear on 10 slides ☐

b) Placental Blood collected

☐ 10 ml Blood in Alsever's

☐ 6 ml Blood in 15 ml tubes

☐ 4 ml Blood in EDTA

☐ Blood Smear on 10 slides

c) Cord Blood collected

☐ 10 ml Blood in Alsever's

☐ 6 ml Blood in 15 ml tubes

☐ 4 ml Blood in EDTA

☐ Blood Smear on 10 slides

3. Foetal examination
   a) Sex of the baby _____ (M/F)

   b) Birth weight _____ g

   c) Condition of the baby

d) Abnormalities _____

_____

4. Placenta Examination

a) Weight of the placenta _____g

b) Colour of placenta _____

c) Cord insertion to placenta _____
_____

d) Estimated length of cord _____cm

e) Number of umbilical vessels _____

f) Number of lobes _____

g) Placental lobes fused Yes/No _____

h) Placenta diameter _____cm

i) Tearing
Quadrant 1: _____

Quadrant 2: _____

Quadrant 3: _____

Quadrant 4 _____

j) Perfused volume_____ ml

k) Volume recovered_____ ml

l) Fibrinoids: Marked ☐ Normal ☐ None ☐

m) Calcification: Marked ☐ Normal ☐ None ☐

n) Infracts: Yes ☐ No ☐ Size_____ cm

Superficial ☐ Deep ☐

o) Meconium staining: None ☐ Acute ☐ Chronic ☐

p) Placenta praevia: Complete ☐ Partial ☐ Marginal ☐

q) Membranes: Intact ☐ Marginal ☐ Circumvalate ☐ Colour

r) Retro-placental Hemorrhage: Present/Absent _____
(Note the age, size and extent of clotting)

s) Retro-Membranous Hemorrhage: Present/Absent _____
(Note the age, size and extent of clotting)

5. Foetal surface
   a) Blood vessel distribution: Sparse ☐ Normal ☐ Dense ☐

   b) Thrombin (#)_____

   c) Subchorionic fibrosis: Marked ☐ Normal ☐ None ☐

d) Cord length _____

6. Cord Insertion:

Central ☐ Eccentric _____ cm from margin

Marginal ☐ Valamentous ☐ Furcated ☐ Vasa Previa ☐

5. Placental Tissues
   a. Placental snap frozen tissue (store in LN2) ☐
   b. Tissue in OCT medium ☐
   c. Tissues in formalin ☐

**Appendix VI: Histopathology Data Collection Form**

ANIMAL N0……. SLIDE NO……. DATE……

| | CHARACTERISTIC FEATIRES | SEVERITY SCORE |
|---|---|---|
| **1** | **Fibrinoid necrosis of the villi (Score 0-4)** | |
| **2** | **Infarcts (Score 0-4)** | |
| **3** | **Chorionic plate thrombosis (CPT) (Score 0-4)** | |
| **4** | **Syncytiotrophoblast disruption (SD) (Score 0-4)** | |
| **5** | **Chorionic plate syncytiotrophoblast disruption (Score 0-4)** | |
| **6** | **Total placental damage** | |
| **7** | **Malaria pigment (hemozoin) (Score 0-4)** | |
| **8** | **Macrophages containing malaria pigment (Score 0-4)** | |
| | **(a) Decidua** | |
| | **(b) Basal plate** | |
| | **(c ) Intravillious space** | |
| **9** | **Total pigment score** | |
| **10** | **Inflammatory cells in sections** | |
| **11** | **Parasites in sections** | |

| | | |
|---|---|---|
| **12** | **Others** | |

**PUBLICATIONS**

Onditi *et al. Malaria Journal* (2015) 14:118
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MALARIA JOURNAL

RESEARCH Open Access

# Parasite accumulation in placenta of non-immune baboons during *Plasmodium knowlesi* infection

Faith I Onditi[1,2], Onkoba W Nyamongo[1], Charles O Omwandho[2], Naomi W Maina[3], Fredrick Maloba[1], Idle O Farah[1], Christopher L King[4], Julie M Moore[5] and Hastings S Ozwara[1*]

**Abstract**

**Background:** Placental malaria (PM) causes adverse pregnancy outcomes in the mother and her foetus. It is difficult to study PM directly in humans due to ethical challenges. This study set out to bridge this gap by determining the outcome of PM in non-immune baboons in order to develop a non-human primate model for the disease.

**Methods:** Ten pregnant baboons were acquired late in their third trimester (day 150) and randomly grouped as seven infected and three non-infected. Another group of four nulligravidae (non-pregnant) infected was also included in the analysis of clinical outcome. Malaria infection was intravenously initiated by *Plasmodium knowlesi* blood-stage parasites through the femoral vein on 160$^{th}$ day of gestation (for pregnant baboons). Peripheral smear, placental smear, haematological samples, and histological samples were collected during the study period. Median values of clinical and haematological changes were analysed using Kruskal-Wallis and Dunn's Multiple Comparison Test. Parasitaemia profiles were analysed using Mann Whitney U test. A Spearman's rank correlation was run to determine the relationship between the different variables of severity scores. Probability values of $P < 0.05$ were considered significant.

**Results:** Levels of white blood cells increased significantly in pregnant infected (34%) than in nulligravidae infected baboons (8%). Placental parasitaemia levels was on average 19-fold higher than peripheral parasitaemia in the same animal. Infiltration of parasitized erythrocytes and inflammatory cells were also observed in baboon placenta. Malaria parasite score increased with increase in total placental damage score ($r_s = 0.7650$, $P < 0.05$) and inflammatory score ($r_s = 0.8590$, $P < 0.05$). Although the sample size was small, absence of parasitized erythrocytes in cord blood and foetal placental region suggested lack of congenital malaria in non-immune baboons.

**Conclusion:** This study has demonstrated accumulation of parasitized red blood cells and infiltration of inflammatory cells in the placental intravillous space (IVS) of baboons that are non-immune to malaria. This is a key feature of placental falciparum malaria in humans. This presents the baboon as a new model for the characterization of malaria during pregnancy.

**Keywords:** Baboons, Non-immune, Placental malaria, *Plasmodium knowlesi*, Accumulation

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# *Plasmodium knowlesi* Ligand-receptor Process in Baboon (*Papio anubis*) Placenta

Joab O. Nyamagiri[1, 2*], Faith I. Onditi[1, 3], Lucy Ochola[1], Rebecca Waihenya[2], Hastings S. Ozwara[1]

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**Abstract**

Pregnancy associated malaria poses many risks to both women and their infants. It is characterized by the accumulation of infected erythrocytes in the intervillous spaces of the placenta leading to adverse reactions. Studies using the *P. knowlesi*-Olive baboon model of pregnancy malaria have demonstrated this phenomenon though the mechanisms and molecules involved are not known. This study sought to identify the ligands and receptor molecules that permit accumulation of infected erythrocytes in the placenta of *P. knowlesi* infected Olive baboons and to further test placental isolates for adhesion to purified receptors. Sequences of known *Plasmodium* erythrocyte binding antigens and human placental receptors were BLASTed against the genome of *P. knowlesi* and *P. anubis* respectively. Hits generated were analysed and characterized to determine the prospective ligands and receptors in *P. knowlesi* and *P. anubis* respectively. Also, four adult female baboons (*P. anubis*) were infected with *P. knowlesi* parasites and their placentas sampled. Infected erythrocytes isolated from these placentas were tested for binding against purified receptors. We identified Predicted CSPG 4 partial and Predicted HAPLN 1 as the putative receptor molecules in the Olive baboon. Further, the *P. knowlesi* erythrocyte binding proteins (EBP-*alpha*, EBP-*beta* and EBP-*gamma*) matched closely to the placental *P. falciparum* ligand *Var2csa*. However, static binding assays with *P. knowlesi* infected erythrocytes did not show any binding to purified receptors. This study has identified and proposed receptors and ligands involved in the adherence process in *P. knowlesi* infected Olive baboons during pregnancy.

**Keywords:** *Plasmodium knowlesi*, Olive baboon, receptor, ligand, malaria, pregnancy

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# *Plasmodium berghei* is immunomodulated by transgenic mouse interferon gamma leading to enhanced malaria protection in mice

Simeon Mogaka[1, 2], Caroline Muriithi[1], Faith Onditi[1], Ruth Mumo[1], Naomi Maina[2], Rebecca Waihenya[2] and Hastings Ozwara[1]

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***Abstract:*** *The aim of this study was to explore immunomodulatory potential of immunopotentiated Plasmodium berghei parasites in a murine malaria model with a view of contributing to malaria vaccine development strategies. Transfection was used to generate immunopotentiated Plasmodium berghei parasites through expression of mouse interferon gamma (mIFN-γ) in wild-type Plasmodium berghei parasites. Mice were inoculated with mIFN-γ expressing Plasmodium berghei parasites and treated. Another group of mice was inoculated with the parasite expressed mIFN-γ culture supernatants. The mice were later intraperitonially challenged with wild-type parasites. Sampling for cytokine and antibody assays was done and ELISA performed on the collected samples. Parasitaemia was monitored daily and survival time (days) recorded for the two sets of experiments. Analysis of variance (ANOVA) was used to analyze the results using graphpad instat software. There was a significantly higher level of IFN-γ ($p < 0.001$). The level of IL-4 was significantly low ($p < 0.05$). There was no significant difference in the levels of IgG ($p = 0.0682$). There was a 3 to 4 day delay in patent parasitaemia accompanied by reduced mean parasitaemia and improved survival of the mice. This study showed that interferon gamma expressing Plasmodium berghei immunomodulates malaria infection in mice leading to enhanced protection during challenge infection.*

***Key words:*** *immunomodulation, interferon gamma, parasitaemia, Plasmodium berghei, transfection.*

ABSTRACTS PRESENTED AT SEMINARS

1. The **1st Africa International Biotechnology & Biomedical Conference and the 8th International Workshop on Approaches to Single-Cell Analysis** held at Safari Park on 10th to 11th September 2014.

**Title: Baboon infants are protected *in utero* against *P. knowlesi* infection**

**Faith Onditi[1]** , Esther Kagasi[1] , Charles Omwandho[2], Fredrick Maloba[1], Idle Farah[1], Julie Moore[3],Hastings.S.Ozwara[1]
[1]Institute of Primate Research, National Museums of Kenya, P.O. Box 24481– 00502, Karen, Kenya, [2]University of Nairobi, P.O. Box 30197–00100, Nairobi, Kenya, [3]Center for Tropical and Emerging Global Diseases & Department of Infectious Diseases N330C Paul D. Coverdell Center 500 DW Brooks Drive University of Georgia Athens, GA 30602

**ABSTRACT**:

Infants born to mothers infected with placental malaria (PM) is a common occurrence in malaria endemic areas like sub-Saharan Africa. This could either result in foetal immune priming or tolerance in utero. To determine the effect of PM infection in protecting baboon infants against progression of malaria, we measured IgG levels in serum samples from infants born from PM positive and PM negative mothers in addition to cord, maternal peripheral circulation and placental circulation. Infants were infected with *P. knowlesi* ($1x10^5$ parasites) and progression of parasitaemia compare in the two groups. Infants born from malaria PM-positive baboons had higher IgG titers compared to infants born from PM-negative baboons. This change was not significant (P= 0.4000). At peak parasitaemia infants born from PM-negative mothers had significantly higher parasitaemia levels (P= 0.0287) compared to infants born from PM-positive mothers. This study supports the hypothesis that in utero exposure to malaria infection is associated with foetal immune priming in non-immune baboons.

**Key words**: Baboon (*Papio anubis*), In utero, Placental malaria, *Plasmodium knowlesi*

2. The 9th **Federation of African Immunological Societies (FAIS) conference** held at Safari Park hotel on 30th November to 4th December 2014.

**Title: IN UTERO SENSITIZATION OF *PLASMODIUM KNOWLESI* IN BABOON INFANTS**

**Faith I. Onditi[1, 2]**,Charles O.A. Omwandho[2] , Fredrick Maloba[1] ,Christopher L. King[3] ,Julie M. Moore[4], Hastings S. Ozwara[1]

[1]Department of Tropical and Infectious Diseases, Institute of Primate Research, P. O Box 24481-00502 Karen, Nairobi-Kenya. [2]Department of Biochemistry, University of Nairobi, P. O Box 30197-00100, Nairobi-Kenya. [3]Center for Global Health and Disease, Case Western Reserve University, Wolstein Research Building 4-132, 2103 Cornell Road, Cleveland, OH 44106,. [4]Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia Athens, GA 30602-7387

**ABSTRACT**:

Infants born to mothers infected with placental malaria (PM) is a common occurrence in malaria endemic areas like sub-Saharan Africa. This could either result in foetal immune priming or tolerance in utero. To determine the effect of PM infection in protecting baboon infants against progression of malaria, we measured IgG levels in serum samples from infants born from PM positive and PM negative mothers in addition to cord, maternal peripheral circulation and placental circulation. Infants were infected with *P. knowlesi* ($1x10^5$ parasites) and progression of parasitaemia compare in the two groups. Infants born from malaria PM-positive baboons had higher IgG titers compared to infants born from PM-negative baboons. This change was not significant (P= 0.4000). At peak parasitaemia infants born from PM-negative mothers had significantly higher parasitaemia levels (P= 0.0287) compared to infants born from PM-positive mothers. This study supports the hypothesis that in utero exposure to malaria infection is associated with foetal immune priming in non-immune baboons.

**Key words**: Baboon (*Papio anubis*), In utero, Placental malaria , *Plasmodium knowlesi*

3. **Point of Care Workshop** held at the Institute of Primate Research on 25th -29th June 2012.

Title: **Baboon (*Papio anubis*) as a model for developing future diagnostic tools for placental malaria in humans**

**Onditi F[1,2], Omwandho C[2], , Farah IO [1], Moore J [3], Ozwara H[1]**
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Abstract

Malaria infection during pregnancy leads to adverse pregnancy outcomes affecting both the mother and the child/foetus. In humans, comprehensive studies cannot be carried out directly due to confounding variables hence lack of effective diagnostic tools for placental malaria (PM). Reproducible animal models are required to overcome these challenges. The human-like structure of the baboon placenta and the cyto-adherent property of *Plasmodium knowlesi* justify the *baboon-P. knowlesi* model. In our approach, pregnant baboons were experimentally infected with *P. knowlesi* H strain parasites ($1x10^6$). They were monitored for parasitaemia, clinical symptoms (from day 2 post infection) and analyzed for haematology and immunology. Placental tissues (collected via caesarean section) were analyzed for pathological findings. Our findings show that parasitaemia development was associated with clinical symptoms and abortion. Pathology demonstrated sequestration of *P.knowlesi* parasites and massive congestion in placental layers, and widespread aggregation of erythrocytes. Immunological findings showed mixed Th1/Th2 responses and reduced acquired immune responses in these baboons. These findings in the *baboon-P. knowlesi* model correlate well with PM observed in humans. Consequently, the baboon- *P. knowlesi* model of malaria is a promising model of choice that can be used in the development of diagnostic and intervention strategies against PM in humans.

4. **Kenya Society for Immunology Conference** held at Kenya Medical Research Institute on 24th-25th May 2012.

**Title: Baboon (*Papio anubis*) as a potential model for understanding immunological mechanism of placental malaria**

**Onditi F[1,2], Onkoba N[1], Omwandho C[2,], Ozwara H[1], Farah IO [1], Moore J [3]**
[1]Institute of Primate Research, National Museums of Kenya, P.O. Box 24481– 00502, Karen, Kenya, [2]University of Nairobi, P.O. Box 30197–00100, Nairobi, Kenya, [3]Center for Tropical and Emerging Global Diseases & Department of Infectious Diseases N330C Paul D. Coverdell Center 500 DW Brooks Drive University of Georgia Athens, GA 30602

## Abstract

The human-like structural of the Olive baboon (*Papio anubis*) placenta and the cyto-adherent property of *Plasmodium knowlesi* have informed choice for *baboon-P. knowlesi* model of placental malaria (PM). The Olive baboon model of PM is thus useful in understanding pathophysiology of PM in humans. This study sought to develop and utilize the baboon model of placental malaria. Pregnant baboons were experimentally infected with *Plasmodium knowlesi* H strain parasites ($1x10^6$). They were monitored for parasitaemia and clinical observations recorded from day 2 post infection. Peripheral blood was collected on a weekly basis for haematological and immunological analysis while placental tissues were collected via caesarean section, processed and analyzed for pathological changes. Our findings show that parasitaemia development was associated with clinical symptoms leading to abortion and still birth. Pathological findings demonstrated sequestration of *P.knowlesi* parasites in baboon placenta coupled with placental damage (massive congestion in placental layers and widespread aggregation of erythrocytes). Immunological findings showed mixed Th1/Th2 responses as well as reduced acquired immune responses in pregnant baboons infected with *P. knowlesi*. In conclusion this study demonstrates that PM in baboons (experimentally infected with *P. knowlesi*) leads to clinical symptoms that are associated with poor pregnancy outcomes and high levels of parasitaemia. Immunological and pathological findings in this study correlate well with those observed in humans. Consequently, the baboon- *P. knowlesi* model of malaria is a promising model of choice in the study malaria during pregnancy.

**Key Words:** *Papio anubis,* placental malaria, *Plasmodium knowlesi*

**AWARDS**

# BEST POSTER AWARD

## BABOON INFANTS ARE PROTECTED IN UTERO AGAINST P. KNOWLESI INFECTION

By

Faith Onditi, Esther Kagasi, Charles Omwandho, Fredrick Maloba, Idle Farah, Julie Moore and Hastings Ozwara

*Presented during The 1st Africa International Biotechnology and Biomedical Conference and The 8th International Workshop on Approaches to Single-Cell Analysis*

*Nairobi, Kenya*

*10th - 12th September 2014*

Hideki Kambara

*Hideki KAMBARA, Conference Chair*