

CHARACTERISATION OF PLACENTAL MALARIA IN OLIVE BABOONS
(*PAPIO ANUBIS*) INFECTED WITH *PLASMODIUM KNOWLESI* H STRAIN //

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placental malaria in*



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DECLARATION

I Mustafa Barasa declare that this thesis is my original work and has not been presented for a degree in any other university or for any other award

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DEDICATION

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ACRONYMS AND ABBREVIATIONS

ARD	Animal Resources Department
BSA	Bovine Serum Albumin
CSA	Chondroitin Sulfate A
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Foetal Bovine Serum
IFN-γ	Interferon Gamma
IEs	Infected Erythrocytes
Ig G	Immunoglobulin G
Ig M	Immunoglobulin M
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-12	Interleukin 12
IPR	Institute of Primate Research
LBW	Low Birth Weight
MEM	2-Methoxyethoxymethyl
OD	Optical Density
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCV	Packed Cell Volume
PM	Placental Malaria
PRBC	Parasitised Red Blood Cells

RBC	Red Blood Cells
rpm	Revolutions Per Minute
SPAS-BPS	School of Pure and Applied Sciences – Board of Postgraduate Studies
SI	Stimulation Index
T h 1	T Helper 1
T h 2	T Helper 2
TNF α	Tumor Necrosis Factor Alpha

ABSTRACT

Pregnant women have increased susceptibility to malaria infection. In these women, malaria parasites are frequently found sequestered in the placental intervillous spaces, a condition referred to as placental malaria (PM). Placental malaria threatens the health of the mother and the child's life by causing intrauterine growth retardation, abortions, still births and reduction in gestational age. An estimated 24 million pregnant women in Sub-Saharan Africa are at risk. Mechanisms responsible for increased susceptibility in pregnant women are not fully understood. Baboons are susceptible to *Plasmodium knowlesi* and have similar host pathogen interactions and reproductive physiology similar to humans, making them attractive for the development as a model for studying mechanisms underlying development of placental malaria. This study exploited the susceptibility of baboons to *Plasmodium knowlesi* infection to develop a non-human primate (baboon) model for studying PM. The main objective of the study was to demonstrate PM and characterize immunological mechanisms underlying the pathogenesis of PM in baboons infected with *Plasmodium knowlesi*. The pregnancies of three time mated adult female baboons and their gestational levels (one in its second trimester and two in their third trimester) were confirmed by ultrasonography. On the 150th day of gestation, the pregnant baboons were infected with *Plasmodium knowlesi* H strain parasites together with four non pregnant controls. Peripheral parasitaemia development was monitored on a daily basis from two days post inoculation. Collection of sera, plasma, mononuclear cells and haematological samples was done on a weekly basis. Peripheral blood mononuclear cells (PBMC) were stimulated in culture with concanavalin A and *P. knowlesi* antigens and their proliferation quantified. Sera cytokine and immunoglobulin concentrations were measured by ELISA using respective enzyme conjugated antibodies. Two pregnant baboons aborted (one on day 6 and the other on day 7 post infection) and cesarean section was only done on one baboon. Smears prepared from placental blood demonstrated the presence *Plasmodium knowlesi* parasites in all the sampled placentas. On average, the pregnant baboons had more than 29 fold higher placental parasitaemia than simultaneous peripheral parasitaemia. This shows that *Plasmodium knowlesi* preferentially sequesters in the baboon placenta just like *Plasmodium falciparum* does in humans. Two baboons that had high placental parasitaemia experienced abortion, which is a sequelae of human placental malaria. Results indicate that PM in this model is associated with significant ($P < 0.05$) suppression of immunoglobulin G, Interferon gamma, and interleukin 6 responses. Tumour necrosis factor alpha responses were significantly upregulated ($P < 0.05$) while immunoglobulin M, interleukin 10, interleukin 12, interleukin 4 and PBMC proliferation responses did not differ from controls ($P > 0.05$). These data are consistent with some findings from human studies, showing the feasibility of this model for studying mechanisms underlying placental malaria. The study has contributed valuable data to be used in further studies and the development of preventative, control and therapeutic measures against PM in women.

CHAPTER ONE: INTRODUCTION

1.1 Background Information

Many experiments have demonstrated that there is increased susceptibility to *Plasmodium falciparum* malaria infection in pregnant women (Malhora *et al.*, 2002). In these women, malaria parasites frequently sequester in placental intervillous spaces, a condition referred to as placental malaria (PM). Malaria during pregnancy leads to many complications in women and their infants, threatening the lives of both the mother and the child. These complications include stillbirths, pre-term births, low birth weights, reduction in gestational period, anaemia and high fever (Menendez *et al.*, 2000; Malhora *et al.*, 2002). An estimated 24 million pregnant women in sub Saharan Africa are at risk of suffering from PM and prevalence may exceed 50% among primigravidae and secundigravidae in endemic areas (Phillips-Howard *et al.*, 1999). There is no vaccine and currently the best therapeutic measure involves the use of artemisinin-based combinational therapy (www.who.com).

The causes of increased susceptibility to *P. falciparum* in pregnant women are not fully understood. Recent studies have shown that parasite-induced inflammatory-like reactions produced in the placental foetal-maternal interface are responsible for the adverse effects of PM and that different cytokine profiles may influence protection from PM (Moore *et al.*, 1999; Davison *et al.*, 1998). Thus, understanding the underlying cell-mediated immune responses can help in the development of mechanisms of control. This study characterised clinical aspects and underlying immune responses during placental malaria in baboons experimentally infected with *P. knowlesi* H strain parasites. Generated data characterized PM and can help in understanding the immunopathogenesis of PM in pregnant women.

1.2 Problem Statement

Placental malaria (PM) has been known to predispose infected women to still births, pre-term births, low birth weights and reduction in gestational period (Fievet *et al.*, 2001). Malaria during pregnancy leads to many complications in women and their infants including anaemia, high fever, hypoglycaemia, pulmonary oedema, renal failure, cerebral malaria, abortion, intrauterine growth retardation, congenital infection, and increased foetal and maternal mortality and morbidity (Menendez *et al.*, 2000; Steketee *et al.*, 2001). Although PM is to an extent studied in pregnant women, the studies have shortcomings resulting from confounding inherent variables such as the mother's health status, inaccurate estimation of gestational age, inadequate tissue for analysis. Other problems include patient compliance, socio-economic conditions, moral, ethical and financial limitations (Moore *et al.*, 1999; Steketee *et al.*, 1996). As a result, many questions are not satisfactorily addressed during human studies.

Neither the immune response mechanisms involved during placental malaria nor the immunopathophysiologic mechanisms underlying adverse fetal outcomes are fully understood. In addition, other mechanisms such as transplacental transmission, congenital infections and effects of maternal anaemia have not been well understood (Fievet *et al.*, 2001). Previous studies have shown that the foetal-maternal interface parasite-mediated induction of different cytokine profiles influences the development of an inflammatory - like reaction that seem to be involved in the immunopathogenesis of placental malaria (Fievet *et al.*, 2001; Ordi *et al.*, 2001).

1.3 Justification

Animal models that mimic the human PM are required to give a clearer understanding of mechanisms of PM immunopathogenesis in order to formulate better control measures. Although rodent models are well established (Odoula *et al.*, 1982; Van Zon *et al.*, 1986; Vinayak *et al.*, 1986; Odoula *et al.*, 1986; Desowitz *et al.*, 1989; Tegoshi *et al.*, 1992;), their relevance to malaria in human pregnancy is questionable because of the many differences between rodent and human pregnancies. Rats cannot be used as models of the human maternal fetoplacental unit because progesterone is produced by the corpus luteum of the ovary in rats rather than the placenta as in humans and because the rodent placenta is labyrinthine hemodichorial, rather than villous hemomonochorial like the human placenta (Bernische and Kaufmann, 1995; Pepe and Albrecht, 1995). The usefulness of rodents in the study of congenital malaria is also questionable (Fischer, 1996).

An effective disease model should closely mimic the pathogenesis of malaria in pregnant women. Non human primates are ideal candidates because they are susceptible to many species of *Plasmodium* and have humoral and cellular responses similar to those of humans (Coeneyi *et al.*, 1971). In addition to macaques, great apes and baboons have discoid villous hemochorial placentas similar to those of humans. Great apes cannot be used because they are endangered species. Currently, an olive baboon *Plasmodium knowlesi* model has been developed. The baboon placenta has also been studied extensively. Thus the baboon is a logical choice for these studies. There are only three previous reports on malaria during pregnancy in non human primates. One pregnant rhesus monkey was

inoculated with *P. knowlesi* twice during the third trimester (Das Gupta, 1934). The monkey became severely anaemic and was killed 17 days post infection. Although the placenta was heavily parasitized, the foetus did not become congenitally infected. Rhesus monkeys at gestational day 56-70 have previously been infected with *P. cynomolgi* (Saxena *et al.*, 1988). The rhesus monkey *Plasmodium coatneyi* model of malaria during pregnancy was established by inoculating pregnant rhesus monkeys (*Macaca mulatta*) with *Plasmodium coatneyi* during the first trimester (Davison *et al.*, 1998).

4.4 Research Questions

4.4.1 Does *Plasmodium* ...

Results of experimental infection of baboons may give a strong indication of what could happen in a human situation. This is because these non-human primates have similar host-pathogen interactions and reproductive physiology to humans (D' Hooghe *et al.*, 2004). The menstrual cycle of baboons is about 28 days and the gestation period is on average 180 ± 7 days compared to 165 ± 10 days in rhesus monkeys. Non-human primates like baboons are of particular interest because, other than the armadillo, they are the only animals with a discoidal, villous, hemochorial placenta like that of humans (Davison *et al.*, 1998). Baboons (*Papio anubis*) are susceptible to *P. knowlesi* and are extensively used in reproductive studies (D' Hooghe *et al.*, 2004; Escalante *et al.*, 1995; King, 1993).

Plasmodium knowlesi, a parasite whose entire genome has been sequenced to 5-fold coverage (<http://www.sanger.ac.uk>), is an ideal experimental system for malaria research because it is a parasite of monkeys, which have immune and metabolic systems that are

physiologically very similar to those of humans (King, 1988; Mons *et al.*, 1990). It is a pathogen known to infect humans and is an excellent model parasite for studies of malaria in pregnancy, since, like *P. falciparum* it is a cytoadherent parasite (Ozwarra *et al.*, 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 studies into the immunopathogenesis of PM.

1.4 Research Questions

- i) Does *Plasmodium knowlesi* cause placental malaria in the olive baboon ?
- ii) If so what are the clinical effects and immune response mechanisms underlying placental malaria in the olive baboon-*Plasmodium knowlesi* model?

1.5 Null Hypothesis

Baboons infected with *Plasmodium knowlesi* H strain parasites do not develop placental malaria, disease symptoms and immune responses associated with human placental malaria.

1.6.0 Objectives

1.6.1 General Objective

Characterisation of placental malaria in baboons (*Papio anubis*) infected with *Plasmodium knowlesi* H strain

1.6.2 Specific Objectives

- i) To determine whether pregnant olive baboons infected with *Plasmodium Knowlesi* develop placental malaria
- ii) To determine clinical effects of disease in olive baboons infected with *Plasmodium knowlesi*
- iii) To determine antibody responses in olive baboons infected with *Plasmodium knowlesi*
- iv) To determine cytokine responses in olive baboons infected with *Plasmodium knowlesi*

CHAPTER TWO: LITERATURE REVIEW

2.1 Historical Background of Malaria

Malaria is an ancient scourge as evidenced by early Chinese and Hindu writings. During the fourth century B. C., the Greeks noticed association with exposure to swamp and began drainage process to control the disease. The Italians gave the disease its name, malaria, which means 'bad air' in the seventeenth century. Malaria ranged as far north as Siberia and as far south as Argentina. In 1902, Donald Ross received a nobel prize for demonstrating the life cycle of the protozoan cause of malaria (Nester *et al.*, 2004).

Malaria is the most common serious infectious disease worldwide. In 1955, the World Health Organisation (WHO) began a worldwide campaign aimed at elimination of malaria (Nester *et al.*, 2004). Initially there was great success, as WHO employed insecticides against the mosquito vector, detected infected patients by obtaining blood smears, and provided treatment for those who were infected. Fifty—two nations undertook control programs and, by 1960, 10 of them had eradicated the disease. Unfortunately, strains of *Anopheles* mosquitoes resistant to insecticides began to appear, and in cooperation with bureaucracy and complacency, malaria began a rapid resurgence. In 1976, the World Health Organisation acknowledged that the eradication program was a failure (Nester *et al.*, 2004). Today there are 300 to 500 million infected people annually worldwide, with about 3 million deaths (Nester *et al.*, 2004). More people are dying from the disease than when the eradication programs first began. Of all the parasitic diseases, malaria is not only the greatest killer but is also the most widely spread. It is a threat to approximately 40% of the

human population (Nester *et al.*, 2004). Efforts to control malaria are made through treatment of infected people and by physical and chemical strategies to control the mosquito vector. However, it is to be expected that, due to the rapid increase in multi-drug resistant parasites and reduced investment in developing new drugs, the number of malaria cases and casualties will rise in the coming years. These factors necessitate the development of new and alternative approaches to resolve the current malaria crisis (Ozwarra *et al.*, 2005).

The development of new and alternative strategies for malaria control is hampered by the complex life cycle of the parasites and their interactions with human hosts and insect vectors. Therefore, a more rational approach to resolving the malaria crisis requires a deeper understanding of the biology of malaria parasites. Malaria is a parasitic disease affecting red blood cells (RBC) and hepatocytes and is transmitted by female mosquitoes of the genus *Anopheles*. *Anopheles gambiae* and *A. funestus* are the main vectors of the disease in Africa. Human malaria is caused by protozoa of the genus *Plasmodium*. Four species are involved- *P. vivax*, *P. falciparum*, *P. malariae*, and *P. Ovale*. These species differ in microscopic appearance and, in some instances, life cycle, type of disease produced, severity, and treatment. *Plasmodium falciparum* is the most common and the most virulent, causing between 700 000 and 2.7 million deaths (approximately 90% of deaths) annually, most of which are in children and pregnant mothers (www.cdc.gov). The millions of pregnant women residing in areas where malaria is endemic are particularly vulnerable to the effects of *P. falciparum* infection. The severity of malarial infections is

also considerably increased in pregnant women and their unborn children (www.cdc.gov).

The first symptoms of malaria are flu-like, with fever, headache, and pain in the joints and muscles. The symptoms begin generally about two weeks after the bite of an infected mosquito, but in some cases they can begin many weeks after ward. After 2 or 3 weeks of these symptoms, the pattern changes and symptoms tend to fall into three phases highly suggestive of malaria. The patient abruptly feels cold and develops shaking chills that can last for as much as an hour (cold phase). Following the chills, temperature begins to rise steeply, often reaching 40°C (104°F) or more (hot phase). After a number of hours of fever, the temperature falls and drenching sweat occurs (wet phase). Except for fatigue, the patient feels well until 24 to 48 hours later, depending on the causative species, when the pattern of symptoms repeats (Nester *et al.*, 2004).

2.2 The Life Cycle of the Simian Malaria Parasite, *Plasmodium knowlesi*

Malaria infection in the monkey begins when an infected mosquito injects sporozoites into the blood circulation. The sporozoites circulate in the blood for less than one hour before invading liver cells, and develop into tissue schizonts in less than one week. The liver schizonts release thousands of merozoites into the blood stream, where they invade erythrocytes in less than 30 minutes. The erythrocyte invading merozoites quickly develop into ring forms. The late ring forms grow and differentiate into actively feeding forms known as trophozoites. A number of the ring forms differentiate into male and female

sexual forms referred to as gametocytes.

As the trophozoites feed and develop, the nucleus undergoes between 8 and 16 rounds of division (schizogony), without subsequent cell division. The multinucleate trophozoite (schizont) starts to divide its cytoplasm producing a similar number of haploid merozoites. The fully mature schizont (segmenter) ruptures the erythrocyte, releasing free merozoites that invade other erythrocytes, thereby completing the blood stage cycle. When a mosquito ingests blood with mature gametocytes, they emerge from the erythrocytes and differentiate into gametes. The male gametocyte (microgametocyte) undergoes three rounds of nuclear division, producing 8 flagellated microgametes (exflagellation), while the female gametocyte differentiates into a single gamete called macrogamete (Ozwarra *et al.*, 2005).

In the mosquito midgut, the microgamete immediately fertilizes the macrogamete to form a diploid (2N) zygote. The diploid zygote undergoes one more round of genome division into a polydiploid (4N) zygote. The zygote then undergoes meiotic division and transforms into a motile ookinete, which penetrates the midgut wall, and rounds up below the midgut epithelium as an oocyst. The oocyst enlarges and the nucleus divides frequently, followed by multiple cell fission to form thousands of midgut sporozoites. The sporozoites are released into the haemocoel where they migrate into the salivary glands, waiting for being injected into a new vertebrate host (Ozwarra *et al.*, 2005). The mosquito hosts for *P. knowlesi* include *Anopheles dirus*, *A. balabacensis* and *A. hackeri* all of which are found in

Asia. Other possible vectors include *A. freeboni*, *A. maculates*, and *A. quadrimaculatus*. Development in the mosquito (sporogony) takes 10-12 days at a temperature of 26 to 28°C while the exo-erythrocytic phase lasts 5.5 days (Garnham *et al.*, 1966). *Plasmodium falciparum* (Fig 2.1) and *P. vivax* (Fig 2.2) both have similar life cycles to *P. knowlesi* (Fig 2.3).

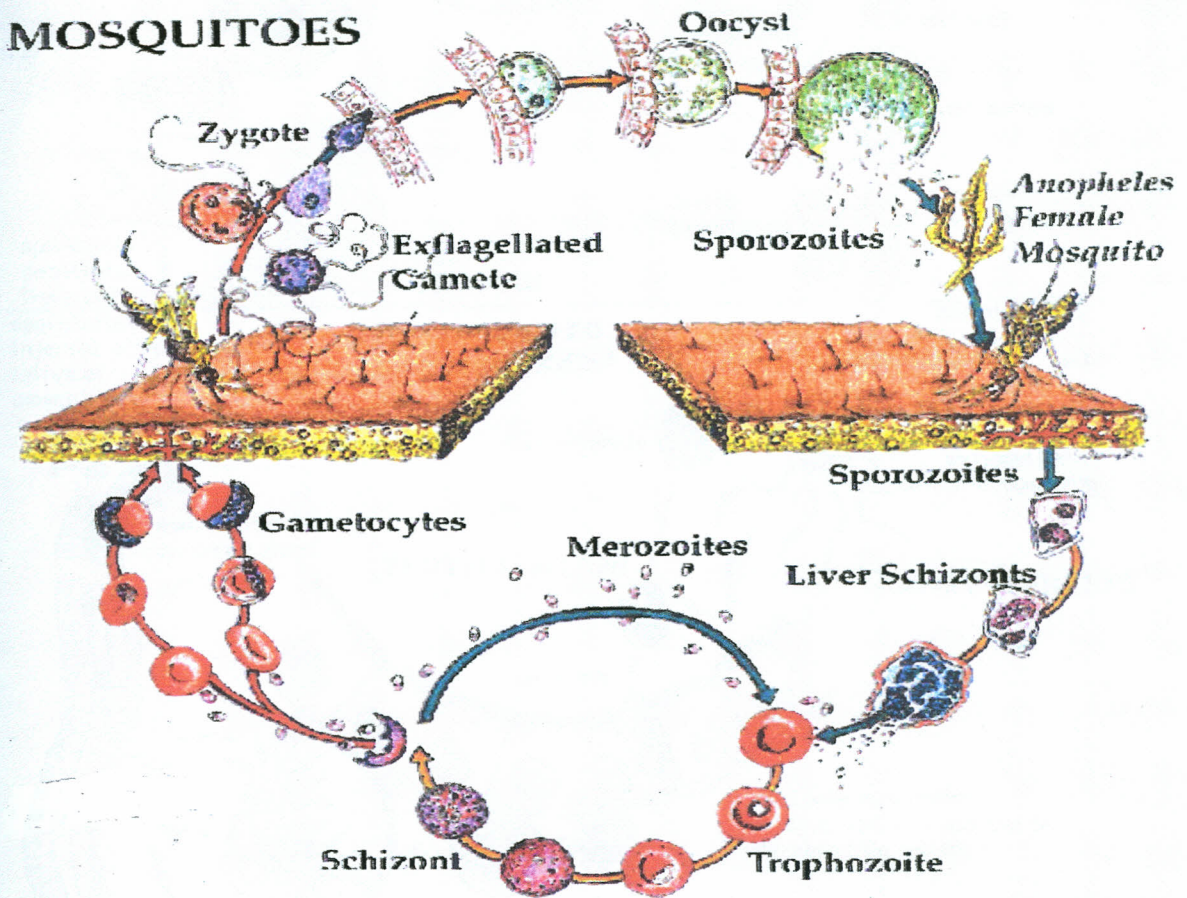


Fig 2.1: The life cycle of the human parasite, *Plasmodium falciparum*. The invasion of erythrocytes by erythrocytic merozoites unfolds in four steps: (1) initial recognition and attachment of the merozoite loosely to the erythrocyte membrane; (2) reorientation and junction formation between the apical end of the merozoite and the release of rhoptry-microneme substances with vacuole formation; (3) movement of the junction and invagination of the erythrocyte membrane: around the merozoite accompanied by removal of the merozoite's surface coat; and finally (4) resealing of the parasitophorous vacuole membrane and erythrocyte membrane after completion of merozoite invasion. Source: www.ijib.com/parasitelifecycles.

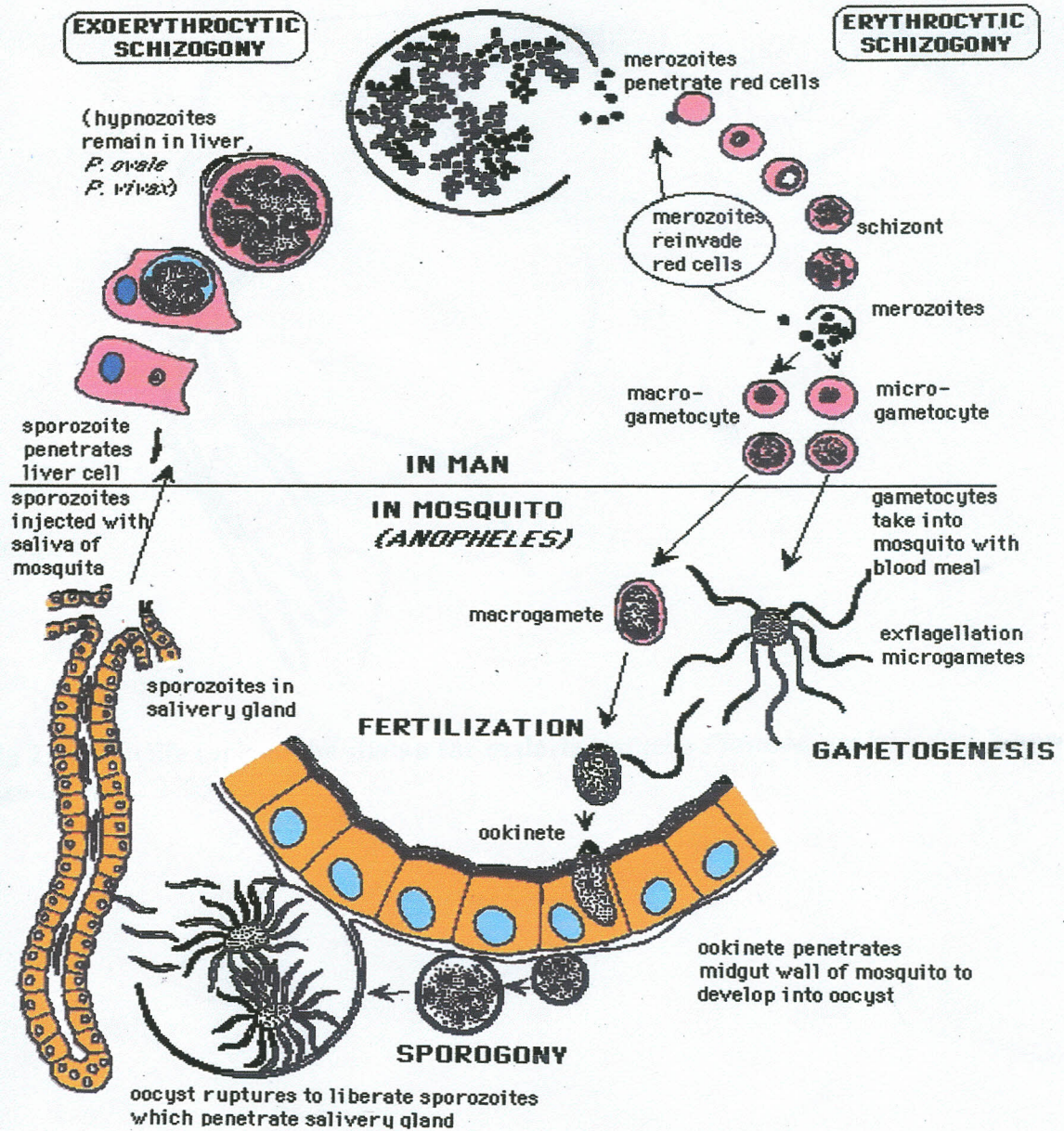
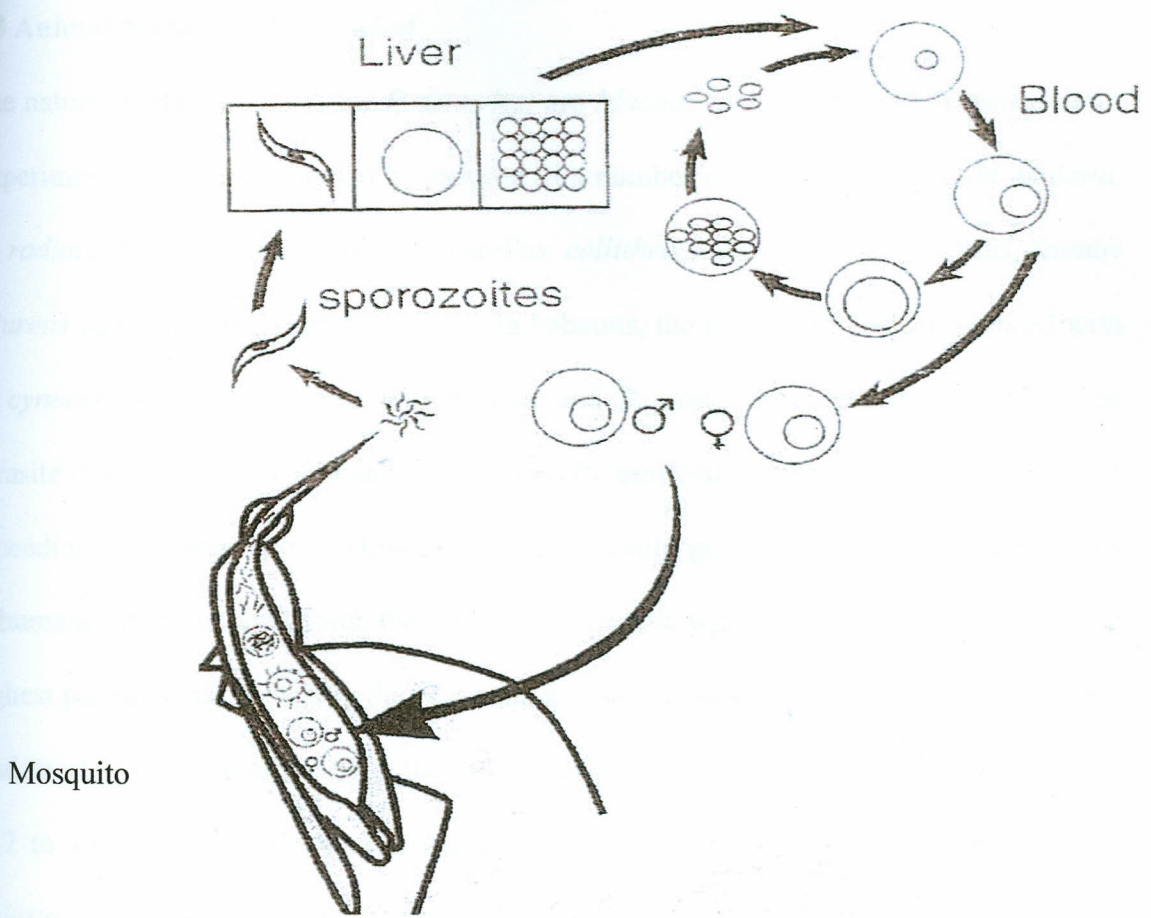


Fig 2.2: The life cycle of the human parasite, *Plasmodium vivax*. *Plasmodium vivax* is phylogenetically close to the monkey parasite, *Plasmodium knowlesi*. Source: www.ijib.com/parasitelifecycles.



Mosquito

Fig 2.3: The life cycle of the simian the malaria parasite *Plasmodium knowlesi*. Source: Ozwara *et al.*, 2005.

2.3 Animal Models of *P. knowlesi*

The natural vertebrate hosts for *P. knowlesi* are *Macaca fascicularis* and *M. nemestrina*. Experimental infection can also be induced in a number of monkeys such as *M. mulatta*, *M. radiata*, *M. assamensis*, *Presbytis entellus*, *Callithrix jacchus*, *Aotus trivigatus*, *Saimiri sciureus* and baboons (Garnham, 1966). In baboons, the infection has been induced with *P. cynocephalus*, *P. doguera*, *P. jubilaeus* and *P. papio* (Ozwarra *et al.*, 2005). The parasite does also infect humans. In humans, *P. knowlesi* causes mild infection seldom exceeding 1 % parasitaemia. However, infection could get virulent on repeated passages in humans. In the natural hosts, the infection is chronic with several relapsing peaks. The highest parasitaemia is usually the first peak and rarely passes 5 %. In the rhesus monkey (and most experimental models), the infection is acute and usually fatal, killing animals in 7 to 14 days post-infection. It requires several infections and cure in order for *M. mulatta* to develop immunity against *P. knowlesi* (Miller *et al.*, 1977).

The availability of natural and experimental hosts for *P. knowlesi* offers the possibility to study the biology of the malaria parasite and its antigens in a natural host parasite combination and in hosts whose systems are predictable of the human situation. In addition, the dichotomous disease profile of *P. knowlesi* (a chronic infection in natural hosts and an acute disease in artificial hosts) provides opportunities for understanding the mechanisms of immunity to malaria. Experimental *P. knowlesi* infection has now been characterized in *P. anubis*, the most commonly used baboon for biomedical research (Ozwarra *et al.*, 2005).

2.4 *Plasmodium knowlesi* as a Model Parasite for Malaria Research

Although human malaria parasites are seen as a major priority for research because of the mortality associated with this infection, they are not always the optimal system in which to study the biology and immunology of *Plasmodium*. The host specificity of human malaria parasites represents a major constraint on *in vivo* studies. The parasites can not be maintained in convenient small laboratory animals. As a result, numerous species of rodent (e.g. *P. berghei*) and avian (e.g. *P. gallinaceum*) malaria parasites have been widely used as laboratory models to study the biology of *Plasmodia*. Although there is proximal phylogenetic relationship between *P. falciparum* and avian *Plasmodia* such as *P. gallinaceum*, differences in their lifecycles, insect hosts and in the immune systems of their vertebrate hosts limit their usefulness as models for human malaria. A good laboratory model should be relevant for human malaria and offer the ability to study the biology of the parasite at the cellular and molecular level. Biological, chemotherapeutic and immunological studies have made use of the fact that a number of *Plasmodium* species found naturally in monkeys are very similar biologically and antigenically and have similar host-parasite relationships to the human *Plasmodia* in man. This is in part due to the close phylogenetic relationships between the nonhuman primate and human malaria parasites.

Plasmodium knowlesi, a parasite whose entire genome has been sequenced to 5-fold coverage (<http://www.sanger.ac.uk>), is an ideal experimental system for malaria research because it is a parasite of monkeys, which have immune and metabolic systems that are

physiologically very similar to those of humans (King, 1988; Mons *et al.*, 1990). It is a pathogen known to infect humans and is an excellent model parasite for studies of malaria in pregnancy, since it is a cytoadherent parasite (Ozwarra *et al.*, 2005) and is likely to sequester in the placenta. This parasite is phylogenetically close to *P. vivax*, sharing many vaccine candidate molecules (<http://www.sanger.ac.uk>).

2.5 Malaria During Pregnancy

In all areas where malaria is endemic, the frequency and severity of malarial infections are greater in pregnant women than in nonpregnant (Gilles *et al.*, 1969; Brabin, 1983). The effects of malarial infection on the mother and the foetus, under different levels of malarial endemicity, have recently been summarized by Menendez (1995). Where malaria is unstable, pregnant women are susceptible to all the manifestations of severe *Plasmodium falciparum* infection and have 2-10-fold higher mortality than non-pregnant women (Brabin, 1983, 1991). Abortion, stillbirth, premature delivery and low birth weight of the infant are also common in pregnant women infected with malaria. In areas of stable malaria transmission, maternal morbidity is mainly represented by anaemia (Brabin, 1983), and the major effect on the foetus is a reduction in birthweight (Oeliffe, 1968; Gilles *et al.*, 1969; McGregor *et al.*, 1983; Watkinson and Rushton, 1983; Cot *et al.*, 1992, Matteelli *et al.*, 1996; Steketee *et al.*, 1996a). In this setting, prime gravidae are much more likely to be affected. The prevalence of infection and the parasite density in peripheral blood both peak in the first half of the gestation period and then decrease progressively until

delivery (Brabin, 1983).

2.6 Occurrence, Distribution and Consequences of Placental Malaria

Placental infection is a well described complication of malaria in pregnancy. Brabin (1983) summarised the results of the relevant studies conducted up to 1978, which showed similar rates of placental parasitisation and peripheral blood parasitaemia. Despite similar rates of occurrence, there is a frequent absence of correlation between placental infection and peripheral parasitaemia (Walter *et al.*, 1981; Watkinson and Rushton, 1983). In a series of 357 women at delivery, Matteelli *et al.* (1994) observed 123 discordant results: 93 women had placental infection without peripheral parasitaemia and 30 had peripheral parasitaemia without placental infection. In their cohort study, Watkinson and Rushton (1983) observed that peripheral parasitaemia had been detected during pregnancy in 12 of 27 mothers with pigmented placentas and in 12 of 38 with non-pigmented placentas.

These results may be explained by the fact that examination of peripheral blood is not always sensitive enough to detect infections when the parasites in the subject are concentrated in the placenta (and is certainly not sensitive if only performed at delivery). The results of Watkinson and Rushton (1983) also show that a malarial infection acquired early in the gestation period may leave no sign of placental disease at delivery. Placental infection can occur in all areas of malaria transmission but its frequency and severity are greatest in highly endemic regions. Primigravidae suffer the most severe consequences of placental infection. After the first report by Bruce-Chwatt (1952), several other studies

have demonstrated a strong association between placental malaria and low birthweight (McGregor *et al.*, 1983; Watkinson and Rushton, 1983; Cot *et al.*, 1992, Matteelli *et al.*, 1996; Steketee *et al.*, 1996a), although other factors, including anaemia (Brabin *et al.*, 1990), probably contribute to the production of small neonates. In rural Malawi, attributable risk for low birthweight was 13.6% for placental malarial infection compared with 5.3% for parasitaemia in blood from the umbilical cord (Steketee *et al.*, 1996a). In another cross-sectional study, in urban Zanzibar, placental malaria was responsible for 61.4% of the total, estimated, attributable low birthweight (Matteelli *et al.*, 1996). Malaria can cause low birthweight by intrauterine growth retardation (IUGR), premature delivery, or both. There is, however, current consensus that IUGR is the predominant mechanism (McGregor, 1984; Steketee *et al.*, 1996a) and that it is associated with placental infection.

Placental malaria may influence the risk of death and disease during the first year of life (infant mortality and morbidity) by inducing low birthweight. In a prospective study in rural Malawi, for example, the hazard ratios for the rate of infant mortality were 1.4 and 5.0 for babies weighing 2000-2499 g and < 2000 g, respectively. The observation, made on the mothers of these children in Malawi, that placental malaria does not contribute to perinatal mortality and that it is, in fact, possibly protective (McDermott *et al.*, 1996) still needs to be explained.

2.7 Immunopathogenesis of Placental Malaria

Several hypotheses have been proposed to explain the increased susceptibility of pregnant women to malaria and the high frequency of placental infection. It has been proposed that pregnancy exacerbates malaria through a non-specific, hormone-dependant, depression of the immune system (Weinberg, 1984; Brabin, 1985). Basically, two mechanisms have been suggested. The first is that pregnancy is a period of generalized immunosuppression which is mainly sustained by increased blood levels of cortisol; it has been shown that cortisol levels are higher in pregnant women with malarial infection than in those without, and that cell-mediated immune responses to malarial antigens are more markedly suppressed in first pregnancies than subsequent ones (Vleugels *et al.*, 1989). However, this hypothesis does not explain the preferential replication of parasites within the placenta. In addition, serum cortisol levels increase linearly during gestation whereas susceptibility to malaria peaks in the second trimester and then decreases.

The second proposed mechanism is that immunosuppression is mainly a local phenomenon at the placental level, sustained by increased local concentrations of oestrogen. Oestrogen production reduces with parity and, possibly, during gestation in infected women, because of decreased production of the hormone by the damaged infected placenta (Watkinson *et al.*, 1985). However, further studies are required to associate placental oestrogen concentrations with placental infection and pregnancy outcome. Recently, an alternative immunological hypothesis to explain the enhanced susceptibility

to malaria during pregnancy has been proposed (Smith, 1996). It has been observed that non-specific effector mechanisms play an important role in limiting the replication of *P. falciparum* in non-immune individuals, by activating type-1 cytokine responses (interferon- γ , interleukins 2 and 12, and tumour necrosis factor). However, the acquisition of specific protective immunity in areas of high endemicity for malaria is associated with the activation of type-2 cytokine responses (interleukins 10, 4 and 6).

It is proposed that pregnant women have an immune system which is biased towards type-2 humoral defence mechanisms and away from type-1 cellular responses because the latter would compromise the viability of the foetal-placental unit (Smith, 1996; Deloron and Maubert, 1995). Pregnant women would therefore be more susceptible to malaria because of the inhibition of type-1 cytokine responses. Although this hypothesis, if correct, would explain why the effects of malaria on the mother and foetus are more severe in areas of unstable malaria transmission than in areas where transmission is stable, the effects on foetal intra-uterine growth and the higher susceptibility of primigravidae in areas of high endemicity are harder to explain.

More than a decade ago, McGregor *et al.* (1983) suggested that the placenta is a preferential site of multiplication of malarial parasites because of (unidentified) immunological factors. Recently, this hypothesis has received further support from the discovery of a subpopulation of *P. falciparum* parasites which is possibly responsible for maternal

malaria (Fried and Duffy, 1996). The incriminated subpopulation adheres to chondroitin sulphate A (CSA), a glycosaminoglycan receptor. By performing *in-vitro* assays that measure adhesion to immobilized extracellular-matrix protein, the researchers have identified a distinct *P. falciparum* subpopulation with a specific CSA-binding phenotype (which does not bind to other receptors). In another set of *in-vitro* experiments, this subpopulation of parasites was seen to bind preferentially to trophoblastic villi, extracellular villi and syncytial bridges from placental tissue; a binding inhibited by the presence of free CSA.

Although CSA is a common component of the extracellular matrix and is found widely in the body, it is proposed that the placenta is the only site where interaction between CSA and the parasitised erythrocytes can actually occur. Since the CSA-binding phenotype was absent from samples of peripheral blood from nonpregnant women (Fried and Duffy, 1996), it may be uncommon in the non-pregnant host and common only in pregnant women. In the proposed model, women only provide a substrate for the CSA-binding phenotype of parasites when they are pregnant, at a time when sequestration of the parasites determines the picture of placental malaria. In areas of high endemicity, primigravidae are presumably the pregnant women most affected by malaria because, despite humoral recognition of an array of parasite antigens by the time they are adults, they are almost naive towards the CSA-binding parasite population. Multigravidae are presumably less affected because immunological memory from the first pregnancy is retained. As specific immunity would start developing as soon as the parasites sequester

in the placenta, infections acquired early in the gestation period might be cleared (both from the placenta and the peripheral circulation) by the end of the third trimester; this is consistent with the pattern of late pregnancy recovery from infection described by Brabin (1983).

The hypothesis of Fried and Duffy (1996) is also in line with the recent recognition of the diversity of parasite genes encoding for proteins that bind to receptors on various surface molecules of the host cell. This diversity indicates that the parasites have an enormous repertoire of potential host-binding sites and that subpopulations with tropisms for specific organs are likely to occur. Fascinating as this model is, it still needs to be confirmed by further studies. A few questions remain to be answered. Why, for example, in all the relevant histological studies conducted so far (Bray and Sinden, 1979; Walter *et al.*, 1981; Yamada *et al.*, 1989), has no concentration of infected erythrocytes been detected on the placental villi?

2.8 Histopathological Studies on Infected Placentas

The diagnosis of placental malaria is based on the identification of parasites or malarial pigment. Parasitised red blood cells have consistently been described in the intervillous spaces (on the maternal side of the bloodstream). The predominant stage is the trophozoite, but schizonts have also been reported (Miller *et al.*, 1998). Infected red blood cells do not line along or attach to the trophoblast. Pigment is present in several

sites; during the early phases of the infection it concentrates in the macrophages in the intervillous spaces, in the trophoblast, in Hoffbauer cells, and in fibrin deposits in the villous stroma. Clearance of pigment may be complete after active infection is resolved, but fibrin deposits are last to disappear (Bulmer *et al.*, 1993). It is assumed that clearance of pigment occurs within months, and that pigment identified in term placentas arises from infections acquired in the second half of the pregnancy. Parasites and pigment are not the only characteristic abnormalities of placental malaria.

Key histological observations were made by Galbraith *et al.* (1980) and Walter *et al.* (1981), who showed that malarial infection of the placenta results in several histopathological changes in the chorionic villi, of diminishing severity from the intervillous space to the villous mesenchymal stroma. The three main changes observed were: (1) monocytes abundant within the intervillous spaces; (2) cytotrophoblastic cells more common (reflecting a non-specific response to trophoblast damage); and (3) evident thickening of the trophoblastic basement membrane (Galbraith *et al.*, 1980; Walter *et al.*, 1981).

Similar histological changes (numerous monocytes in the intervillous spaces, trophoblastic damage and focal necrosis, partial loss of microvilli and thickening of the trophoblastic basement membranes) were described by Yamada *et al.* (1989), in another series of 20 placentas. In addition, Yamada *et al.* (1989) reported excessive syncytial

knotting and chronic basal villitis. Although they failed to demonstrate any correlation between the severity of the pathological changes and the birth weight of the related infant, the series they studied was very small and they made no attempt to quantify the histological damage. The first, published, semi-quantitative study of histological abnormalities in placental malaria was apparently that of Bulmer *et al.* (1993). In this study, fibrinoid necrosis was found to be a feature of active infection, thickening of the trophoblastic basement membrane was seen to occur in both infection categories (active and past), and perivillous fibrin and cytotrophoblast prominence were not associated with malarial infection. Unexpectedly, no association was seen between placental infection and low birth weight; the effects of histological changes on birth weight could not therefore be assessed. It might be important to note that, in this series, where placental infection was apparently not associated with low birthweight, accumulation of mononuclear cells in the intervillous spaces was uncommon.

Recently, Leopardi *et al.* (1996) reported the results of a study in which use of morphometry and quantification of parasites and white blood cells using a grid minimised any bias and showed general consistency among 372 placentas from urban Zanzibar. Morphometric techniques were applied to determine the quantitative (rather than qualitative) histological abnormalities which occurred in the placentas as the result of malarial infection. In this study, active placental infection (API) depicted by presence of parasites and pigment, but not past placental infection (PM) depicted by presence of pigment only or no infection (NM), was strongly associated with low birth weight. The

findings of this study may be summarized as follows: (i) infected placentas were characterized by a significant increase in fibrin deposits which was not, however, associated with low birth weight; (ii) the volumes of villi and of the intervillous spaces were similar in infected and non-infected placentas; (iii) the surface area of the respiratory villi covered by the syncytiotrophoblast was relatively large in placentas with active infection whereas the surface area of the blood capillaries within the third-order villi was relatively small; (iv) the numbers of leucocytes in the intervillous space of each of the placentas from women of the PM or NM groups (≤ 15 cells/20 fields) were significantly lower than in the AM placentas.

When the intervillous inflammatory reaction was scored from 0 to 4, on the basis of the numbers of leucocytes seen in 20 microscope fields, the level of the reaction showed an inverse linear relationship with birth weight. As McGregor *et al.* (1983) had observed earlier, the grading of parasitaemia showed no such relationship. Pigment deposits, which were present in placentas of the AM and PM groups, also did not correlate with birthweight. These findings indicate that the persistent presence of parasites (but not of malarial pigment) is necessary for the development of detrimental effects in the newborn. Parasites are unlikely to determine placental pathology directly because they are invariably confined to the intervillous space and because the numbers of them do not correlate with birthweight. Leucocytes in the intervillous space seem to be a hallmark of active placental infection and to be directly associated with the development of low birthweight.

2.9 Mechanisms of Impairment of Foetal Growth

How placental malaria influences birthweight is still unknown. Recent results indicate that the induction of premature labour is of little importance. The alternative is that placental malaria induces Intrauterine growth retardation (IUGR), but there is no conclusive evidence that placental function is impaired by malarial infection. It has been pointed out that the placenta has considerable functional reserve capacity, and that it can rapidly repair ischaemic damage and compensate for toxic injury (Brabin, 1991). In one study (McLaren and Ward, 1962), placental malaria was not associated with abnormal transfer of vitamin A or ergothioneine into the foetal circulation. There is no evidence that the placenta suffers immuno-mediated damage either. Neither the presence of complement fractions Clq, C4, C3 or C9 in the trophoblastic cytoplasm and basement membranes (Galbraith *et al.*, 1980) nor that of C3 and *P. falciparum* antigens (Yamada *et al.*, 1989) could be associated with the degree of parasitaemia in the placentas.

Several hypotheses have been proposed to explain how the histological changes described above may adversely affect the nutritional capacities of the placenta. These include the reduction of oxygen and nutrient transport across the placenta, caused by mechanical blockage arising from thickening of trophoblastic basement membrane, nutrient use by the developing and replicating parasites, and poor oxygen and glucose transfer from the parasitised erythrocytes sequestered in the placenta (Steketee *et al.*, 1996a). Non-chemotactic cytokines produced by the inflammatory reactions in the intervillous space have been proposed as the mediators of the trophoblastic damage and

the thickening of the trophoblastic basement membrane (Leopardi *et al.*, 1996). That malaria can impair placental function has been demonstrated in at least one study by measurement of plasma concentrations of oestradiol (a hormone synthesised by the placenta and by the foetal adrenal pathways); the concentrations in the mothers who later delivered infected placentas were significantly lower from 32 weeks gestation onwards than those in women with uninfected placentas (Watkinson *et al.*, 1985).

2.10 Potential for Prevention

Malaria chemoprophylaxis has been shown to be effective, in several trials, in reducing the incidence of low-birth weight infants and maternal anaemia when given to semi-immune women, particularly primigravidae. Chemoprophylaxis also significantly reduces placental malaria, giving support to the hypothesis that prevention of placental malaria is the mechanism by which chemoprophylaxis increases birthweight and decreases the proportion of low-birth weight infants. This has been demonstrated for chloroquine (Cot *et al.*, 1992), sulphadoxine-pyrimethamine (Schultz *et al.*, 1994) and mefloquine (Steketee *et al.*, 1996b).

Whatever the mechanisms, the fact still remains that at a time when prophylaxis in pregnancy is being questioned for a variety of reasons, several studies demonstrate that there is no justification to abandon the recommendation of antimalarial treatment on the first attendance at an antenatal clinic, followed by continued prophylaxis (Gilles, 1996).

Because of the proven association between low birth weight and infant mortality, interventions during antenatal care, to prevent placental infection by malarial parasites, would be expected to have a great impact in reducing early childhood deaths.

There is evidence to indicate that *Falciparum* malaria affects the foeto-placental unit particularly seriously in the third trimester (Watkinson *et al.*, 1985). This concept is of great importance in determining the most important period at which to optimise malaria-control efforts for pregnant women. If the third trimester is the crucial period, malaria-protective measures may be successful despite the common habit of late booking to antenatal-care clinics in large areas of subSaharan Africa (Steketee *et al.*, 1996c).

2.11 Current Animal Models of Malaria During Pregnancy

There are only three previous reports on malaria during pregnancy in non human primates. One pregnant rhesus monkey was inoculated with *P. knowlesi* twice during the third trimester (Das Gupta, 1934). The monkey became severely anaemic and was killed at 17 days post infection. Although the placenta was heavily parasitized, the foetus did not become congenitally infected. Rhesus monkeys at gestational day 56-70 have previously been infected with *P. cynomolgi* (Saxena *et al.*, 1988). The rhesus monkey *Plasmodium coatneyi* model of malaria during pregnancy was established by inoculating pregnant rhesus monkeys (*Macaca mulatta*) with *Plasmodium coatneyi* during the first trimester (Davison *et al.*, 1998).

Other animal models of malaria in pregnancy are based on pregnant mice and rats infected with *Plasmodium berghei* (Odoula *et al.*, 1982; Van Zon *et al.*, 1986; Vinayak *et al.*, 1986; Odoula *et al.*, 1986; Desowitz *et al.*, 1989; Tegoshi *et al.*, 1992;) and more recently mice infected with *Plasmodium chabaudi* (Jayekumar and Moore, 2006). The following have been achieved using these models: placental pathology in *Plasmodium berghei*-infected rats has been characterized, effects of malaria (*Plasmodium berghei*) on maternal foetal relationship in mice have been investigated, histology, immunocytochemistry and ultrastructure of the placenta in rodent pregnancy malaria have been analysed. From the studies, it has been found that pregnancy-induced recrudescences strengthen malarial immunity in mice infected with *Plasmodium berghei*. The indicated studies have also characterized the influence of malarial infection on the maternal foetal relationship in pregnant mice. *Plasmodium berghei* infection in the white rat has also been characterized.

The recent study using *Plasmodium chabaudi* has found that murine malaria infection induces fetal loss associated with accumulation of *Plasmodium chabaudi*-infected erythrocytes in the placenta. Peak parasitemia following inoculation with 1,000 parasite-infected murine erythrocytes and survival were similar in infected pregnant and nonpregnant mice, although development of parasitemia and anemia was slightly accelerated in pregnant mice. Importantly, pregnant mice failed to maintain viable pregnancies, most aborting before day 12 of gestation. At abortion, maternal placental blood parasitemia was statistically significantly higher than peripheral parasitemia.

Infected mice had similar increases in spleen size and cellularity which were statistically significantly higher than in uninfected mice. In contrast, splenocyte proliferation in response to mitogenic stimulation around peak parasitemia was statistically significantly reduced in both groups of infected mice compared to uninfected, nonpregnant mice, suggesting that lymphoproliferation is not a good indicator of the antimalarial immune responses in pregnant or nonpregnant animals. This study suggests that while pregnant and nonpregnant C57BL/6 mice are equally capable of mounting an effective immune response to and surviving *P. chabaudi* infection, pregnant mice cannot produce viable pups. Fetal loss appears to be associated with placental accumulation of infected erythrocytes. Further study is required to determine to what extent maternal antimalarial immune responses, anemia, and placental accumulation of parasites contribute to compromised pregnancy in this model (Jayekumar and Moore, 2006).

In the rhesus monkey *Plasmodium coatneyi* study, since rapid onset of parasitaemia was initially responsible for some abortions, a decision was made to reduce the size of the effective inoculum in order to study the effects of *Plasmodium* infection throughout pregnancy. The study discovered that pregnancy malaria in this model was associated with low birth weight, low placental weight, asymmetrical intrauterine growth retardation and congenital infection. Placental changes associated with fetal outcome in the *Plasmodium coatneyi*/rhesus monkey model of malaria in pregnancy have also been determined (Davison *et al.*, 2000).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Experimental Animals

Experimental animals consisted of 8 adult female baboons (*Papio anubis*) originally from Kajiado District of Kenya with a mean weight of 10 Kg. These baboons were trapped and maintained in the quarantine facilities at the Institute of Primate Research (IPR; appendix III), Karen, Kenya, for not less than three months. They were examined and confirmed free of hemoprotozoan, gastrointestinal parasites and Simian Immunodeficiency Virus (SIV) before being included in the study. Additionally microbiological examination of effusions, pus, ulcer material and skin specimens was done to detect pathogenic agents which cause infection in wounds and the skin. Before infection the baboons were maintained in the IPR animal resources department's (ARD) baboon colony facilities (Eley and Bambra, 1993).

After infection with *P. knowlesi* malaria parasites the baboons were transferred to the IPR biocontainment facility where each baboon was housed in a squeeze back cage, 0.6 x 0.6 x 0.68 metres high. They were maintained on commercial non human primate diet (Unga Millers Limited, Nairobi), supplemented with fruits, vegetables and additional ascorbic acid. Mineral salts and water were provided *ad libitum*. The biocontainment facility was inspected for proper lighting, ventilation, drainage, temperature, foot bath sterilisation and sprayed with insecticide daily according to IPR standard operating procedures (SOP). For all invasive procedures, the baboons were anaesthetised with ketamine hydrochloride (10mg/kg Bwt).

3.2 Study Design

Ten adult female baboons were maintained in the baboon colony facility in the company of an adult male baboon for mating to occur. Ultrasound tests were used to confirm pregnancy status and gestation periods of the baboons. Baboon groups used were as follows: (1) Pregnant infected; PAN 2724, PAN 2809, PAN 2859 and PAN 2889. (2) Non pregnant infected; PAN 2870, PAN 3023, PAN 2911 and PAN 3035. Unfortunately PAN 2889 died before the infection due to unclear natural causes. Immediate replacement of the pregnant baboon was not possible due to a shortage of baboons. The baboons were infected with *Plasmodium knowlesi* blood stage, overnight cultured parasites on the gestation days 147 and 150 for PAN 2724 and PAN 2809 respectively. PAN 2859 was on its late second trimester when the infection took place.

Overnight cultured *P. knowlesi* parasites (Ozwara *et al.*, 2003) were used to initiate blood stage malaria infections in experimental baboons. Two culture flasks were pooled for parasitaemia counts before being centrifuged at 1200 rpm, 24°C for 10 minutes. The parasites were resuspended to a population of 1.0×10^6 parasites/ml in incomplete RPMI 1640. Parasites were transported in sterile 50 ml tubes from the hood the biocontainment where baboons were kept. Infections were preceded by anaesthetisation and bleeding of the baboons for baseline blood samples. Inoculation sites (femoral vein) were cleaned using 70 % alcohol soaked swabs on the legs of baboons laid on examination tables on their backs before and after the infections. Each of the 7 sedated baboons received 1 ml

inoculum. The parasites were injected through the femoral vein of the baboons' left legs via butterfly needles from 1 ml tuberculin syringes. Parasites were immediately flushed into the baboons using 5 ml saline solution for every baboon. Following infection the baboons were carefully transported to the biocontainment facility and locked in cages (Ozwarra *et al.*, 2005). Following infection the baboons were finger pricked daily for parasitaemia determination. Clinical symptoms were closely monitored on daily basis for both groups. At 5 % level of parasitaemia baboons were intravenously injected with chloroquin sulphate at a dosage of 5 mg /kg Bwt daily for 3 days.

Peripheral blood was obtained from the baboons for the processing of Mononuclear cells (for proliferation and cytokine assays), EDTA treated whole blood (for haematology work) sera, plasma (for cytokine and antibody ELISA). Peripheral blood sampling begun with preinfection bleeding on the day of infection. One baboon underwent cesarean section at day 7 post infection. Placental tissues were processed by paraffin wax embedding methods then stored for future immunolocalisation of parasites in the placental tissues.

3.3 Preparation of Baboon Sera for *Plasmodium knowlesi* *in vitro* Culture

Methods described by Ozwarra *et al.*, (2005) were used. Briefly, baboon sera was required as an ingredient of complete medium for *Plasmodium knowlesi* parasite culture. Blood for sera preparation was collected in sterile 50 ml tubes from a baboon. The blood was

left at room temperature for around 7 hours for it to clot. It was then left overnight at 4 °C in upright position in the fridge. Spinning of the blood directly in the centrifuge tubes followed at 2000 rpm for 10 minutes at 24°C (Sorvall RT 6000D centrifuge). Clear serum was sucked off and divided into 10 ml aliquots. Serum was then heat inactivated in a water bath set at 56° C for 30 minutes. Heat inactivated serum was stored at - 20° C until use.

3.4 Processing Baboon Red Blood Cells for Parasite Culturing

To prepare baboon erythrocyte for malaria culture, methods described by Ozwara *et al.*, (2005) were used. Briefly, alsever diluted baboon whole blood was transferred from a 20 ml syringe into a sterile 50 ml tube and spun down at 1500 rpm for 10 minutes (Sorvall RT 6000D centrifuge). The supernatant was discarded then the cells resuspended in alsever's solution two times the pellet's volume. Centrifugation was done as before to wash. Washing was done three times in twice the RBC pellet's volume of Alsevers solution. The blood was mixed well to ensure proper washing. One final washing step in RPMI 1640 was done by centrifuging as above. After the last wash, an equivalent volume to the pellet of incomplete RPMI 1640 was added to the erythrocyte pellet resulting in 50% baboon RBC PCV solution. The baboon erythrocytes were stored at 4° C and used for a maximum of two weeks.

3.5 *In vitro* Propagation of *Plasmodium knowlesi*

It was important to establish *Plasmodium knowlesi in vitro* cultures in order to propagate the few parasites that were available to get large populations for establishing infection. For *in vitro* propagation of malaria parasites methods described by Ozwara *et al.*, 2005 were followed. Briefly, *in vitro* cultures were initiated with cryopreserved *P. knowlesi* parasites previously isolated from *P. knowlesi* infected baboons. For retrieval, parasite vials were removed from liquid nitrogen (-196°C) and quickly thawed at 37°C in a water bath. They were then transferred into 50 ml centrifuge tubes. Equivalent volumes of 3.5% NaCl (at room temperature) were added and mixed with the parasites before centrifugation at 1200 rpm, (Sorvall RT 6000D) at 24°C for 10 min.

The supernatants were sucked off and 1/2 original volumes of 3.5% NaCl added before centrifugation again as before. RPMI 1640 with 10 % baboon serum (a volume equivalent to the first 3.5 % NaCl) was added, mixed and centrifuged again as before. RPMI-10 (5x the original volume) was then added and mixed with the parasites for a final washing step by centrifugation as before. The parasite pellets were transferred into culture to a starting erythrocyte PCV of 2.5%. The complete culture medium consisted of 2.5% baboon erythrocyte PCV, 20% baboon serum, 15 µg/ml gentamycin solution and the rest RPMI 1640. All baboon sera for use had been heat inactivated previously at 56°C. One hundred to two hundred microlitres of culture was used for thin smear preparation (for starting and daily parasitaemia). Cultures were mixed gently and transferred into sterile labeled T 25 culture flask. New gassing needles were heat sterilized before

connection to a gas pipe fitted with a 0.2 µm filter and the cultures were gassed for 25 minutes. The gases in the mixture were (5% CO₂, 5% O₂, 90% N₂). The flasks were tightly capped and transferred to an incubator (37 °C). The incubators and exterior of culture flasks were swabbed with 70 % ethanol to ensure sterility.

3.6 Observation of Parasitaemia and Giemsa staining

Parasitaemia was one of the parameters monitored following infection of baboons with *Plasmodium knowlesi* parasites. For determination of parasitaemia, methods described by moore and Jayekumar (2005) were used. Briefly, in cultures, parasitaemia was evaluated every 24 hours for every culture flask. Labelled duplicate slides were prepared. Cultures were first gently mixed. One hundred to two hundred microlitres of culture was used for thin smear preparation. The volume was centrifuged for 1 minute at 3500 rpm in a microcentrifuge, supernatant removed then pellet. A drop (5-10 µl) was then placed on a microscope slide close to the frosted end of the slide. The blood film was touched with another slide inclined at 45° and after the blood had spread on its edge it was moved in the forward direction to make a smear. The smear was air dried in for 5 minutes then fixed in 100% methanol. Fixed slides were stained for 10 min in 10% Giemsa solution (appendix I). Ten microlitres of erythrocyte pellet was used to prepare a thick smear as follows. The erythrocytes were spread gently on a slide using the edge of another slide then the smear air dried properly. The smear was fixed in acetone for 20 seconds with agitation and stained in the same way as thin smear. Smears were observed microscopically at x 100 magnification. At least 2000 red blood cells (RBC) were

counted in every parasitaemia count session. This was done by counting erythrocytes from a quarter of each field and multiplying by four. Counting was done with the aid of manual lab counters. Parasitaemia was calculated as follows: count the number of infected erythrocytes in a minimum of 2000. Parasitaemia % = $(\text{Total counted parasites} \div \text{Number of erythrocytes counted}) \times 100$. To determine levels of developmental stages of the parasites differential count were performed (for rings, trophozoites and schizonts). All the slides were stored in standard slide storage racks at room temperature. Medium was changed every 24 hours and fresh baboon erythrocytes were 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 parasitaemias exceeded 5 %. Parasites were preserved at the young ring stage of development (Ozwara *et al.*, 2005).

Daily parasitaemia in infected baboons was observed on thick and thin smears preparations (finger prick method) every 24 hours beginning on day 2 post infection. Double smears were prepared for each thick and thin smear preparation session. A baboon's finger to be pricked was cleaned by alcohol swabbing followed by pricking with a sterile needle. A drop of blood from a pricked finger was used to prepare thick and thin smears as described above after being transferred onto a slide using a capillary tube. Determination of parasitaemia and parasite stage differential counts was done daily as described above. Baboon general agility, playing habit and appetite were recorded on daily basis.

3.7 Preparation of *P. knowlesi* Antigens for Recall Proliferation and Enzyme Linked Immunosorbent Assays

Plasmodium knowlesi parasite antigen was required for use in recall proliferation and enzyme linked immunosorbent assays. *Plasmodium knowlesi* parasites from culture and infected baboons were used for the preparation of saponin-lysed and sonicated antigens for recall proliferation and ELISA assays respectively. For the preparation of antigens via sonication, parasites were first washed twice by centrifugation at 1200 rpm, 24°C for 10 minutes with an equivalent volume of alsever's solution (Gicheru *et al.*, 1995). The parasites were suspended at a final concentration of 10^9 parasites/ml in incomplete RPMI 1640 and sonicated at 14-18 amplitude microns for 5 periods of 45 seconds each in ice. The parasites suspension was centrifuged at 10 000 g for 30 minutes. Aliquots of soluble and crude parasite antigens were stored at -70°C. For the preparation of saponin-lysed antigen, 50 % PCV of parasite pellets were lysed using 0.15% saponin in RPMI incomplete 1640, added to the parasites in the ratio of 1:4. The mixture was incubated for 10 minutes at 4°C with mixing after every 3 minutes. Centrifugation was done at 4000 rpm at 24°C for 10 minutes. The pellet was loosened and resuspended for step round of saponin mediated lysis and spinning as before. The parasite pellet was then resuspended in incomplete RPMI 1640 (Sigma, USA) and spun as before to wash. This washing was done twice. The crude parasites were resuspended to contain 10^5 parasites per every 20 μ l volume (5.0×10^6 parasites/ml). Aliquots of saponin-lysed crude parasite antigen were stored at -70°C until use (Gicheru *et al.*, 1995).

3.8 Peripheral Blood Sampling

Baboons were bled for peripheral blood samples beginning with preinfection bleeding. For parasitaemia analysis blood drops were extracted by the finger prick method. Venous blood was drawn via the alcohol-sterilised femoral vein from anaesthetised baboons. Needles used in the procedures were 21 G sized. Blood for isolation of peripheral blood mononuclear cells (PBMC) and plasma (10 ml) was drawn into 10 ml of alsever solution in a sterile 20 ml syringe. Blood for isolation of serum was drawn into a 10 ml syringe and then used to fill up 6 ml serum isolation tubes. The remaining blood (4 ml) was transferred into EDTA tubes. EDTA blood was then placed on a roller machine for proper mixing at room temperature (for haematological analysis), while alsever diluted blood and blood for serum were transported on ice to the lab for immunological assays.

3.9 Cesarean Section Procedures and Placental Parasitaemia Analysis

Caesarean sections were performed (as described in IPR Standard Operating Procedures) in order to obtain intact sterile placental tissue from baboons which can otherwise be consumed by the mother. For PAN 2809 (pregnant infected baboon) the procedures were undertaken at 7 days post infection. For the pregnant control baboons the procedures were undertaken during late third trimester of pregnancy in order to collect mature placentas. Peripheral sampling of sedated baboons was followed by shaving and cleaning of the abdominal region before the baboons were taken to the theatre. Veterinary surgeons performed the abdominal incisions with minimum bleeding. Massaging of the baboons' abdomen was done and light pressure applied to the area at the top of the uterus. This manipulation was enough to expel the placenta. After surgery the infants

delivered by control baboons were maintained in the IPR non human primate nursery. PAN 2809's infant was euthanized shortly after delivery using euthatal® (pentobarbital sodium) via cardiac puncture. Freshly extracted placental tissues were placed in sterile petridishes with the maternal side facing up. Sterile forceps were used to prick the maternal side of the placenta after wiping with cotton wool. Drops of blood were used to prepare thick and thin placental blood smears before staining and parasitaemia analysis as described before (Moore *et al.*, 2000; Othoro *et al.*, 2006). Umbilical cords and chorionic membranes were striped off and the placentae rinsed and submerged in sterile saline buffers containing 0.1% heparin and 2% penicillin-streptomycin before processing for OCT and paraffin wax embedding. Antibiotic and chloroquin therapy (for PAN 2809) was administered soon after cesarean section's abdominal stitching. The baboons were then transferred to baboon cages where they were kept under constant clinical monitoring.

3.10 Determination of Haemoglobin Concentration and Packed Cell Volume

All haematological procedures were done according to the IPR pathology laboratory standard operating procedures. Haemoglobin concentration and Packed Cell Volume (PCV) were measured so that levels of anaemia if any could be determined. Two microhaematocrit (capillary) tubes were filled with well mixed EDTA whole blood by capillary action to about three quarters of each tube. One end of the capillary tubes was sealed with the sealant until the plug was 4-6mm long. It was ensured that blood was not forced out of the top of the capillary tubes during this process. Two tubes were placed in the radial grooves of the haematocrit centrifuge head exactly opposite each other with the

sealed ends facing away from the center of the centrifuge the cover replaced firmly. Centrifugation was then done for three minutes. The tubes were removed from the microhaematocrit centrifuge as soon as it stopped spinning. The packed cell volume 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 tube was adjusted such that the bottom line (0%) ran exactly at the interphase of the sealant and the packed cells. The sliding tube holder was slid until the end of the plasma column touched the 100% mark of the PCV reader. The pointer was slid until it was exactly at the inter-phase between the plasma and the packed cells. The packed cell volume of the sample was then read and reported as a percentage. Haemoglobin concentration was calculated by dividing PCV by three basing on the formula $3\text{Hb}=\text{PCV}$ (Moore *et al.*, 1999).

3.11 Isolation of Plasma and PBMC from the Alsever-Diluted Whole Blood

Plasma was required for the determination of TNF alpha responses while PBMC were required for proliferation assays. The 20 ml whole blood-alsever mixture (10 ml alsever mixed with 10 ml blood) was overlaid directly from the syringe onto a 10 ml lymphocyte separation medium (LSM®) layer in a 50 ml centrifuge tube. The LSM and the diluted blood sample were not mixed. Centrifugation followed for 30 minutes in Sorvall RT 6000 D at 20°C, at a speed of 2500 rpm (revolutions per minute) with no brake. The upper plasma layer was then transferred into labeled tubes in 1 ml aliquots. These plasma samples were stored at - 20°C until used. The mononuclear cell layer (buffy coat layer)

was transferred into another 50 ml centrifuge tube. The entire interface was removed but with a minimum amount of LSM and supernatant. Alsevers solution (appendix I) was added (approximately 30 ml) to the isolated PBMC solution and the PBMC were evenly resuspended in the alsevers solution using the serological pipette by drawing in and out. Centrifugation to wash was done for 10 minutes in a Sorvall RT 6000 D centrifuge at 1200 rpm [60–100 x g] at 18° to 20°C. The supernatant was discarded cells resuspend in alsevers solution then the washing repeated once more to remove most of the platelets. Each centrifugation step was preceded by loosening of the pellet and even resuspension in the washing solution before the actual spinning. After the second washing step, the pellet was loosened and resuspended in 10 ml complete RPMI 1640. Approximately 200 µl of the resuspended cells was transferred into a sterile eppendorf microcentrifuge tube for viability check and enumeration. The cells resuspended in 10 ml RPMI 1640 -10 were then centrifuged for 10 min in a Sorvall RT 6000 D rotor at 1500 rpm (500 x g), room temperature/18-20 °C. The supernatant was discarded and the pellet saved. This was the final washing step after which the PBMC pellet was processed for proliferation assays and cryopreservation (Gicheru *et al.*, 2001).

3.12 White Blood Cell Enumeration

It was necessary to enumerate PBMC in order to perform accurate dilution for proliferation assays and have 2×10^5 cells/well distribution. Trypan blue solution (90 µl; 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 well. Gentle aspiration was performed 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 stained non-viable cells blue and left viable cells unstained. With the cover slip in place, a P 20 pipette was used to transfer a small amount of trypan blue-cell suspension mixture to both chambers of the haemocytometer. Each chamber was allowed to fill by capillary action. The chambers were not overfilled.

Starting with chamber 1 of the haemocytometer, all the cells in the four 1 mm corner squares were counted and average number per 1 mm square calculated by dividing by 4. A manual laboratory counter was used in counting. Cells touching the middle line at bottom and right sides were not counted. The procedure was repeated for chamber 2. Accuracy was ensured by taking a second sample and repeating the counting procedure. 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 then resuspended in the right concentration for cryopreservation or proliferation assays. The coverslips and haemocytometer were decontaminated by rinsing with 70% ethanol and then deionized water before being air dried and stored for future use (Gicheru *et al.*, 2001).

3.13 Peripheral Blood Mononuclear Cells Recall Proliferation Assays

Proliferation assays were necessary for the determination of the infected baboons' cell mediated specific immune responses. Ninety six well round bottom microtitre plates were used for the assays. Peripheral Blood Mononuclear Cells (PBMC) were resuspended for delivery of 2×10^5 cells/well dispensed in 100 μ l of complete RPMI 1640 (RPMI with 10 % FBS, 2 mM L-glutamine, 100 μ g/ml gentamycin, 0.05 mM 2-mercaptoethanol). The cells were stimulated in triplicate wells with 10^5 *Plasmodium knowlesi* crude parasite antigen per well or 10 μ g/ml final concentration of Concanavalin A (Con A; for positive control). Triplicate control background wells received 50 μ l of complete media. The plates were then covered and taped around to prevent rapid evaporation. Cultures were incubated at 37°C in a humidified incubator for 5 days for *Plasmodium knowlesi* antigen cultures and for 3 days for Con A cultures. Cells were pulsed with 0.5 μ Ci of [methyl-³] thymidine (20 μ l delivery/well) over the last 18 hours then harvested on a fiber filter paper.

A semi automatic cell harvester fitted with a suction pump and high pressure distilled water delivery system was used to perform the cell harvesting. The fiber filters were pre wet with distilled water each time before cells were harvested from the 96 well plates. Filters were left to dry at least overnight at room temperature before further processing. Punched round filter papers representing each well were transferred into respective scintillation vials using a pair of forceps. Optiphase high capacity scintillation fluid cocktail was then delivered into each vial (2 ml/vial) using a zippette scintillation fluid

dispenser. The vials containing dissolved fiber filters were vortexed for 20 seconds each before being arranged in racks for spectrometry. Incorporation of radionuclide into DNA was measured by scintillation spectrometry using a TriCarb 6000 liquid scintillation analyser. The machine was cleared for rack entry then the system normalized using a C 40 vial loaded on a clip protocol rack. Shortly after normalization samples were read using a protocol 3 clip initial rack. Readings were performed as counts per minute and printed using an Epson™ printer. Triplicate averages were calculated before proliferations were expressed in form of stimulation indices (*P. knowlesi* antigen counts per minute ÷ media/background counts per minute). The data was entered into a microsoft® excel spread sheet program (Gicheru *et al.*, 1997; Gicheru *et al.*, 2001; Olobo *et al.*, 1995; Coligan *et al.*, 2003).

3.14 Immunoglobulin G (IgG) and Immunoglobulin M (IgM) Measurement

Immunoglobulin G (IgG) and Immunoglobulin M (IgM) were measured using ELISA in order to determine the level of humoral immune responses provoked in the baboons. Ninety six well flat bottomed ELISA microtiter plates (Nunclon, UK) were coated using 50 µl/well of carbonate bicarbonate buffer (pH 9.6; appendix I) containing 10^8 (parasites used in antigen preparation) crude sonicated *P. knowlesi* parasite antigen. These were incubated overnight at 4 °C for adsorption. Excess coating buffer was then flicked off and 100 µl/well of blocking buffer (3% BSA in PBS) added using a multichannel pipette followed by incubation for 1 hour at 37 °C. The plates were then washed six times using wash buffer (0.05% tween in PBS; appendix I) delivered by an automatic washing

machine and sera samples added in triplicate (1:100 dilution in 50 µl/well) on ice before incubation for one hour as before. The plates were then washed as before and 50 µl/well of 1:2000 dilution of either antihuman Immunoglobulin G horseradish peroxidase or antihuman Immunoglobulin M horseradish peroxidase (HRP; Sigma, USA) added. A one hr incubation at 37 °C then followed after which the plates were washed ten times using washing buffer. Colour development was achieved by adding 50 µl/well of Tetramethylbenzidine (TMB) substrate (Sigma, USA) and optical densities were read using a Dynatech MRX ELISA reader at 630 nm filter setting after 15, 30 and 45 min incubation at 37 °C. Reactions were then stopped using 50 µl/well of 2 M sulphuric acid per well and the resulting yellow colour was read at a wavelength of 450 nm. Print outs from the ELISA reader were used in subtraction of background readings, calculation of triplicate means. The preparation of graphs showing Immunoglobulin G titers was done using a microsoft® excel spread sheet program (Gicheru *et al.*, 1995).

3.15 Determination of Cytokine Responses in Sandwich ELISA

Cytokine ELISA were done in order to measure the levels of cytokine mediated immune responses induced in the baboons. Ninety six well flat bottomed ELISA microtiter plates were coated with 5 µg/ml of cross reactive antihuman/baboon cytokine (IFN gamma, tumor necrosis factor alpha, IL4, IL6, IL 10, IL 12) capture monoclonal antibody (Becton Dickinson, USA) delivered 50 µl/well. These were incubated overnight at 4 °C. Excess coating buffer was then flicked off and the wells blocked with 100 µl/well blocking buffer (3% BSA in PBS) followed by 1 hr incubation at 37 °C. After washing the plates

six times using ELISA washing buffer (0.05% tween in PBS; appendix I), undiluted sera samples and recombinant cytokine standards were dispensed in duplicate, 50 µl/well and plates incubated for 2 hr at 37 °C. Standards were serial diluted by transferring 50 µl from well to well with mixing beginning with a neat concentration of 500 pg/ ml. The plates were then washed as before and detector mouse biotinylated antibaboon cytokine (IFN gamma, tumor necrosis factor alpha, IL4, IL6, IL 10, IL 12) monoclonal antibodies added 50 µl/well at dilutions of 1:2000. This was followed by a one hr incubation period at 37 °C then washing as before. Streptavidin Horse Radish Peroxidase (HRP) diluted 1:2000 was added 50 µl/well and incubated 1 hr at 37 °C followed by washing six times.

Colour development was achieved by adding 50 µl/well of Tetramethylbenzidine (TMB) substrate and optical densities were read using a Dynatech MRX ELISA reader at 630 nm filter setting after 15, 30 and 45 min incubation at 37 °C. Print outs from were used in subtraction of background readings, calculation of triplicate means and preparation of a standard curve. The preparation of graphs showing IFN gamma concentrations in pg/ml (with values derived from standard curves) was done using a microsoft® excel spreadsheet program (Ozwara *et al.*, 2005).

3.16 Data Analysis and Management

Geometric mean values of parasitaemia, haematological parameters and immunological parameters (stimulation indices, antibody optical densities, cytokine concentrations) of pregnant infected group of baboons were compared with the values of control group of baboons (non pregnant infected baboons) using the non parametric Mann-Whitney U test.

Tabulated probability values of $P < 0.05$ were considered significant. All data were stored in the Microsoft Excel® computer program and visualized using line and bar graphs generated by the program (www.microsoft.com).

CHAPTER FOUR: RESULTS

4.1 Peripheral Parasitaemia and Disease Course in Baboons Inoculated with *P. knowlesi*

Four pregnant baboons in their third trimester were experimentally infected with blood stage *P. knowlesi*. A similar number of non pregnant female animals were also infected. One pregnant baboon was discontinued from the study for reasons not related to the study. Development of peripheral parasitaemia was monitored on a daily basis in both animal groups. Pregnant baboons became parasitaemic (0.02 %) beginning from day 2 post infection while non pregnant ones became parasitaemic (0.04 %) from day 4 post infection. The mean peak peripheral parasitaemia in pregnant baboons (2.73 %) and in non pregnant baboons (6.74 %) differed significantly (Fig 4.1; $P > 0.05$). Some baboons developed acute infection (severe symptoms that included apathy, ruffled hair, loss of appetite and vomiting) or chronic infection (mild asymptomatic malaria showed by having low to moderate clinical symptoms as observed in severely infected animals) irrespective of the pregnancy status. Severely infected baboons became lethargic, developed dyspnoea, and produced dark coloured urine suggesting cholestasis. They had reduced skin turgor and ocular tension indicating dehydration. Parasitaemia in chronically infected baboons ranged from 0 % to 3.54 % while in acutely infected baboons it ranged from 0 % to 10.67 %. Acutely infected baboons (table 4.1) experienced significantly higher peripheral parasitaemia than chronically infected baboons (Fig 4.2; $P < 0.05$).

Table 4.1: Disease Course in *P. knowlesi* Infected Baboons.

Baboon Number	Gravidity	Disease Course
PAN 2724	Pregnant	Acute
PAN 2809	Pregnant	Chronic
PAN 2859	Pregnant	Chronic
PAN 2870	Non Pregnant	Acute
PAN 2911	Non Pregnant	Acute
PAN 3023	Non Pregnant	Chronic
PAN 3035	Non Pregnant	Chronic

PAN = *Papio anubis*

Acute infection: Baboons with severe symptoms that included apathy, ruffled hair, loss of appetite and vomiting.

Chronic infection: Baboons with mild asymptomatic malaria showed by having low to moderate clinical symptoms compared to severely infected animals.

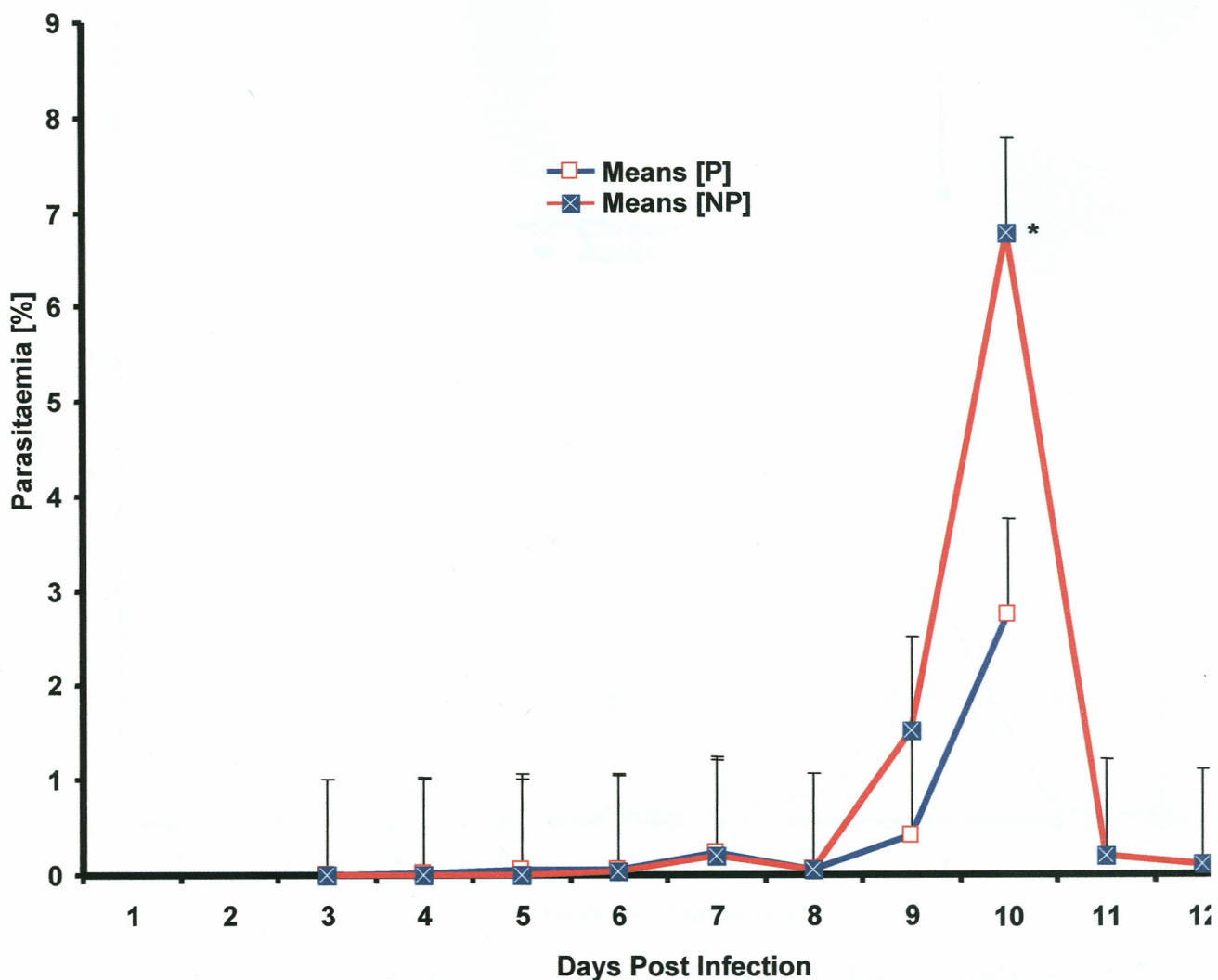


Fig 4.1: Daily Peripheral Parasitaemia in pregnant and non pregnant *P. knowlesi* Infected Baboons. P: Pregnant; NP: Non pregnant.*: Asterisk means significant difference between mean parasitaemia in pregnant and non pregnant baboons at peak parasitaemia, $P < 0.05$; n: P = 3; NP = 4.

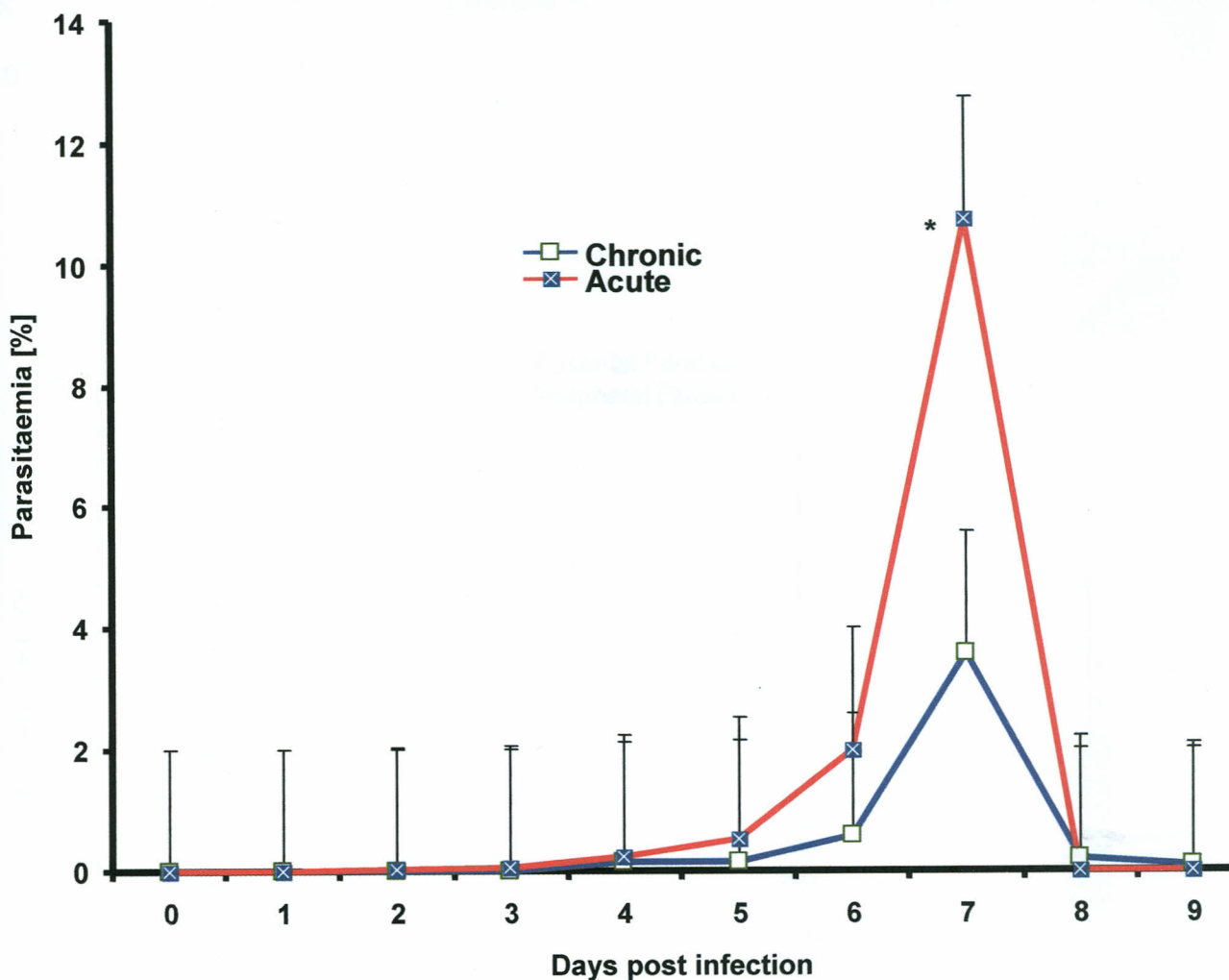


Fig 4.2: Daily Peripheral Parasitaemia in acute and chronic *P. knowlesi* Infected Baboons. *: Asterisk means significant difference in parasitaemia between chronically and acutely infected baboons, $P < 0.05$; n: Acute = 3; Chronic = 4.

4.2 Placental Parasitaemia in Baboons Infected with *P. knowlesi*

Parasites were detectable in the placentas of all the three pregnant baboons in the experiment. Mean placental parasitaemia (11.9 %) was over 29 fold higher than the mean peripheral parasitaemia (0.4 %) in pregnant baboons (Fig 4.3; $P < 0.05$). The placentas of the baboons PAN 2859 and PAN 2724 were sampled following abortions by the baboons on days six and seven post infection respectively. Placental parasitaemia was 14.23 % in PAN 2859, 18.30 % in PAN 2724 and 0.74 % in PAN 2809, sampled after cesarean

section. Simultaneous peripheral parasitaemia in these baboons were 0.39 %, 0.8 % and 0.03 % respectively (Fig 4.3).

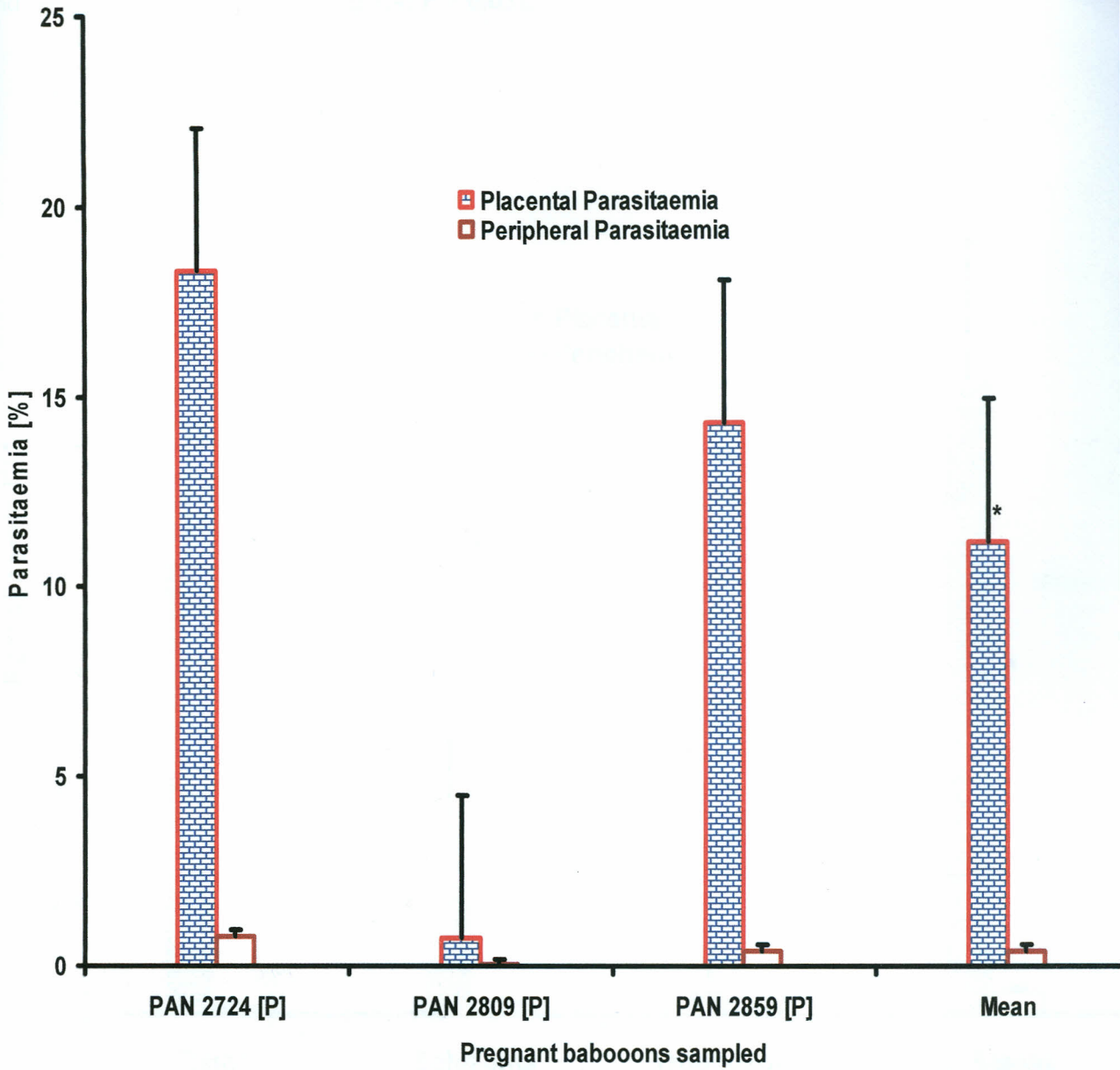


Fig 4.3: Comparison of Placental and Peripheral Parasitaemia. PAN: *Papio anubis*; P: Pregnant. *: Asterisk Means significant difference between peripheral and placental parasitaemia, $P < 0.05$.

When parasite stages were considered, rings (plate 1) and schizonts (plate 3) counted in the placenta (7.93 % and 2.70 % respectively) were found to be significantly ($P < 0.05$)

higher than in peripheral blood (0.13 % and 0.19 % respectively; $P < 0.05$; Fig 4.4). Trophozoites levels (Plate 2) were not significantly different between placental (0.46 %) and peripheral blood (0.10 %; Fig 4.4; $P > 0.05$).

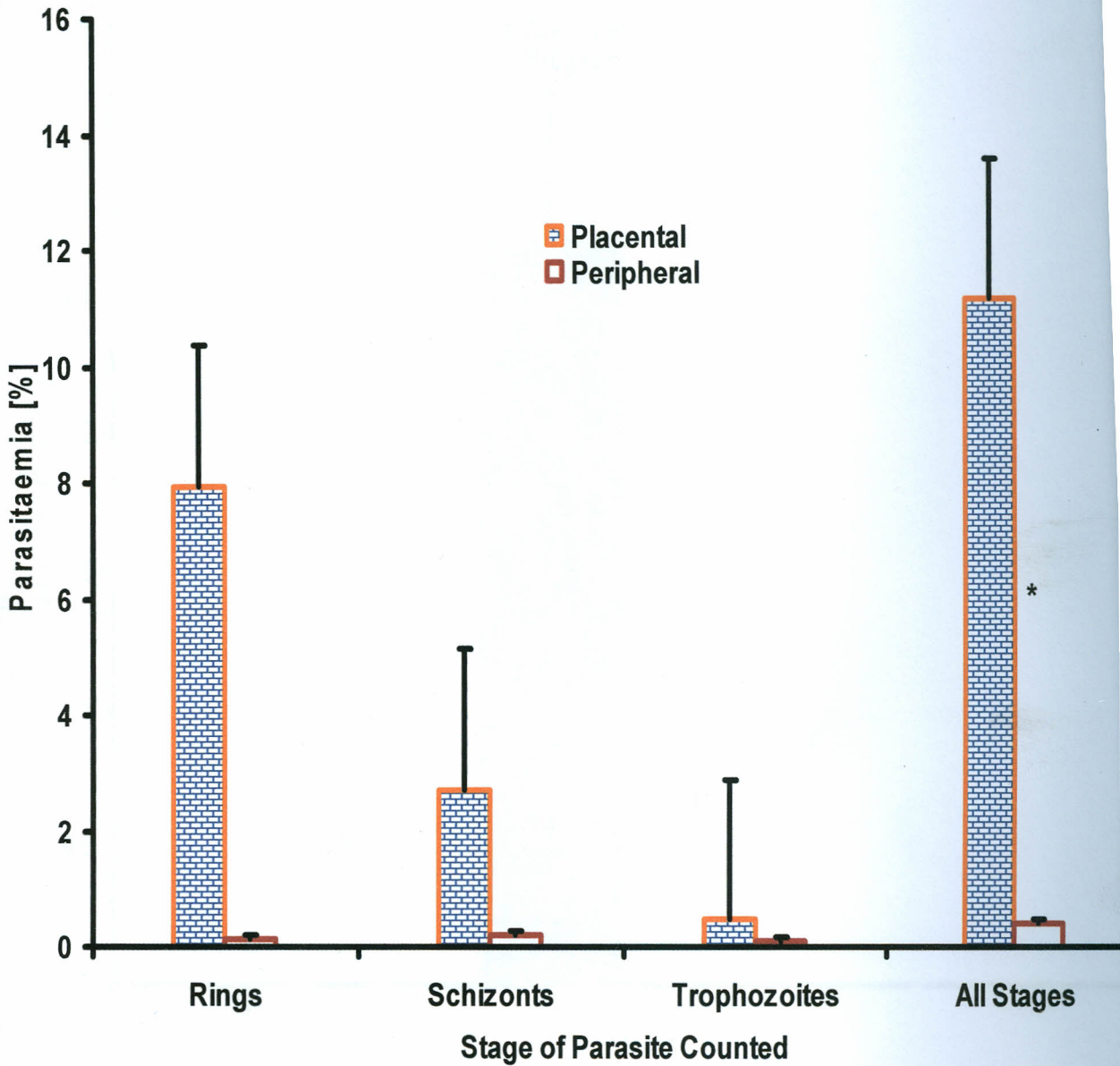


Fig 4.4: Peripheral and Placental Differential Parasite Count. *: Asterisk means significant difference in parasitaemia between placental and peripheral blood, $P < 0.05$.

On the days when placentas were sampled, mean overall peripheral parasitaemia in the non pregnant baboons (2.72 %) was significantly higher than in the pregnant ones of 0.40 % (Fig 4.5; $P < 0.05$).

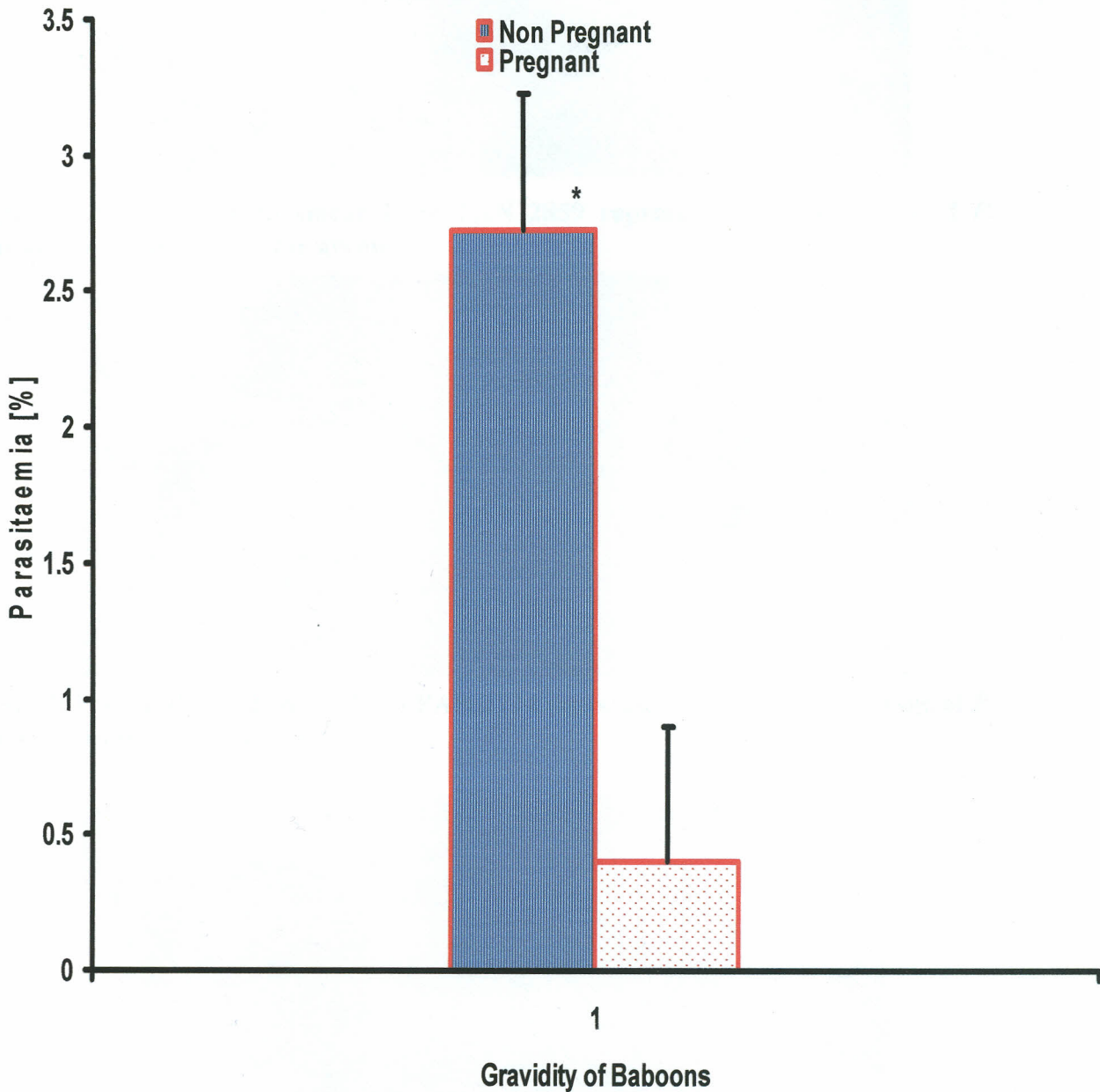


Fig 4.5: Peripheral parasitaemia on abortion/cesarean section days in both pregnant and non pregnant baboons. *: Asterisk means significant difference in peripheral parasitaemia between pregnant and non pregnant baboons, $P < 0.05$. n: Pregnant = 3; Non pregnant = 4.

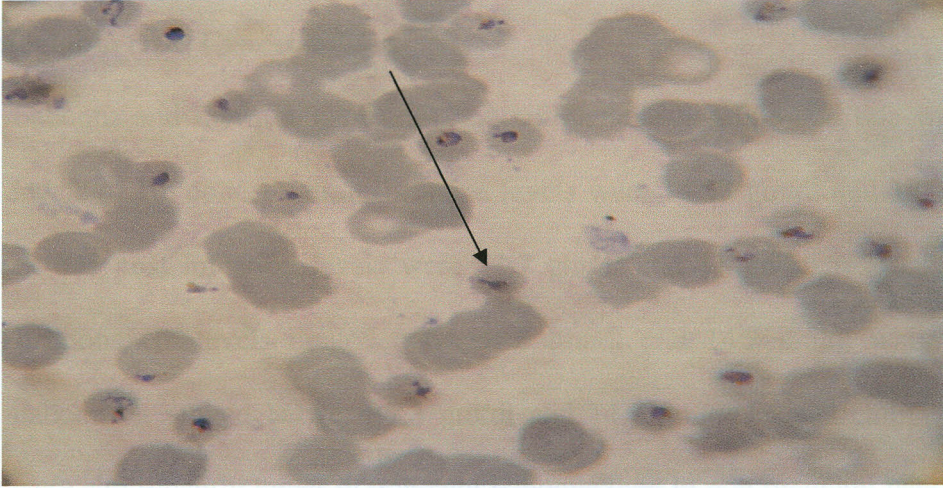


Plate 1: Placental blood smear from PAN 2859 representing a ring stage of *P. knowlesi* as indicated by the arrow.

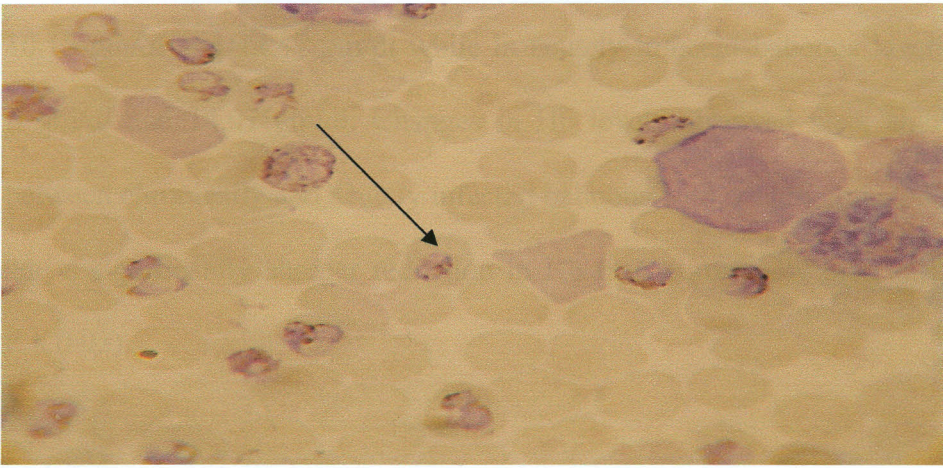


Plate 2: Placental blood smear from PAN 2724 representing a trophozoite stage of *P. knowlesi* as indicated by the arrow.

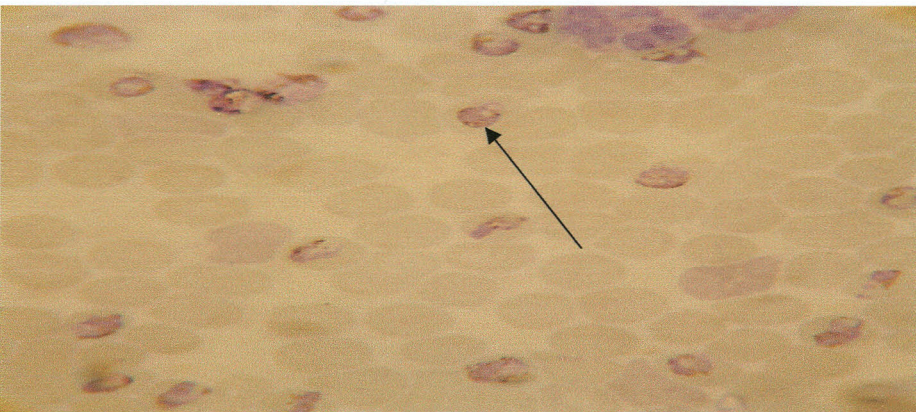


Plate 3: Placental blood smear from PAN 2809 representing a schizont stage of *P. knowlesi* as indicated by the arrow.

4.3 Haemoglobin (Hb) Concentration

Pregnant baboons and non pregnant ones had comparable baseline and one week post infection levels of Hb concentration (baseline levels were 14.23 gm/dl and 14.25 gm/dl while week one post infection levels were 11.2 gm/dl and 11 gm/dl respectively). In pregnant baboons Hb levels ranged between 6 gm/dl and 14.2 gm/dl while in non pregnant baboons it ranged between 7.3 gm/dl and 14.2 gm/dl. Haemoglobin changes observed were significantly lower in pregnant baboons than in non pregnant baboons late during the infection (Fig 4.6; $P < 0.05$). In chronically infected baboons Hb levels ranged between 10.15 gm/dl and 14.47 gm/dl while in acutely infected baboons levels ranged between 6 gm/dl and 14.07 gm/dl. Changes in Hb levels observed were significantly different between chronically and acutely infected baboons with acutely infected baboons experiencing a significantly sharper reduction in Hb than chronically infected baboons (Fig 4.7; $P < 0.05$).

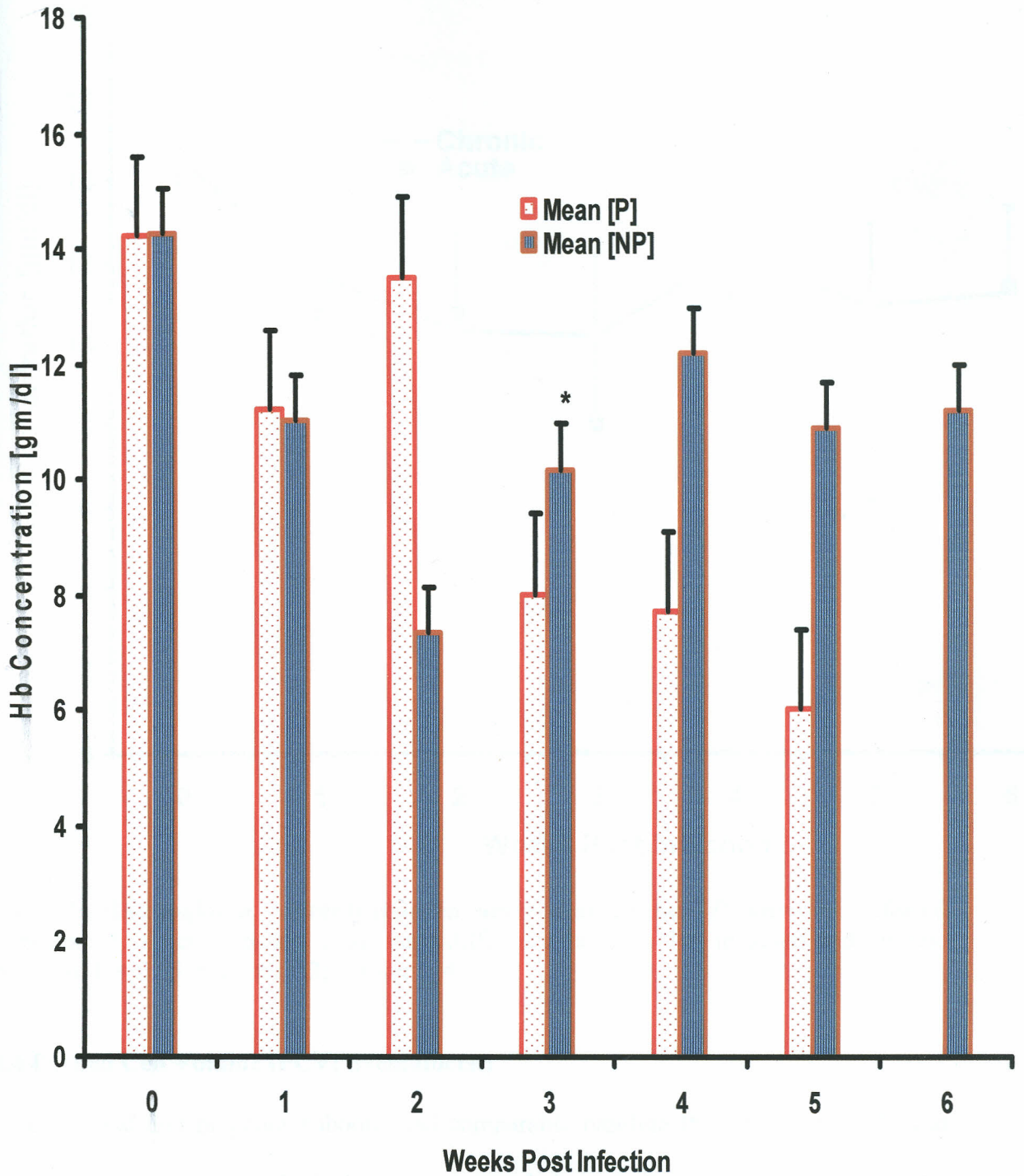


Fig 4.6: Haemoglobin concentrations in pregnant and non pregnant *P. knowlesi* infected baboons. P: Pregnant; NP: Non pregnant; *: Asterisk means significant difference between Hb in pregnant and non pregnant baboons, $P < 0.05$. n: P = 3; NP = 4.

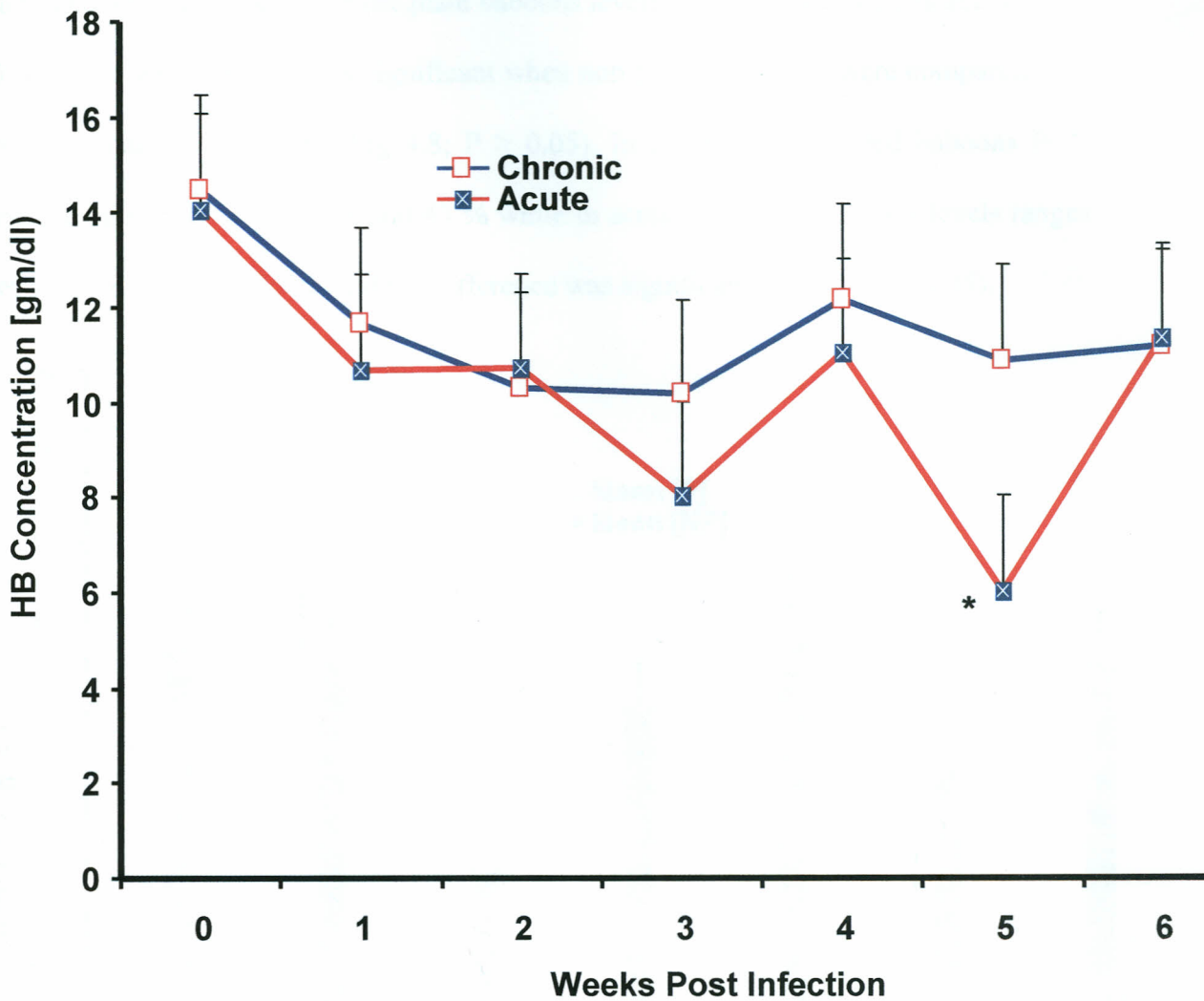


Fig 4.7: Haemoglobin concentration in acute and chronic *P. knowlesi* infected baboons. *: Asterisk means significant difference between Hb in acute and chronic baboons, $P < 0.05$. n: acute = 3; chronic = 4.

4.4 Packed Cell Volume (PCV; haematocrit)

Pregnant and non pregnant baboons had comparable baseline PCV levels (42.67 % in pregnant baboons and 42.75 % in non pregnant baboons). Packed Cell Volume (PCV) levels dropped in all baboons soon after infection. The reduction was from 42.67 % to 33.67 % in pregnant baboons and 42.75 % to 33 % in non pregnant baboons over the first one week of infection. Packed Cell Volume levels in pregnant baboons ranged between

18 % and 42.7 % while in non pregnant baboons levels ranged between 22 % and 42.8 %.

Differences were however not significant when non pregnant means were compared with their pregnant counterparts (Fig 4.8; $P > 0.05$). In chronically infected baboons PCV levels ranged between 30.5 % and 43 % while in acutely infected baboons levels ranges between 18 % and 42.25 %, and the difference was significant (Fig 4.9; $P < 0.05$).

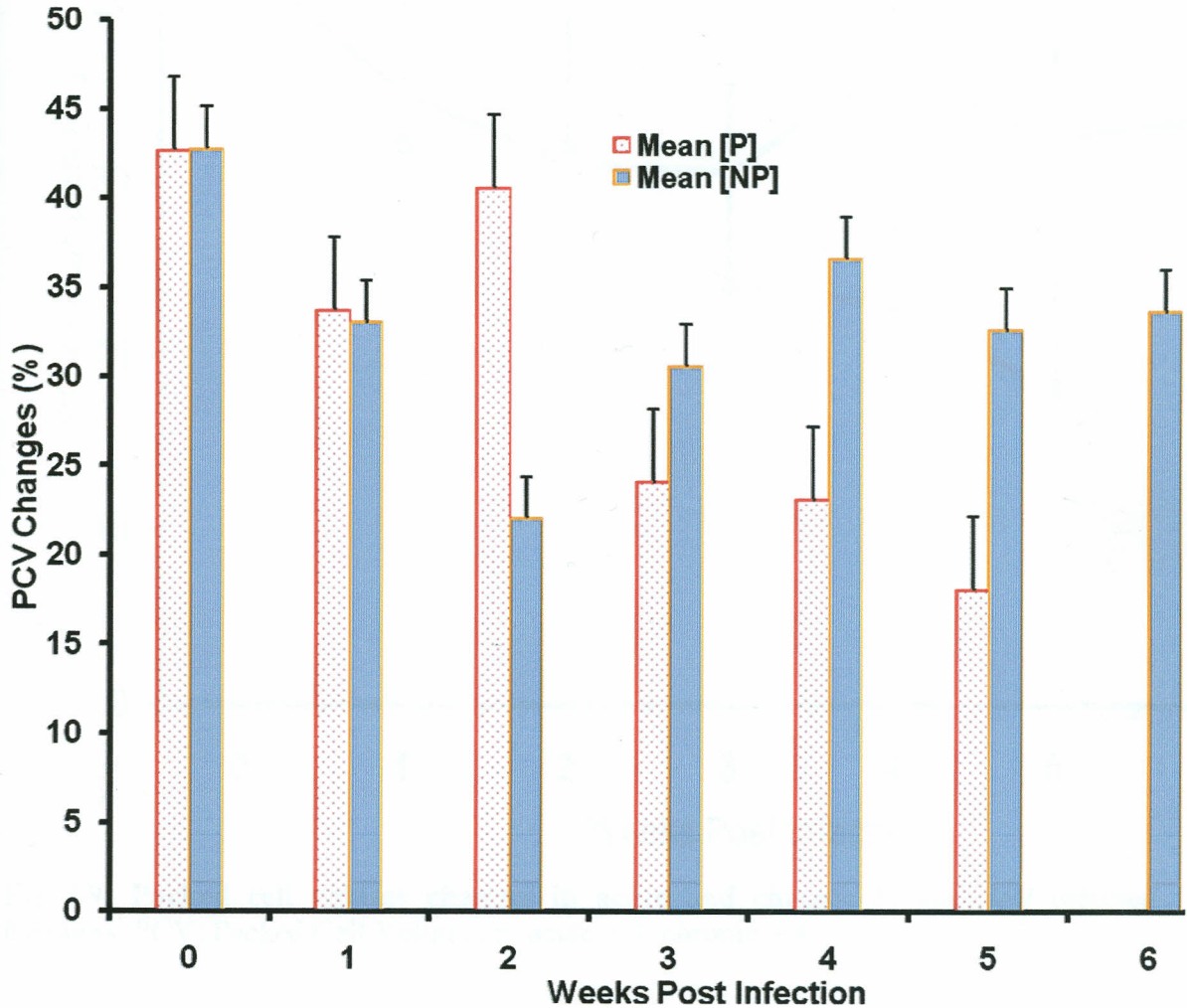


Fig 4.8: Packed cell volume changes in pregnant and non pregnant *P. knowlesi* infected baboons. PCV: Packed Cell Volume; P: Pregnant; NP: Non pregnant. n: P = 3; NP = 4.

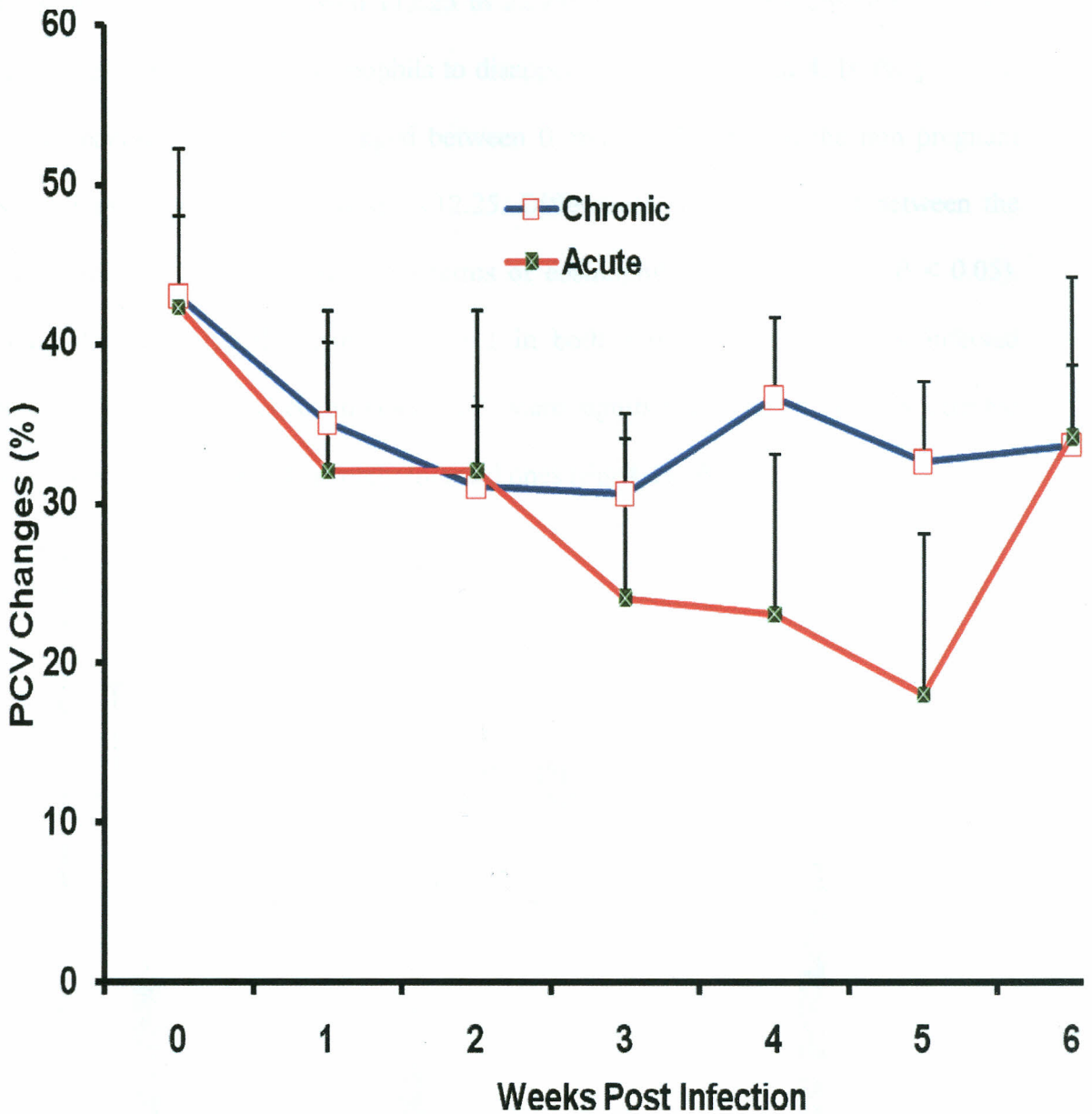


Fig 4.9: Packed cell volume changes in acute and chronic *P. knowlesi* infected baboons. PCV: Packed Cell Volume; n: acute = 3; chronic = 4.

4.5 Eosinophil Levels

On average, pregnant baboons had higher baseline eosinophil levels than non pregnant baboons (177.33 compared to 112.35; Fig 17; $P < 0.05$). Following infection, no eosinophils were observed in the pregnant group henceforth. The non pregnant baboons

had a reduction by week 1 (from 112.25 to 22.75) followed by an increase by week 2 (from 22.75 to 80) only for eosinophils to disappear over week 3 and 4. In the pregnant group of baboons eosinophils ranged between 0 and 177.3 while in the non pregnant baboons they ranged between 0 and 112.25. Differences were significant between the pregnant and non pregnant groups in terms of eosinophil levels (Fig 4.10; $P < 0.05$). Eosinophil levels ranged between 0 and 1 in both chronically and acutely infected baboons (Fig 14). Differences in eosinophils were significant when chronically infected baboons were compared with acutely infected ones (Fig 4.11; $P < 0.05$).

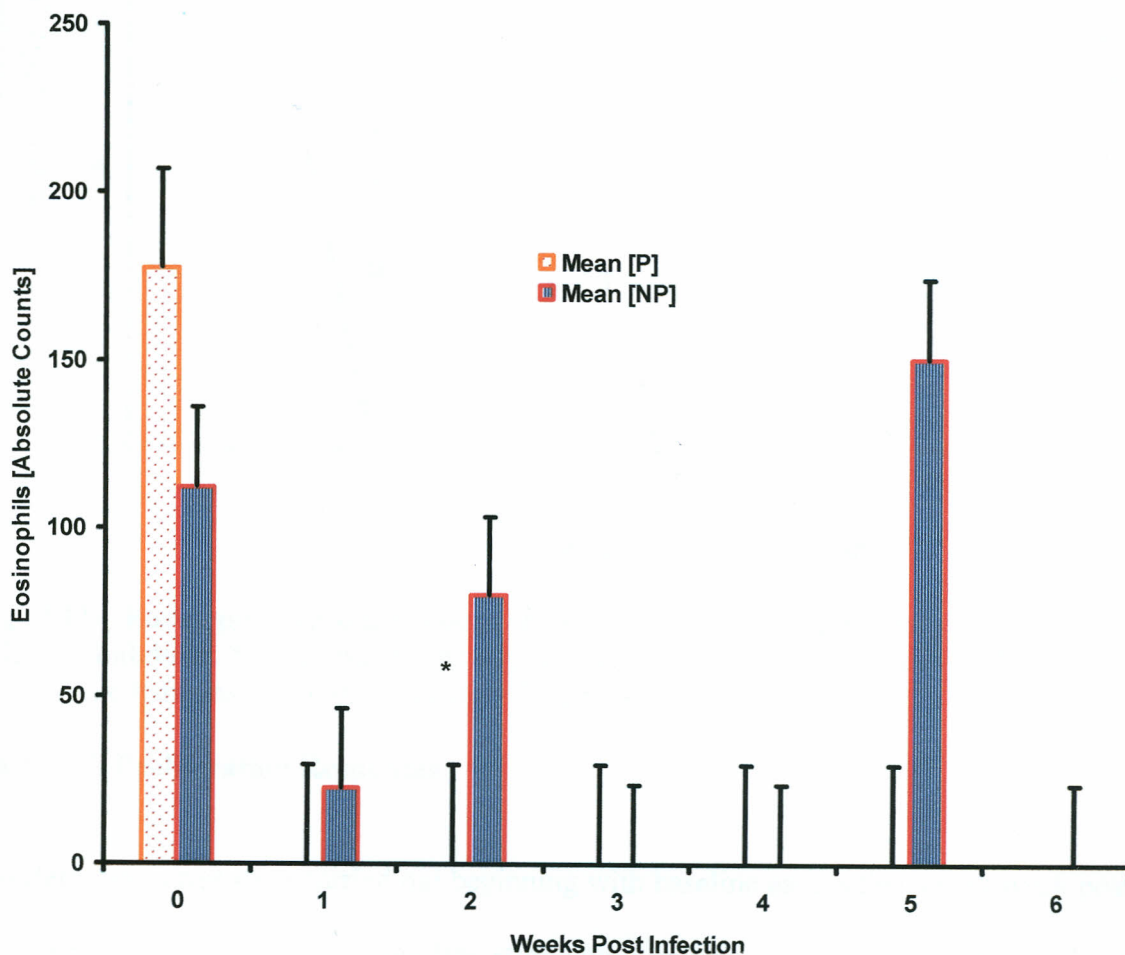


Fig 4.10: Eosinophil absolute count from *P. knowlesi* infected pregnant and non pregnant baboons. P: Pregnant; NP: Non pregnant. *: Asterisk means significant difference between eosinophils in pregnant and non pregnant baboons, $P < 0.05$. n: P = 3; NP = 4.

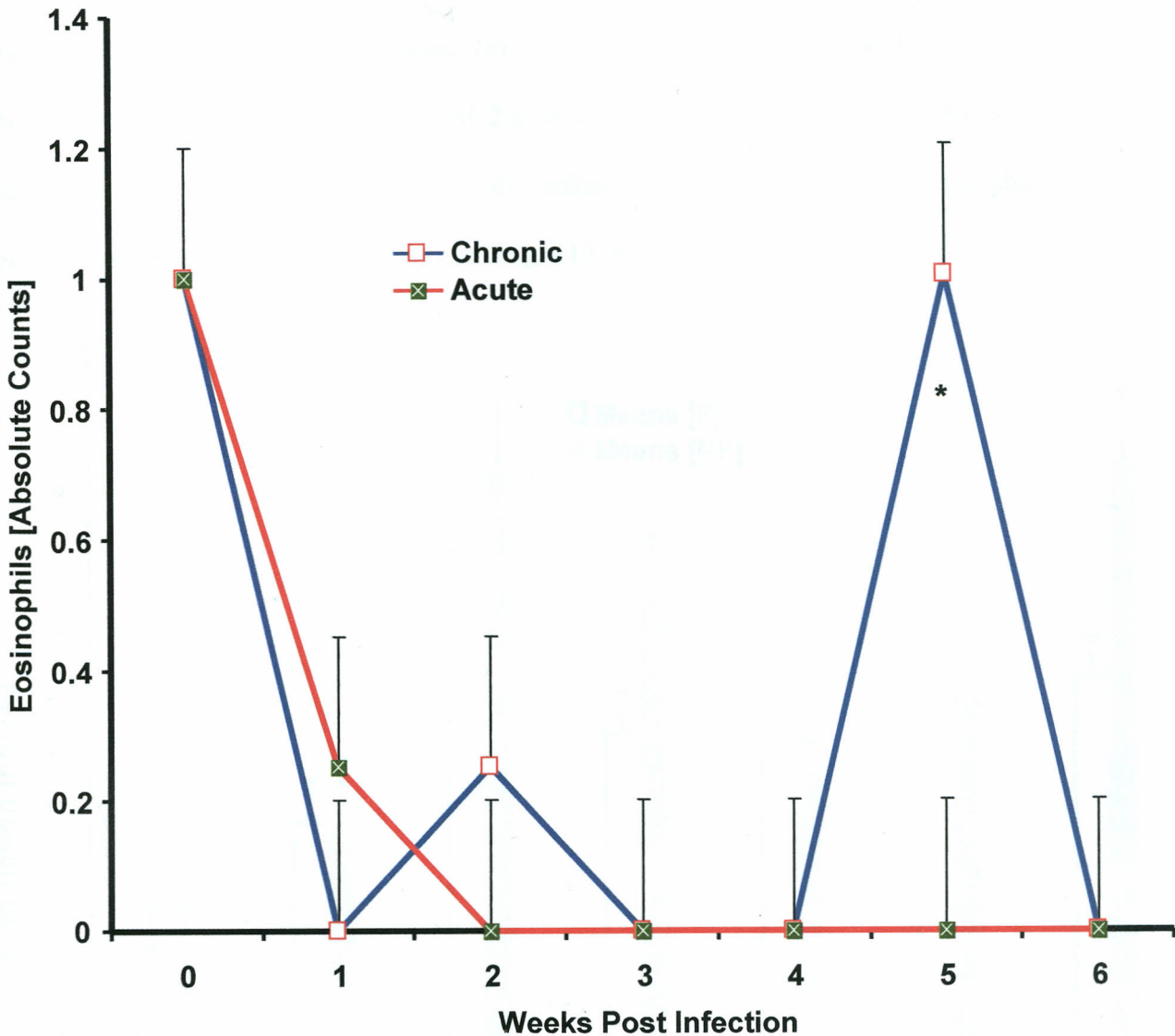


Fig 4.11: Eosinophil absolute count from acutely and chronically *P. knowlesi* infected baboons. *: Asterisk means significant difference between eosinophils in acute and chronic baboons, $P < 0.05$; n : acute = 3; chronic = 4.

4.6 Recall Proliferation Responses

Proliferation assays were carried out beginning with baseline assays then every week post infection for up to six weeks. Baseline stimulation indices detected were comparable in the pregnant and non pregnant baboons. Proliferation in pregnant baboons ranged between 2.67 and 4.87 while in non pregnant baboons it ranged between 2.47 and 6.11.

When mean proliferation was considered, the differences between pregnant and non pregnant baboons were not significant (Fig 4.12; $P > 0.05$). In chronically infected baboons proliferation ranged from SI 2.6 to 8.5 while in acutely infected baboons levels ranged from SI 2.51 to 5.0. Chronically infected baboons had significantly higher proliferation than acutely infected baboons (Fig 4.13; $P < 0.05$).

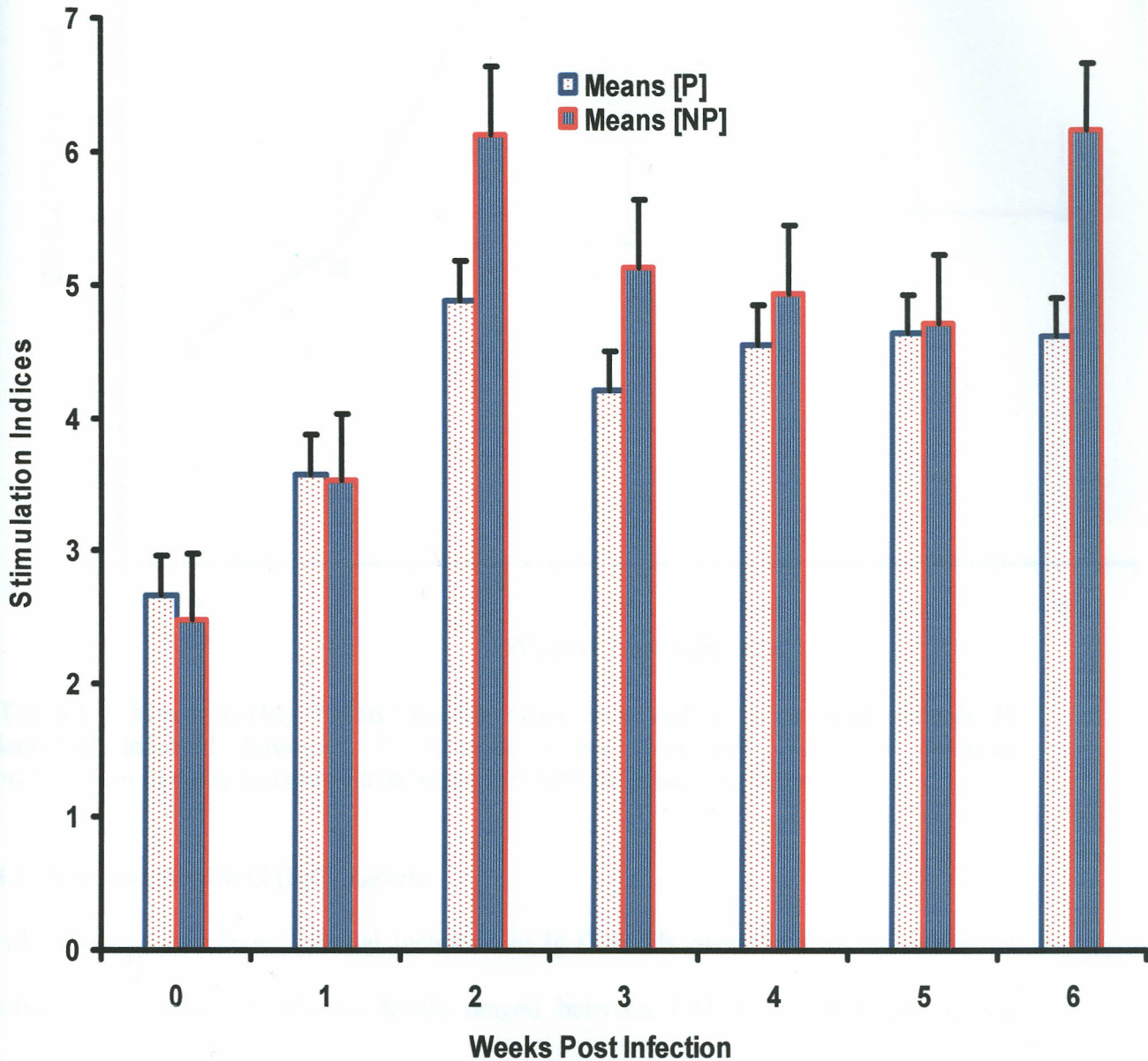


Fig 4.12: Mean weekly PBMC proliferation observed in pregnant and non pregnant *P. knowlesi* infected baboons. P: Pregnant; NP: Non pregnant. n: P = 3; NP = 4.

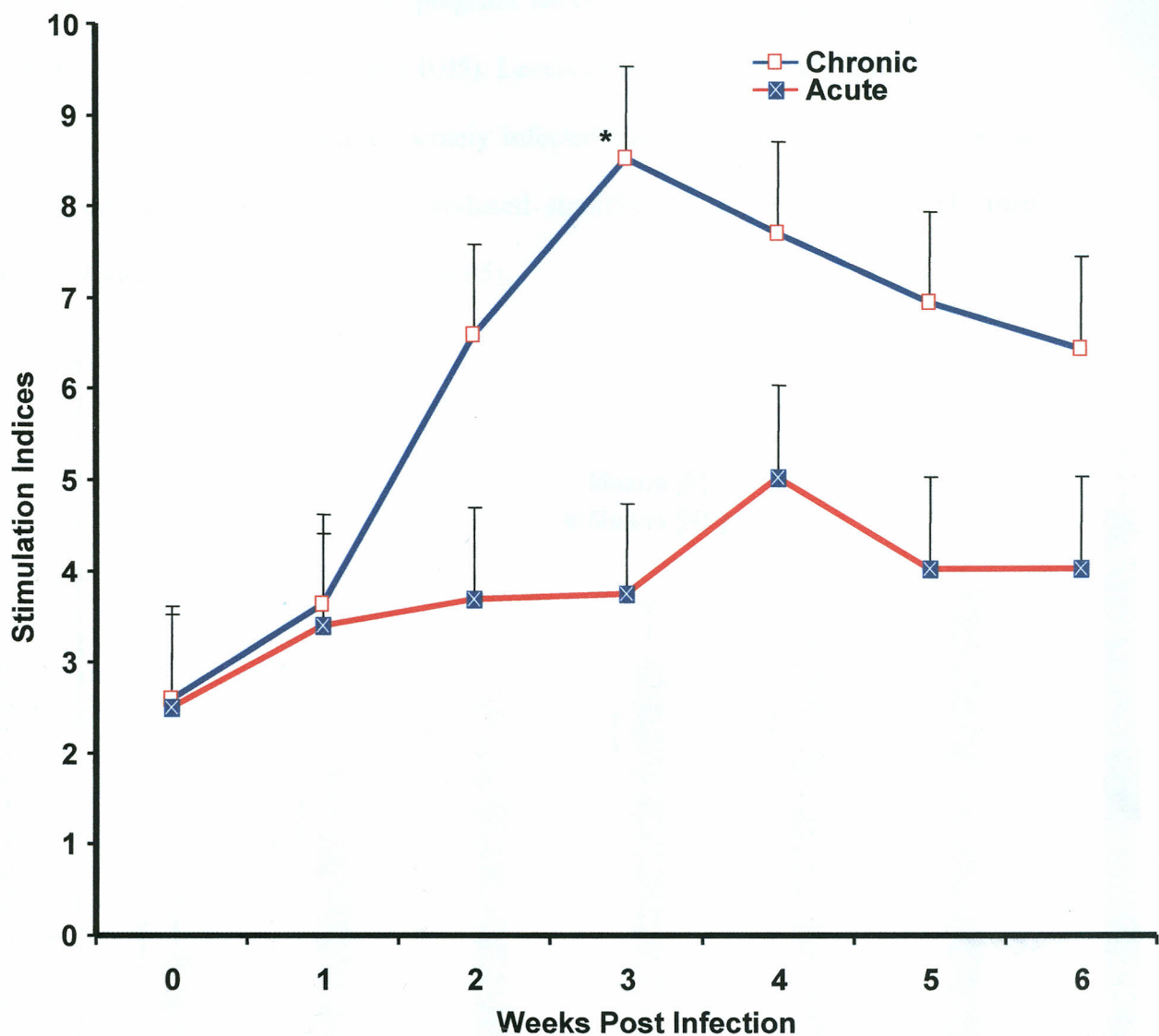


Fig 4.13: Mean weekly PBMC proliferation observed in acute and chronic *P. knowlesi* infected baboons. * Asterisk means significant difference between proliferation in acute and chronic baboons, $P < 0.05$; n: acute = 3; chronic = 4.

4.7 Immunoglobulin G (IgG) Levels

All baboons experienced gradual increases in Ig G levels over the first two weeks of infection. In pregnant baboons levels ranged between 1.41 and 2.58 while in non pregnant baboons levels ranged between 1.45 and 3.36. There was a significant difference in IgG levels between pregnant and non pregnant baboons over the entire

period of experimentation with non pregnant baboons producing higher IgG responses than pregnant baboons (Fig 4.14; $P < 0.05$). Levels of IgG in chronically infected baboon ranged from 1.42 to 4.39 while in acutely infected baboons they ranged from 1.45 to 3.32. Chronically infected baboons produced significantly higher levels of IgG than acutely infected baboons (Fig 4.15; $P < 0.05$).

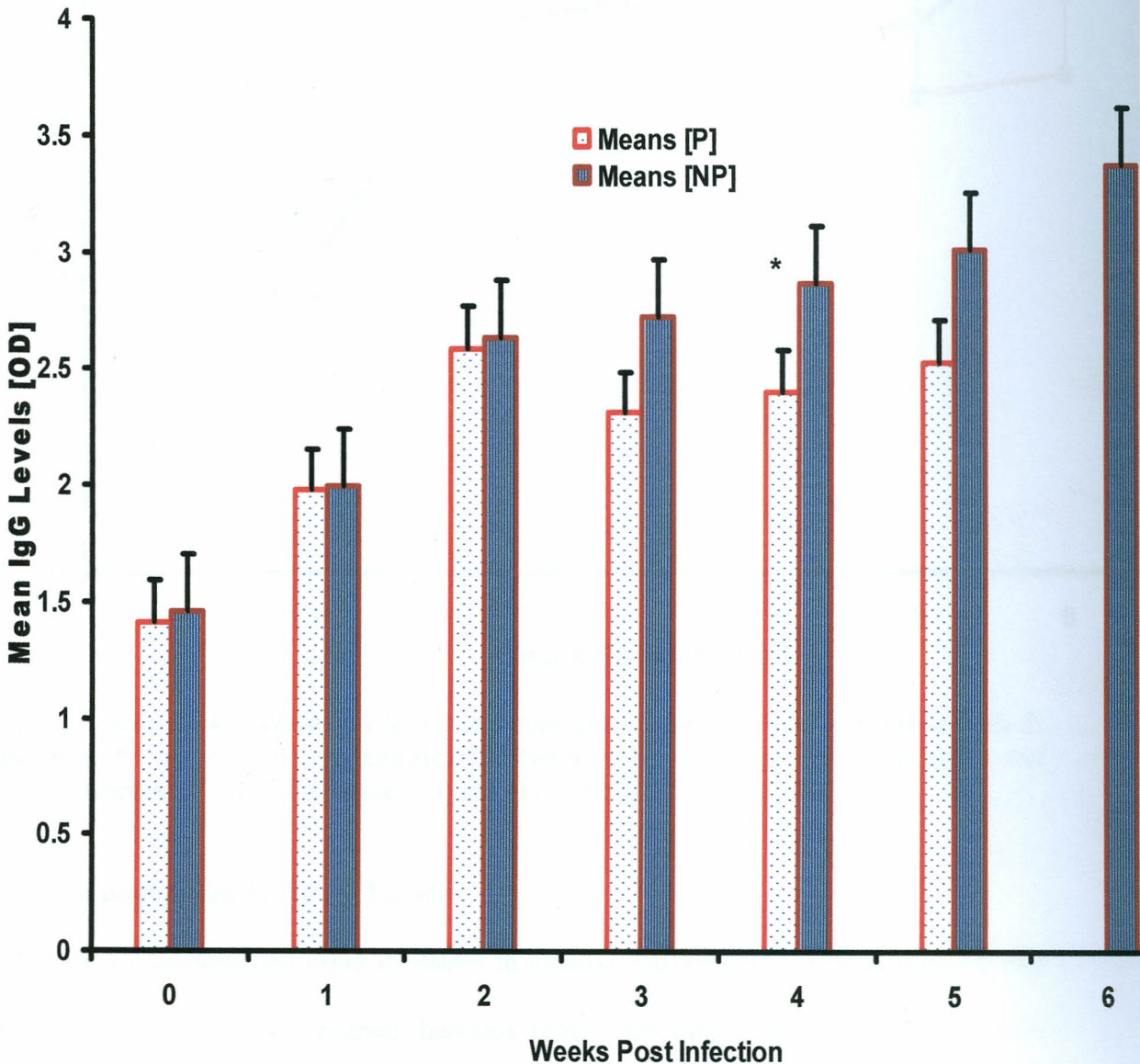


Fig 4.14: Anti *P. knowlesi* IgG levels in pregnant and non pregnant baboons infected with *P. Knowlesi*. OD: Optical Density; P: Pregnant; NP: Non pregnant. *: Asterisk means significant difference between pregnant and non pregnant baboons in IgG responses, $P < 0.05$. n: P = 3; NP = 4.

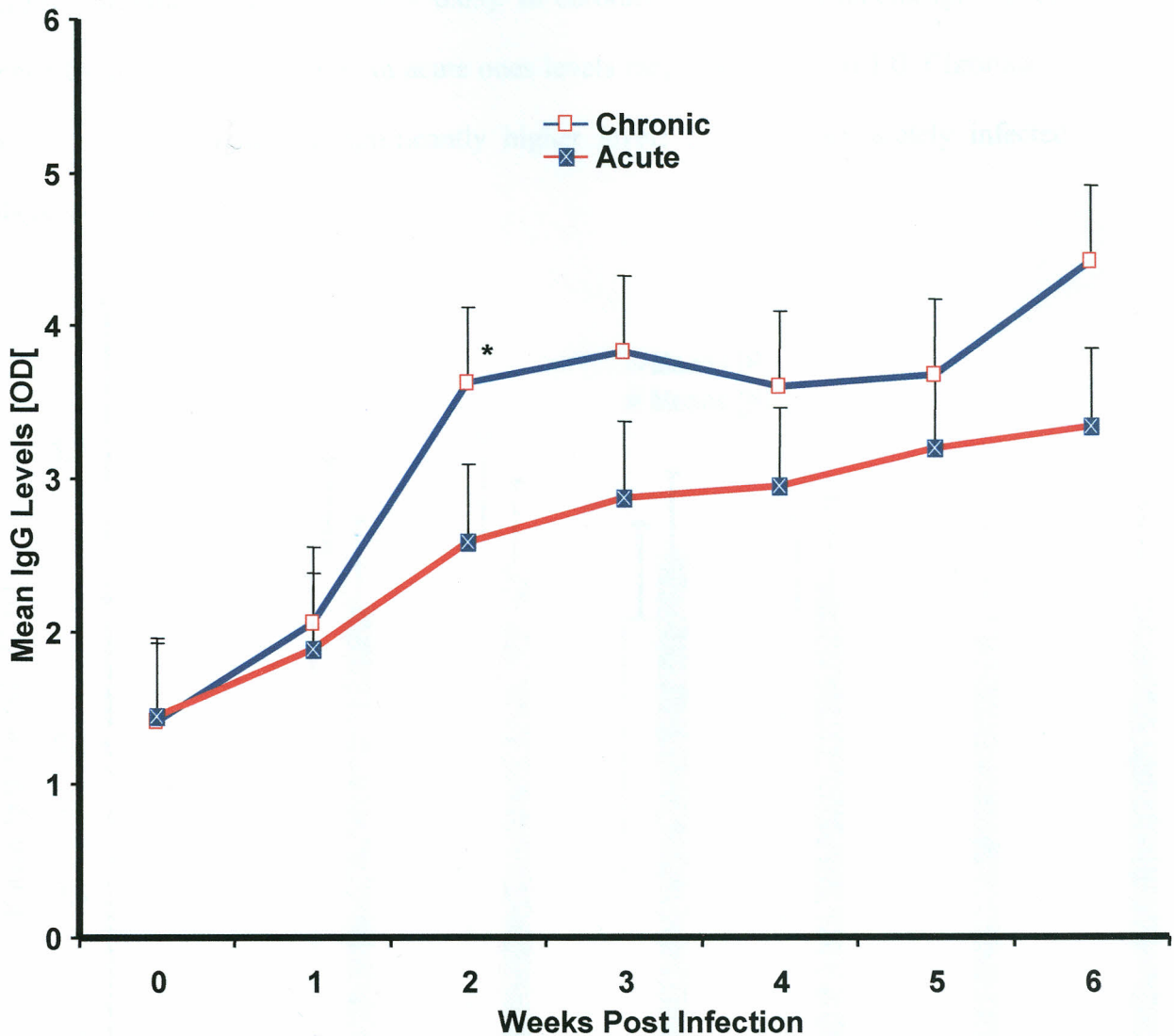


Fig 4.15: Anti *P. knowlesi* IgG levels in acute and chronic baboons infected with *P. Knowlesi*. *: Asterisk means significant difference between IgG levels in acute and chronic baboons, $P < 0.05$; n: acute = 3; chronic = 4. OD: Optical Density.

4.8 Immunoglobulin M (Ig M) Levels

Generally all baboons had rapid increases in malaria antigen specific Ig M titers by one week post inoculation. In pregnant baboons IgM levels ranged between 0.26 and 1.08 while in non pregnant baboons levels ranged between 0.27 and 1.05. Comparison of means of pregnant and non pregnant baboons revealed that differences in IgM production

were not significant (Fig 4.16; $P > 0.05$). In chronically infected baboons IgM levels ranged from 0.23 to 2.06 while in acute ones levels ranged from 0.3 to 1.0. Chronically infected baboons produced significantly higher levels of IgM than acutely infected baboons (Fig 4.17; $P < 0.05$).

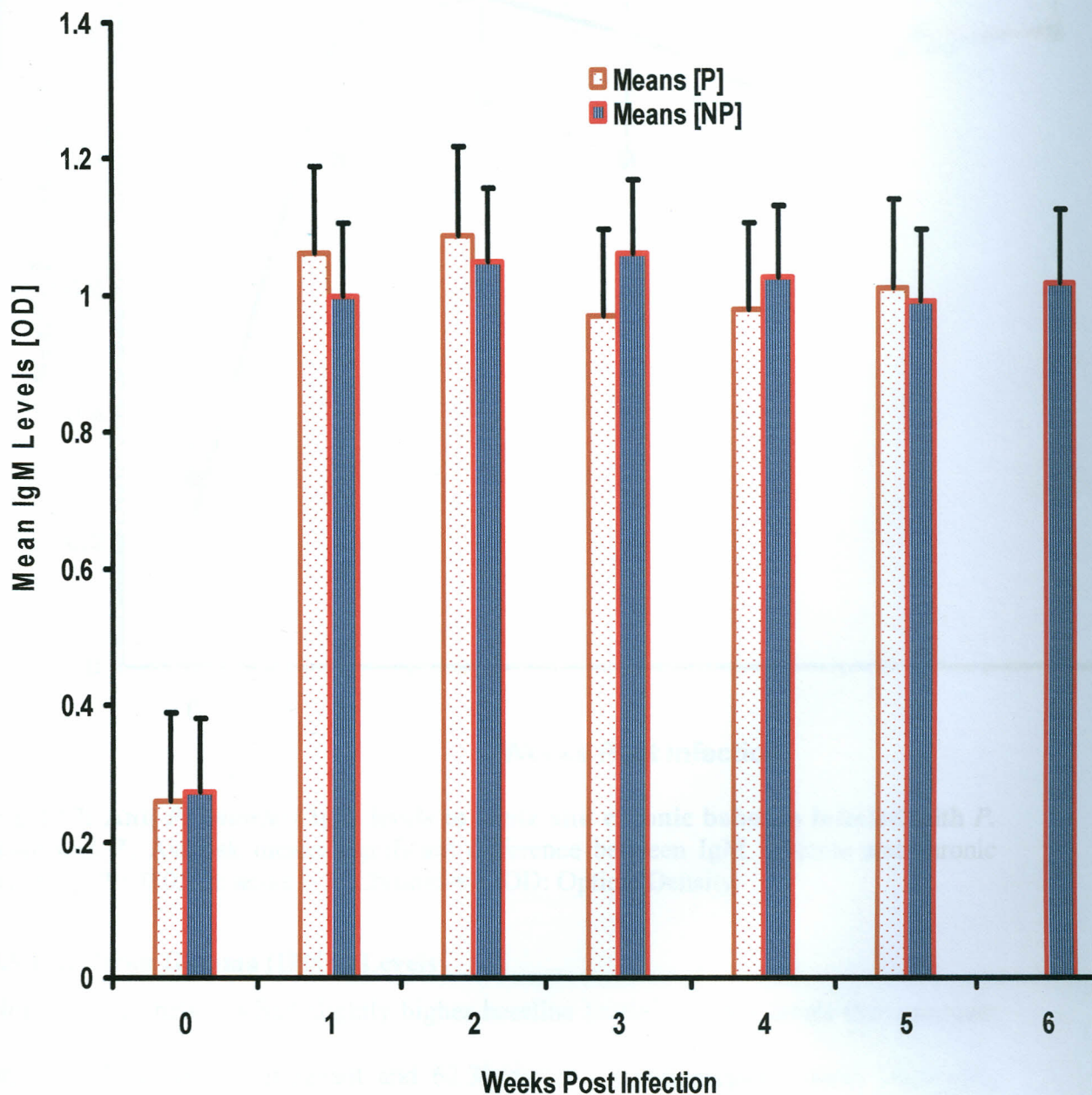


Fig 4.16: Anti *P. knowlesi* IgM levels in pregnant and non pregnant baboons infected with *P. Knowlesi*. OD: Optical Density; P: Pregnant; NP: Non pregnant. n: P = 3; NP = 4.

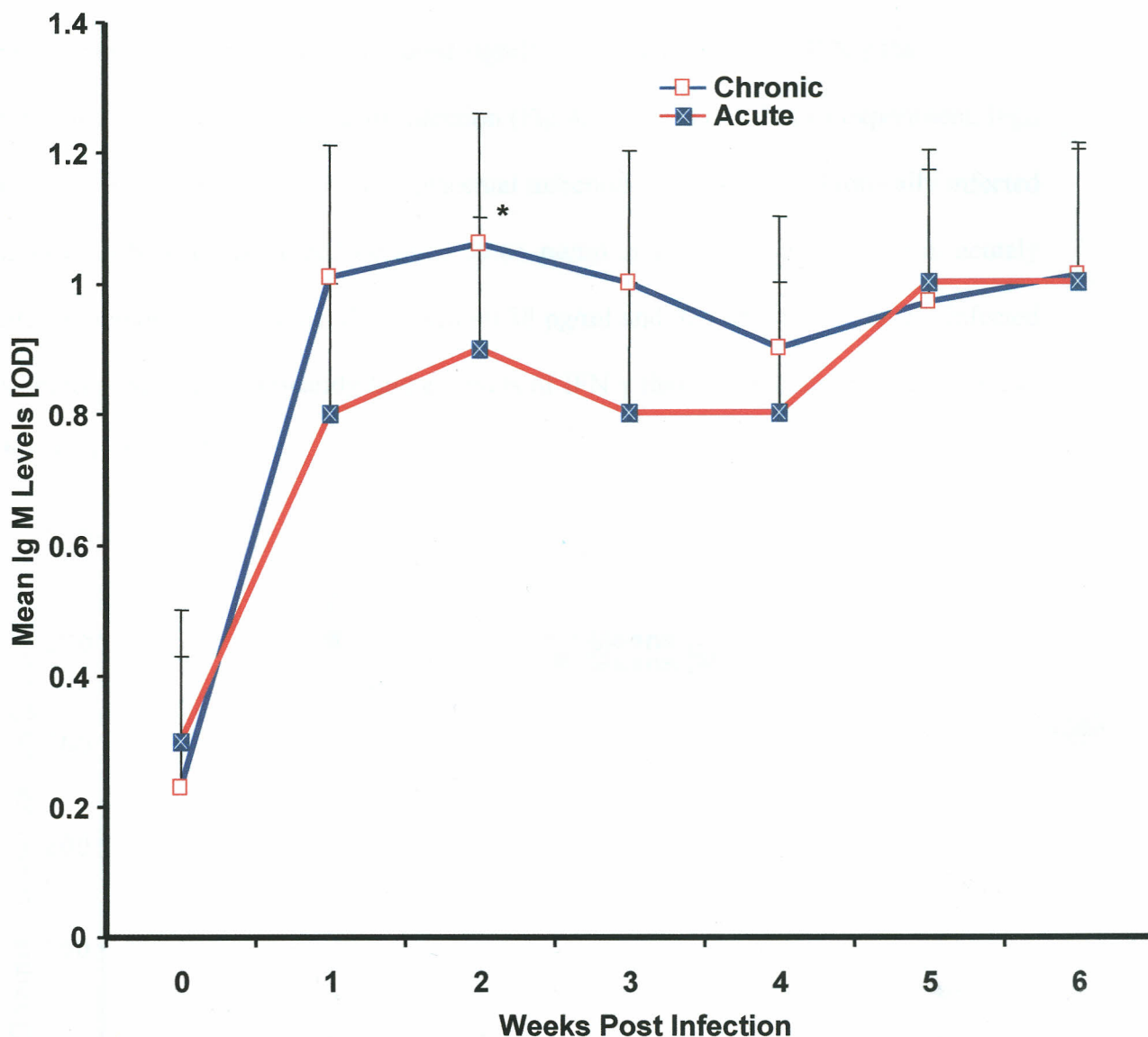


Fig 4.17: Anti *P. knowlesi* IgM levels in acute and chronic baboons infected with *P. knowlesi*. *: Asterisk means significant difference between IgM in acute and chronic baboons, $P < 0.05$; n: acute = 3; Chronic = 4. OD: Optical Density.

4.9 Interferon Gamma (IFN γ) Levels

Non pregnant baboons had slightly higher baseline levels of IFN γ levels than pregnant ones (55.54 pg/ml for pregnant and 62.30 pg/ml for non pregnant ones). Following infection both groups had sharp increases in IFN γ concentration peaking at week one post infection. In pregnant baboons concentration of IFN γ ranged from 36.81 pg/ml to

9.92 pg/ml while in non pregnant baboons levels ranged from 54.98 pg/ml to 141.15 pg/ml. Non pregnant baboons produced significantly higher levels of IFN γ than pregnant baboons during the early phase of infection (Fig 4.18; $P < 0.05$). In this experiment, high levels of IFN γ correlated with low placental infection (table 4.2). In chronically infected baboons IFN γ levels ranged between 39.61 pg/ml and 157.39 pg/ml while in acutely infected baboons levels ranged between 40.38 pg/ml and 325.65 pg/ml. Acutely infected baboons produced significantly higher levels of IFN γ than chronically infected baboons (Fig 4.19; $P < 0.05$).

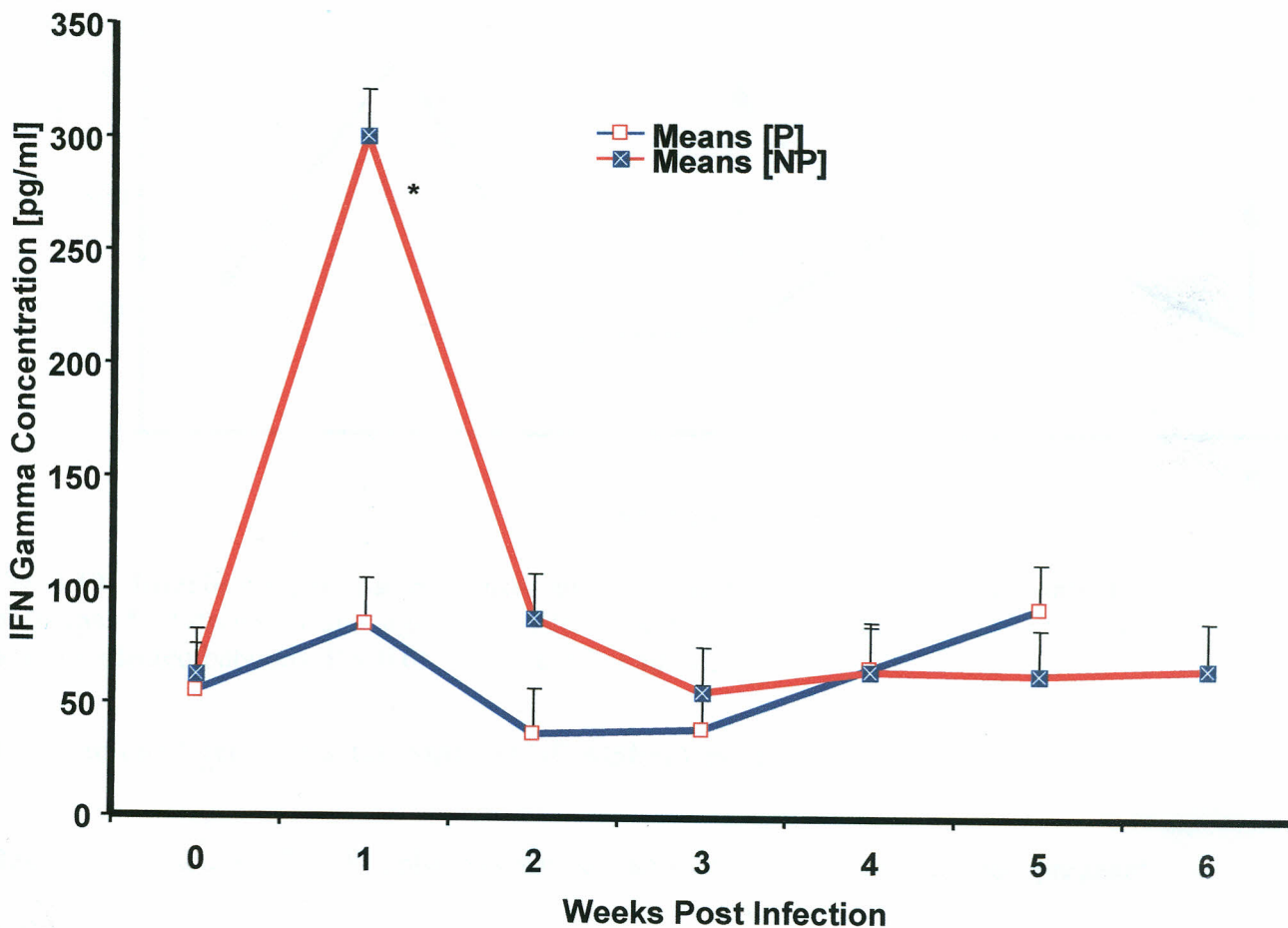


Fig 4.18: Interferon gamma responses in pregnant and non pregnant *P. knowlesi* infected baboons. P: Pregnant; NP: Non pregnant. *: Asterisk means significant difference between pregnant and non pregnant baboons in IFN γ responses, $P < 0.05$. n: P = 3; NP = 4.

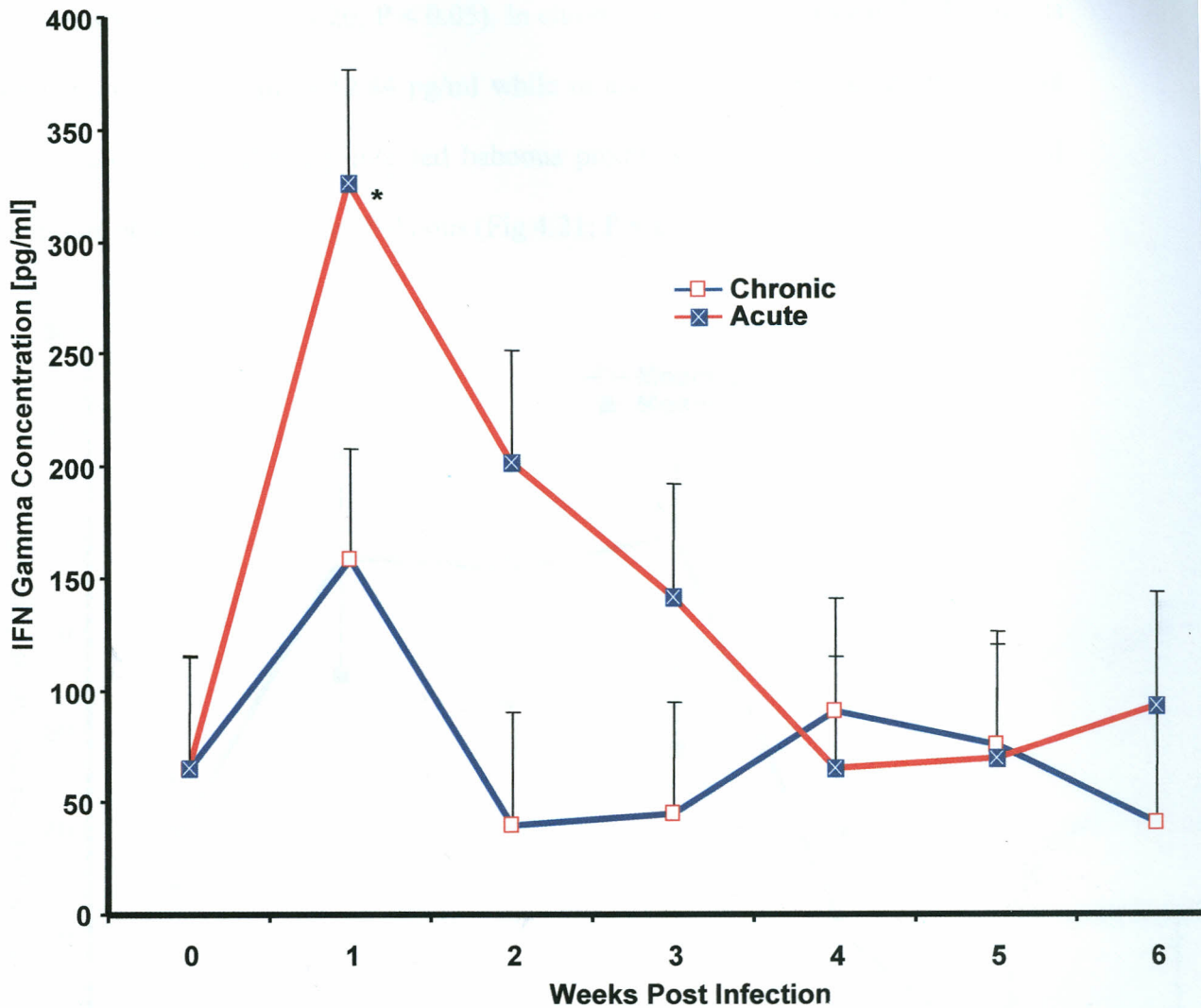


Fig 4.19: Interferon gamma responses in acute and chronic *P. knowlesi* infected baboons. *: Asterisk means significant difference between IFN gamma in chronic and acutely infected baboons, $P < 0.05$. n: acute = 3; Chronic = 4.

4.10 Tumour Necrosis Factor-Alpha (TNF Alpha) Levels

Baseline production of TNF alpha was similar between the pregnant and non pregnant group of baboons (38.46 pg/ml in pregnant baboons and 34.92 pg/ml in non pregnant baboons). In pregnant baboons TNF alpha concentration ranged from 15.18 pg/ml to 69.10 pg/ml while in non pregnant baboons it ranged from 12.97 pg/ml to 55.62 pg/ml

after infection. Pregnant baboons produced significantly higher levels of TNF alpha than non pregnant baboons (Fig 4.20; $P < 0.05$). In chronically infected baboons TNF α levels ranged from 12.88 pg/ml to 52.84 pg/ml while in acute ones levels ranged from 14.48 pg/ml to 69.1 pg/ml. Acutely infected baboons produced significantly higher levels of TNF α than chronically infected baboons (Fig 4.21; $P < 0.05$).

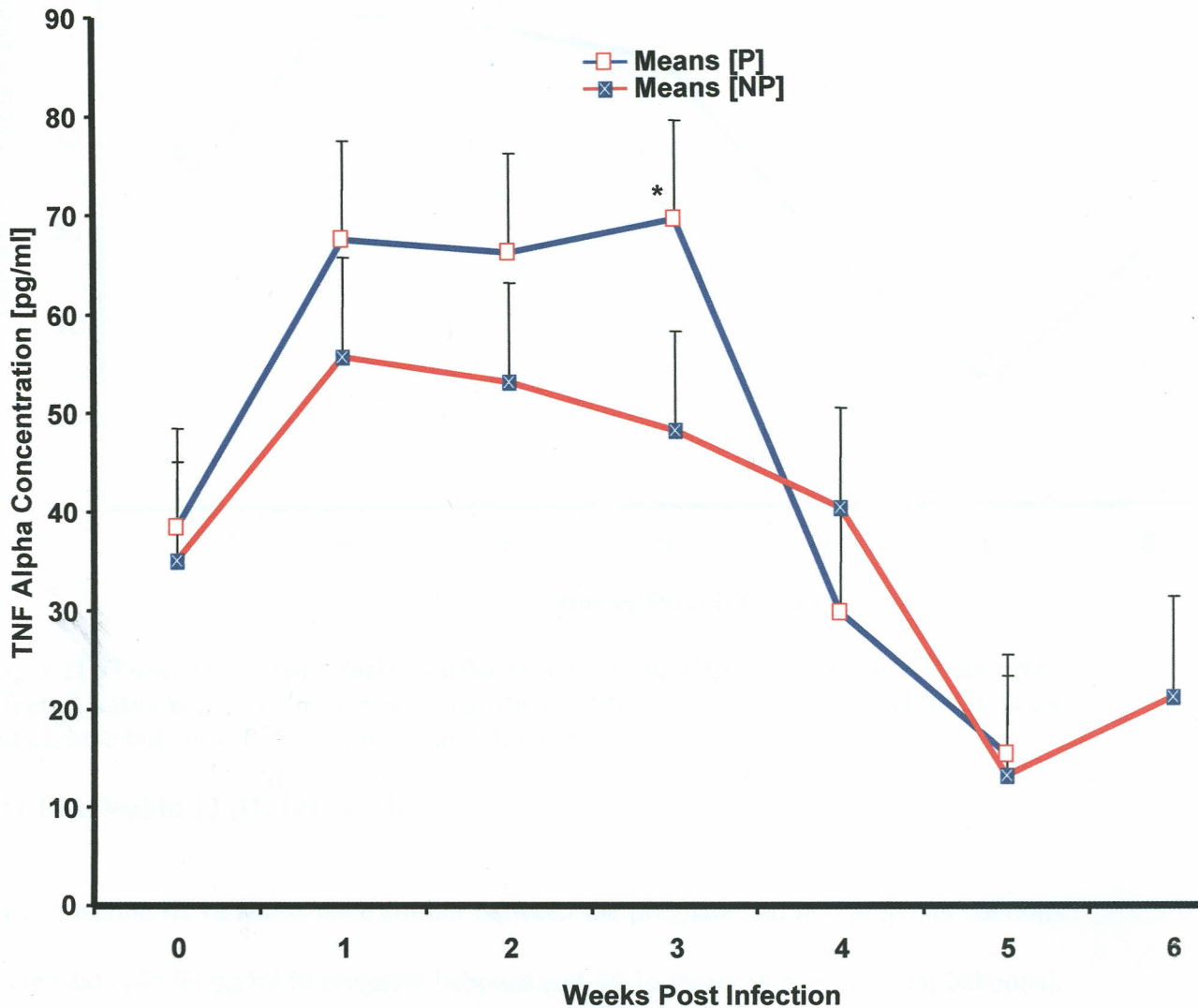


Fig 4.20: Tumour necrosis factor alpha responses in pregnant *P. knowlesi* infected baboons. P: Pregnant; NP: Non pregnant. *: Asterisk means significant difference between pregnant and non pregnant baboons in TNF α responses, $P < 0.05$. n: P = 3; NP = 4.

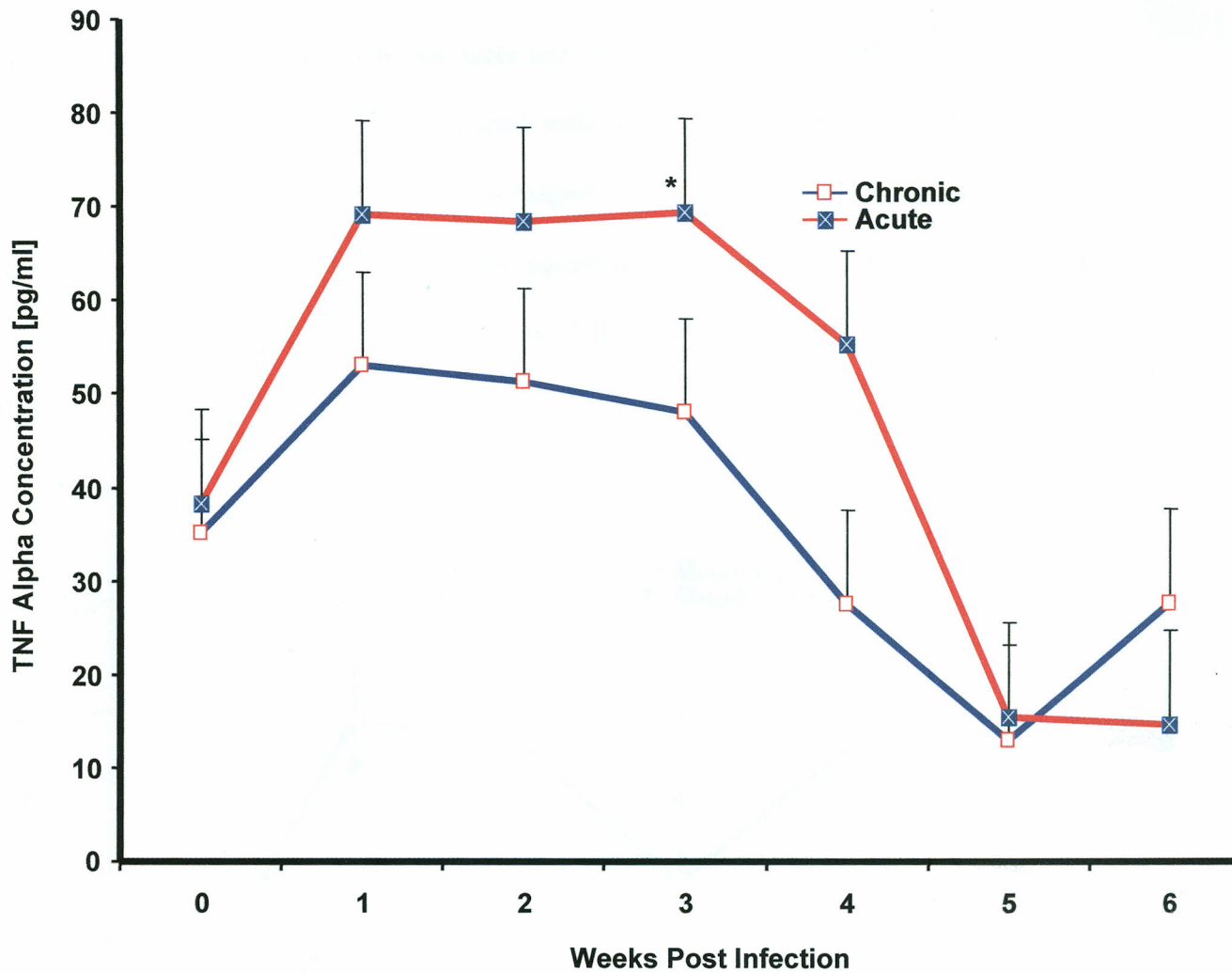


Fig 4.21: Tumour necrosis factor alpha responses in acute and chronic *P. knowlesi* infected baboons. *: Asterisk means significant difference between TNF alpha in acute and chronic baboons, $P < 0.05$; n: acute = 3; Chronic = 4.

4.11 Interleukin 12 (IL 12) Levels

Mean baseline IL 12 levels were similar between the pregnant and non pregnant baboons in the study (46.94 pg/ml in pregnant baboons and 46.16 pg/ml in non pregnant baboons).

Following infection both groups experienced rapid increases in IL 12 concentrations (Fig 4.22). In pregnant baboons IL 12 levels ranged from 46.94 pg/ml to 98.64 pg/ml while in non pregnant baboons levels ranged from 46.16 pg/ml to 94.00 pg/ml over the six weeks

of experimentation. When IL 12 means were considered, the differences between pregnant and non pregnant baboons were not significant (Fig 4.22; $P > 0.05$). In this experiment, low levels of IL 12 correlated with high placental infection (table 4.2). In chronically infected baboons IL 12 levels ranged from 45.77 pg/ml to 98.50 pg/ml while in acute ones levels ranged from 47.44 pg/ml to 93.98 pg/ml. Chronically infected baboons produced significantly higher levels of IL 12 than acute baboons (Fig 4.23; $P < 0.05$).

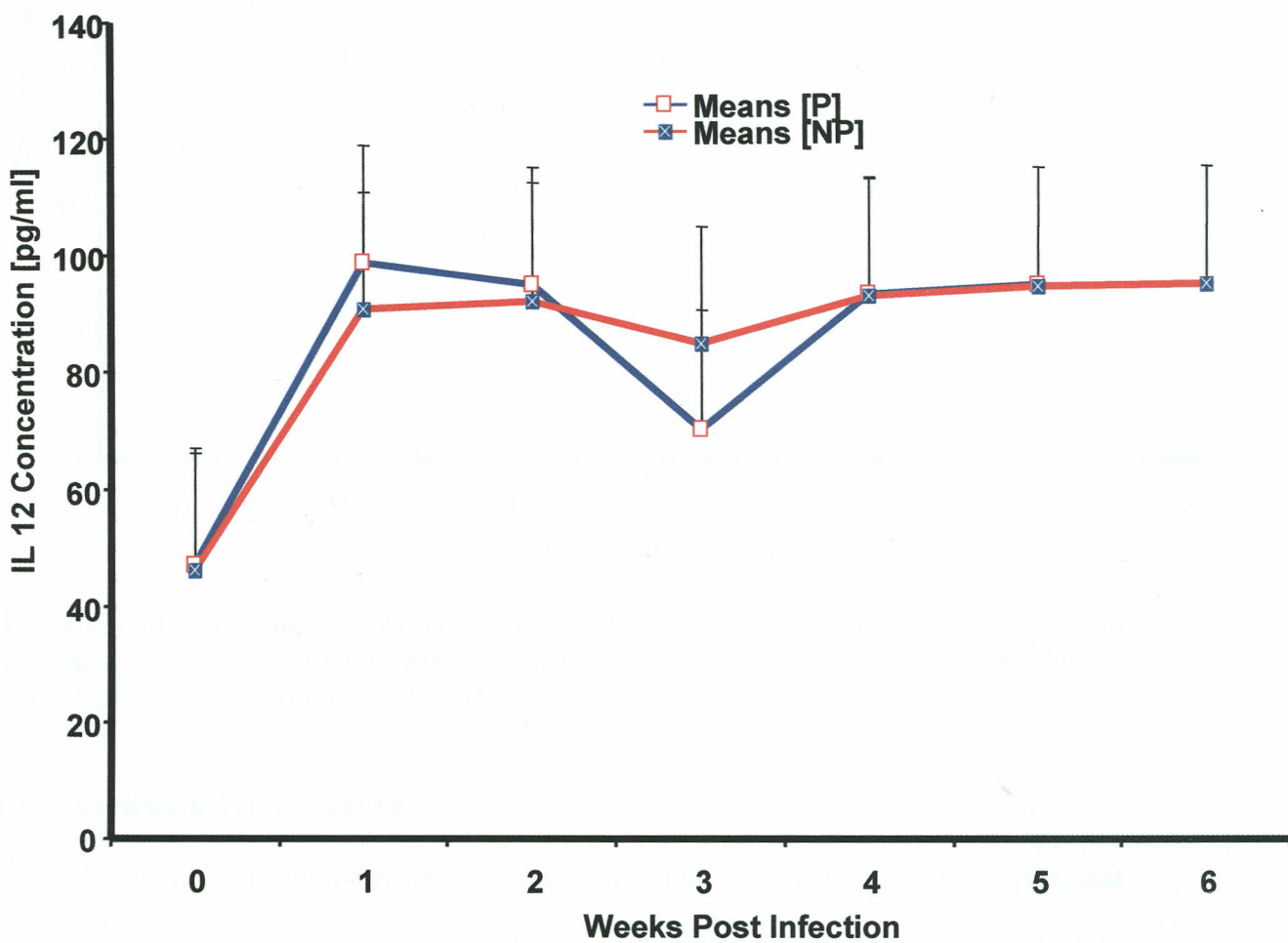


Fig 4.22: Interleukin 12 responses in pregnant and non pregnant *P. knowlesi* infected baboons. P: Pregnant; NP: Non pregnant. n: P = 3; NP = 4.

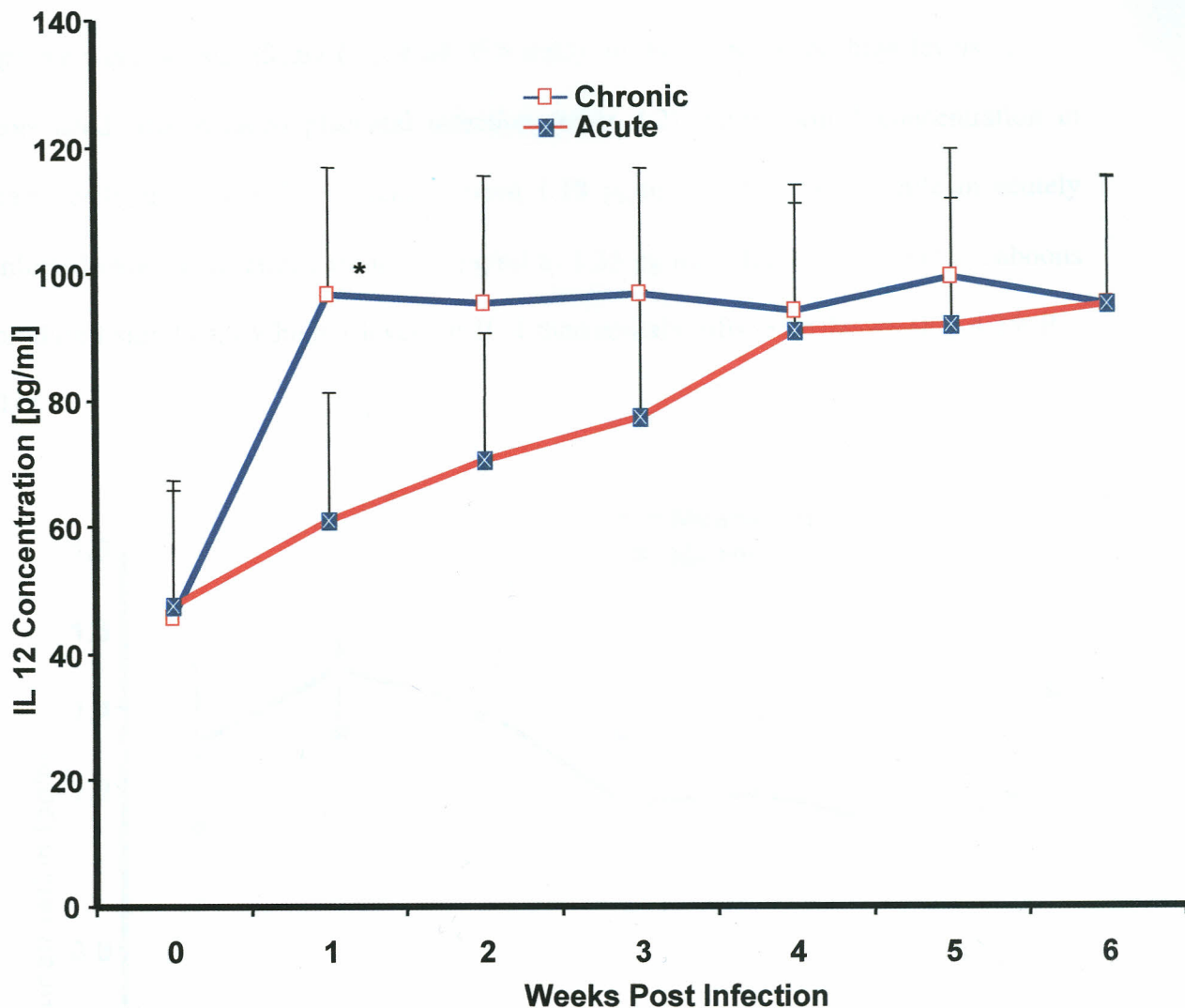


Fig 4.23: Interleukin 12 responses in acute and chronic *P. knowlesi* infected baboons. *: Asterisk means significant difference between IL 12 in acute and chronic baboons, $P < 0.05$; n: Acute = 3; Chronic = 4.

4.12 Interleukin 4 (IL 4) Levels

Pregnant baboons had higher mean baseline interleukin 4 levels than their non pregnant counterparts (1.31 pg/ml compared to 1.10 pg/ml). After infection levels of IL 4 in pregnant baboons ranged from 1.09 pg/ml to 1.49 pg/ml while in non pregnant baboons levels ranged from 1.10 pg/ml to 1.42 pg/ml. Comparison of the mean production of IL 4

between pregnant and non pregnant baboons showed that differences between the two groups were not significant (Fig 4.24; $P > 0.05$). In this experiment, high levels of IL 4 correlated with reduced placental infection (table 4.2). Interleukin 4 concentration in chronically infected baboons ranged from 1.18 pg/ml to 1.91 pg/ml while in acutely infected baboons it ranged from 1.11 pg/ml to 1.33 pg/ml. Chronically infected baboons produced significantly higher levels of IL 4 than acutely infected baboons (Fig 4.25; $P < 0.05$).

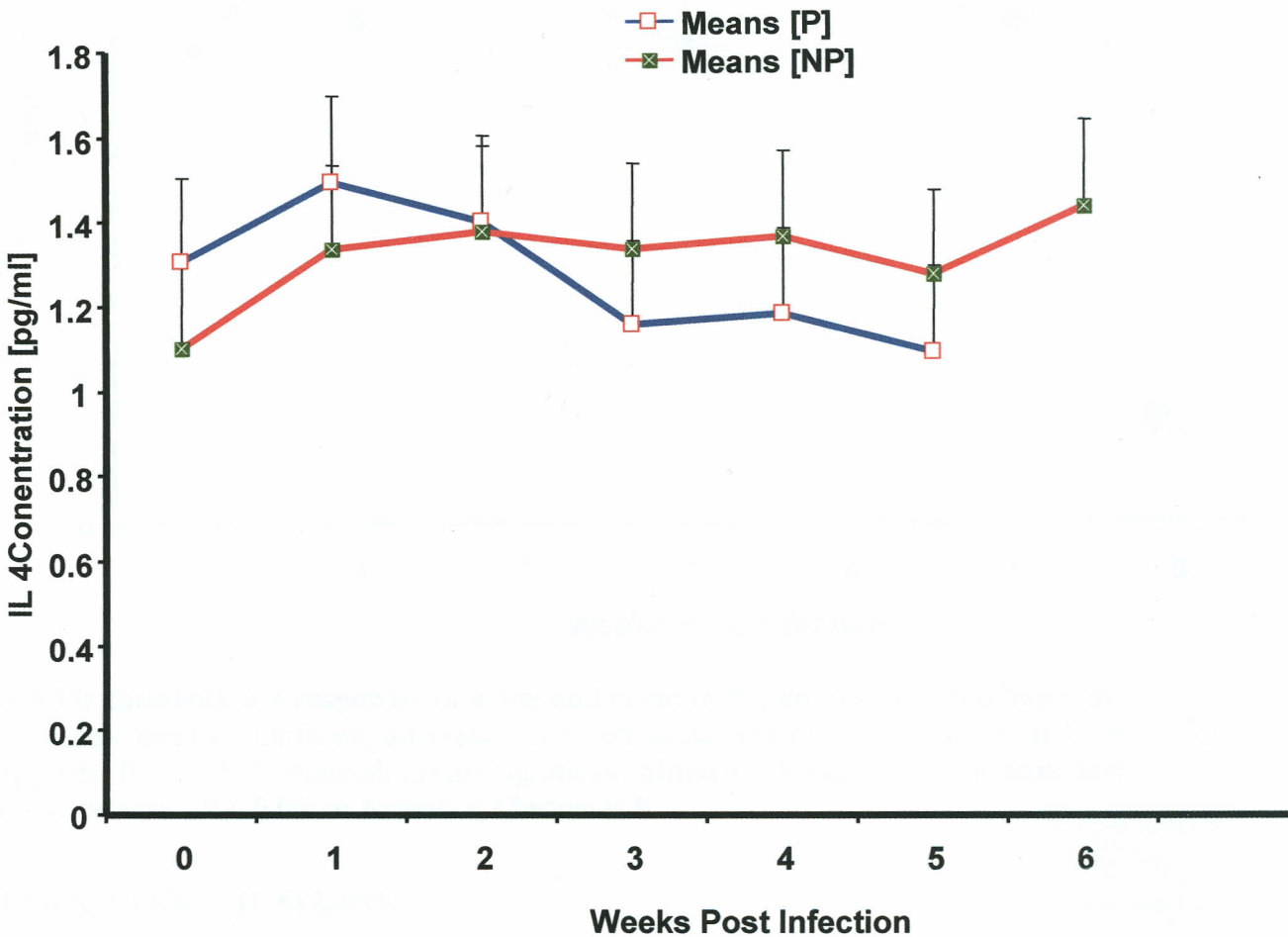


Fig 4.24: Interleukin 4 responses in pregnant and non pregnant *P. knowlesi* infected baboons. P: Pregnant; NP: Non pregnant. n: P = 3; NP= 4.

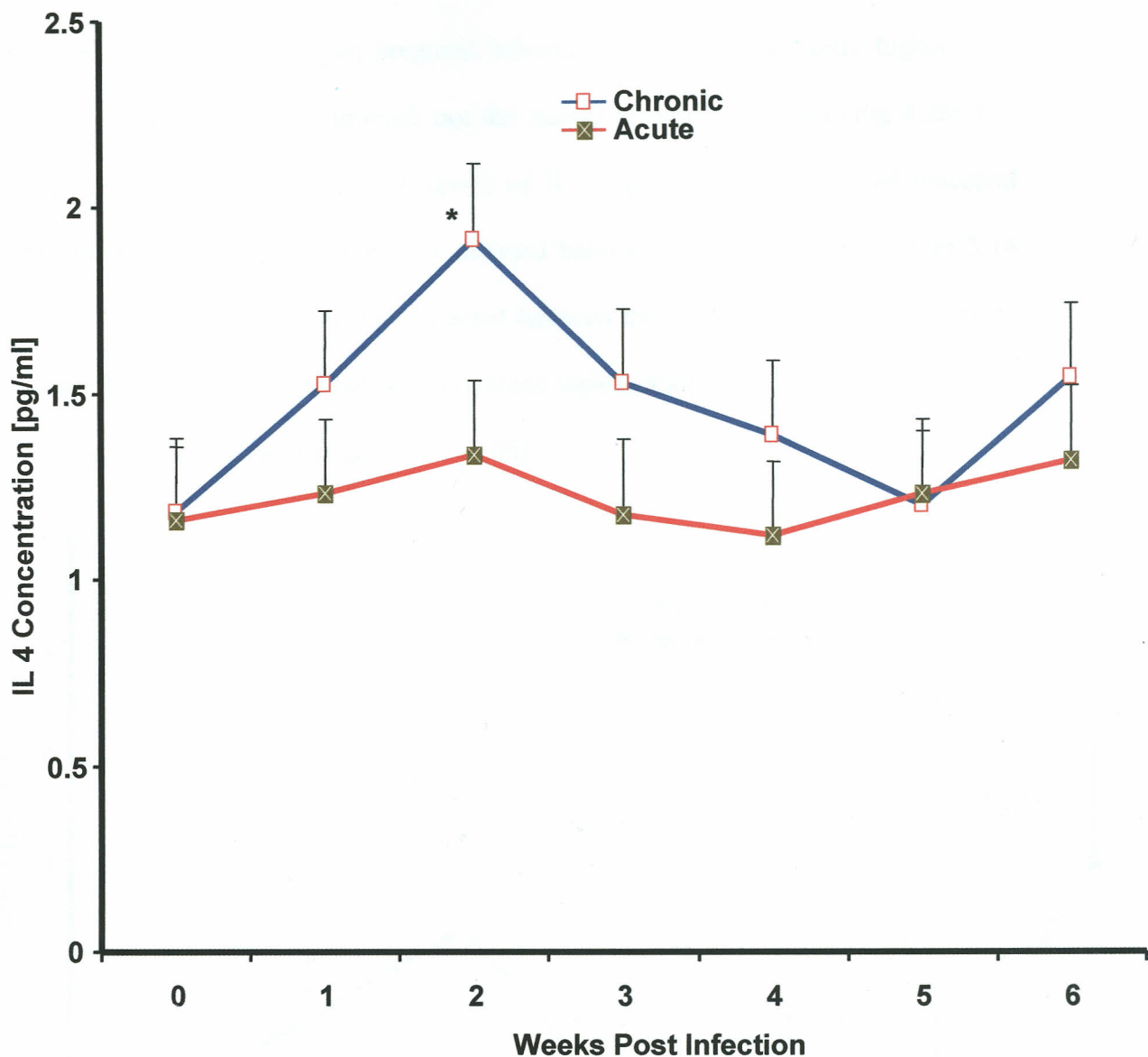


Fig 4.25: Interleukin 4 responses in acute and chronic *P. knowlesi* infected baboons.
 *: Asterisk means significant difference between acute and chronic baboons in IL 4 responses, $P < 0.05$. *: Asterisk means significant difference between IL 4 in acute and chronic baboons, $P < 0.05$; n: Acute= 3; Chronic = 4.

4.13 Interleukin 6 (IL 6) Levels

Pregnant and non pregnant baboons produced similar baseline levels of IL 6 (3.53 pg/ml and 3.56 pg/ml respectively). After infection, pregnant baboons produced IL 6 levels

ranging from 2.98 pg/ml to 6.78 g/ml while in non pregnant baboons levels ranged from 3.46 pg/ml to 11.16 pg/ml. Non pregnant baboons produced significantly higher IL 6 levels than pregnant baboons through out the period of experimentation (Fig 4.26; $P < 0.05$). In the pregnant baboons high levels of IL 6 correlated with reduced placental parasitisation (table 4.2). In chronically infected baboons IL 6 levels ranged from 3.14 pg/ml to 5.36 pg/ml while in acutely infected baboons the range was from 3.31 pg/ml to 11.53 pg/ml. Acutely infected baboons produced significantly higher levels of IL 6 than chronically infected baboons (Fig 3.9; $P < 0.05$).

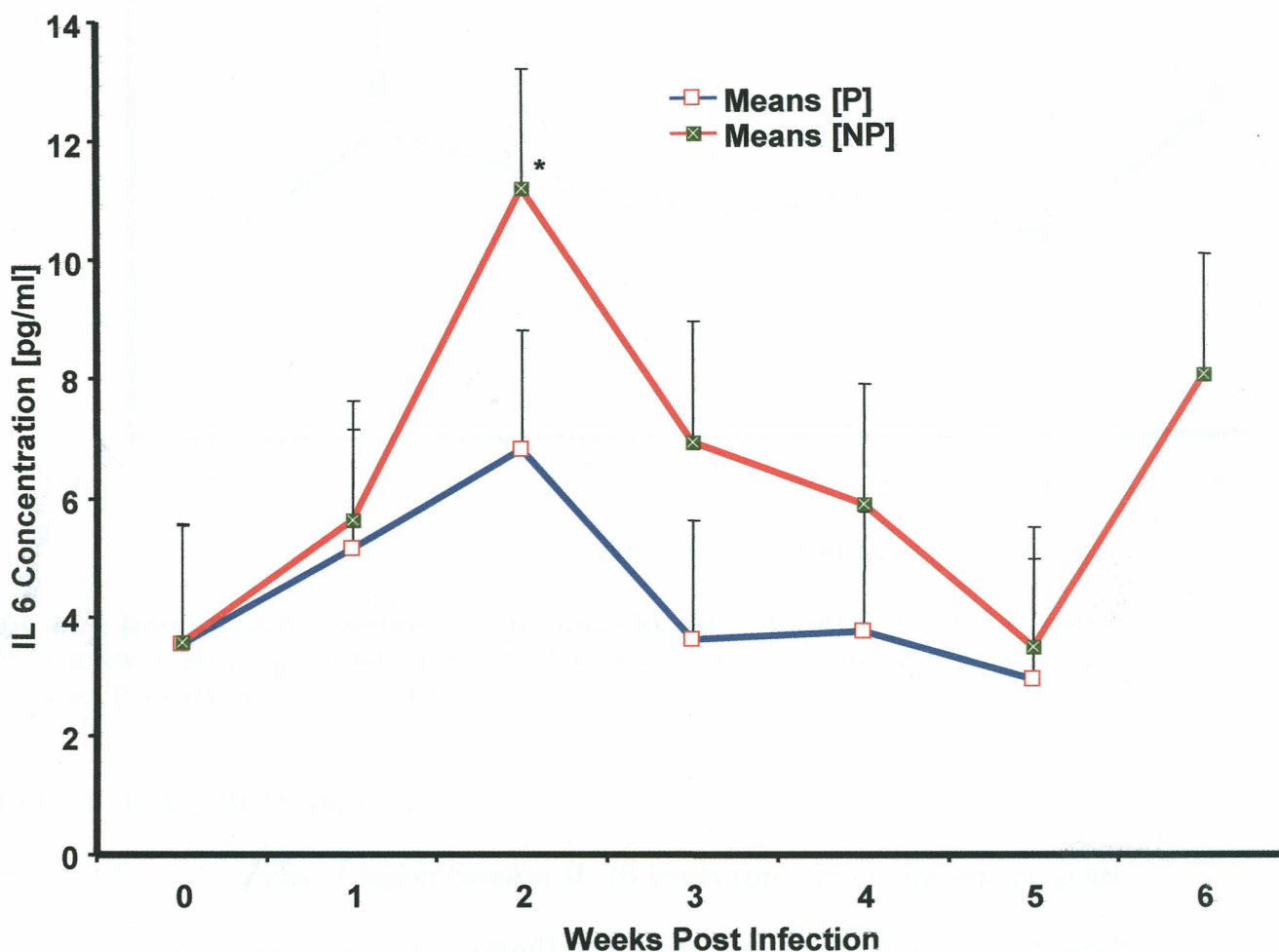


Fig 4.26: Interleukin 6 responses in pregnant and non pregnant *P. knowlesi* infected baboons. P: Pregnant; NP: Non pregnant. *: Asterisk means significant difference in IL 6 responses between pregnant and non pregnant baboons, $P < 0.05$. n: P = 3; NP = 4.

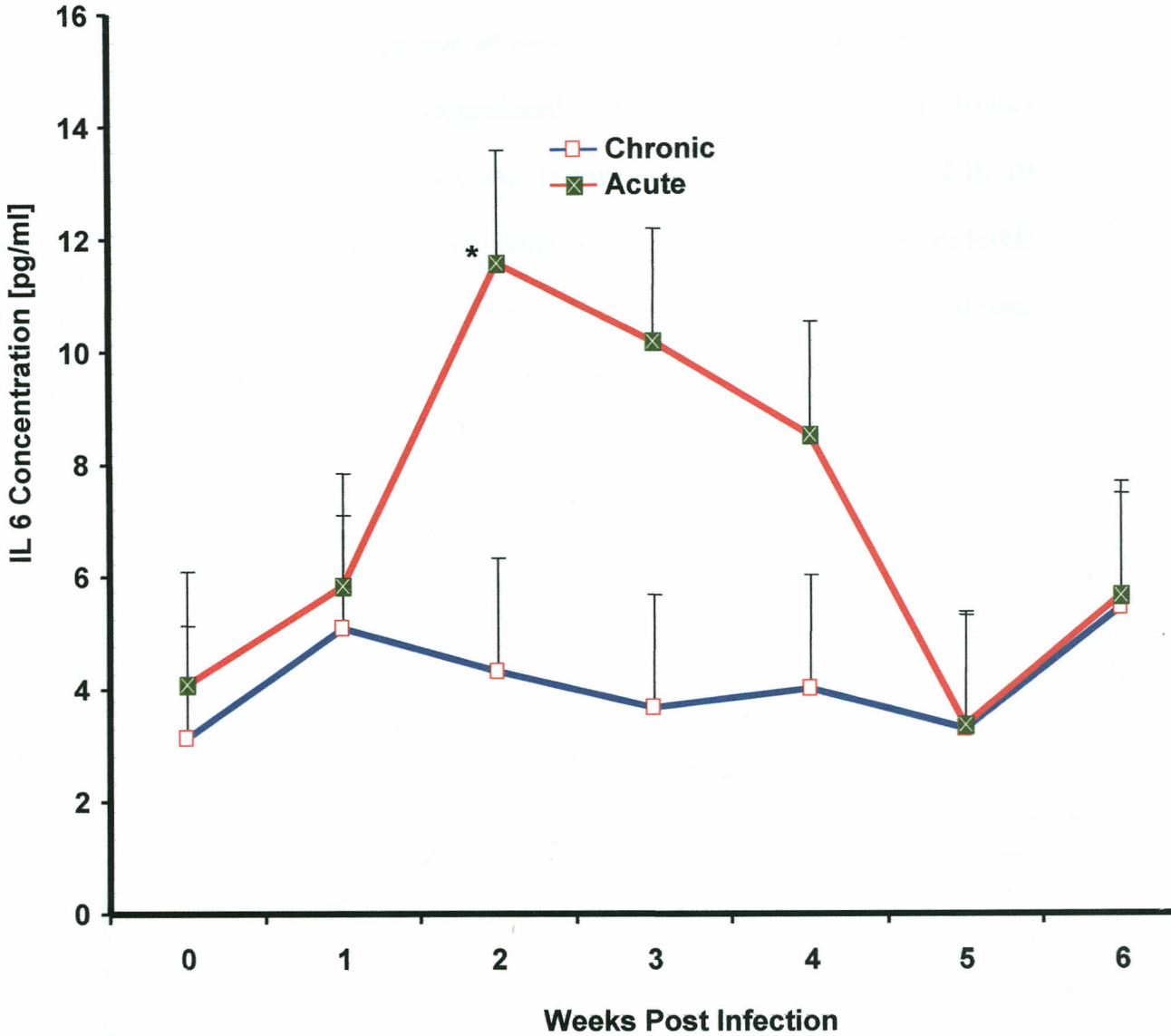


Fig 4.27: Interleukin 6 responses in acute and chronic *P. knowlesi* infected baboons.

*: Asterisk means significant difference between IL 6 levels in acute and chronic baboons, $P < 0.05$; n: Acute = 3; Chronic = 4.

4.14 Interleukin 10 (IL 10) Levels

Pregnant baboons produced higher baseline IL 10 levels compared to the non pregnant ones (15.20 pg/ml compared to 9.48 pg/ml). Following infection both groups had rapid increases in IL 10 levels. In pregnant baboons the concentration of IL 10 ranged from

14.96 pg/ml to 22.16 pg/ml while in non pregnant baboons the concentration ranged from 9.48 pg/ml to 15.96 pg/ml. Comparison of means between pregnant and non pregnant baboons showed that pregnant baboons produced significantly higher levels of IL 10 than non pregnant baboons (Fig 4.28; $P < 0.05$). In this experiment, high levels of IL 10 correlated with low placental parasitisation (table 4.2). In acutely infected baboons levels of IL 10 ranged from 10.48 pg/ml to 14.21 pg/ml while in chronically infected baboons levels ranged from 11.20 pg/ml to 20.19 pg/ml. Chronically infected baboons produced significantly higher levels of IL 10 than acutely infected baboons (Fig 3.10; $P < 0.05$).

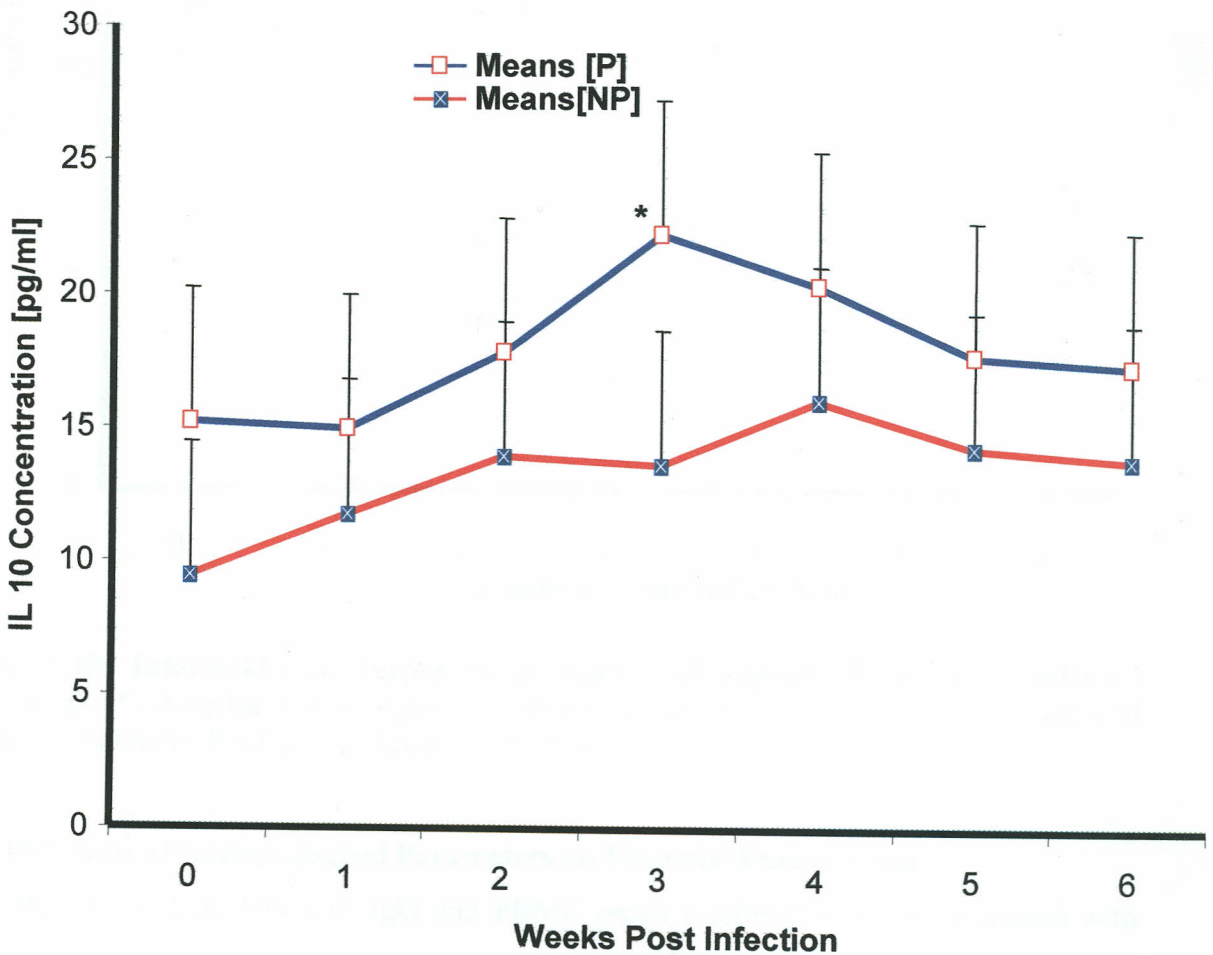


Fig 4.28: Interleukin 10 responses in pregnant and non pregnant *P. knowlesi* infected baboons. P: Pregnant; NP: Non pregnant. n: P = 3; NP = 4.

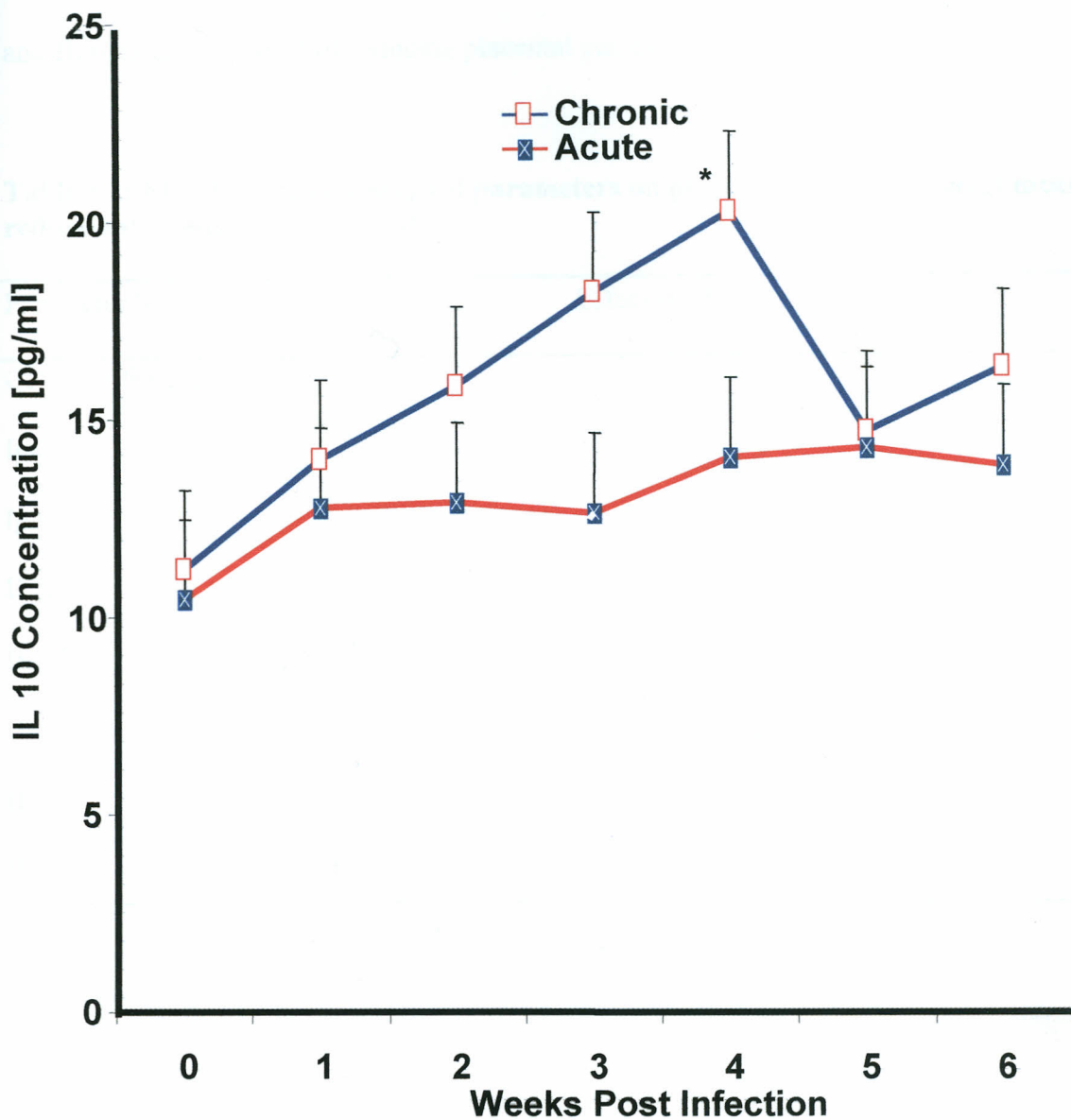


Fig 4.29: Interleukin 10 responses in acute and chronic *P. knowlesi* infected baboons. *: Asterisk means significant difference between IL 10 levels in acute and chronic baboons, $P < 0.05$; n: Acute = 3; Chronic = 4.

4.15 Effects of Immunological Parameters on Placental Parasitaemia

In this study, high levels of IgG and PBMC recall proliferation were associated with reduced placental parasitaemia. On the contrary increased levels of IgM were

accompanied with high placental parasitaemia. The cytokines IFN γ , IL 12, IL 10, IL 4 and IL 6 all had an effect of reducing placental parasitaemia (Table 4.2).

Table 4.2: Effects of immunological parameters on placental parasitaemia. ↓: means reduction; ↑: means increment.

Parameter Measured	Effect on Placental Parasitaemia
Cell proliferation	↓
IgG	↓
IgM	↓
IFN γ	↓
IL 12	↓
IL 10	↓
IL 4	↓
IL 6	↓

CHAPTER FIVE: DISCUSSION

5.1 Overview of Discussion

The aim of this work was to develop a baboon (non-human primate) model for studying human pregnancy malaria. The specific objectives were to demonstrate placental malaria, determine clinical effects, and immune responses following infection of baboons with *P. knowlesi*. Pregnant baboons and non pregnant control baboons were used in the study. The results showed that placental parasitaemia exceeded peripheral parasitaemia. This demonstrates that *P. knowlesi* parasites sequester in the placenta, resulting into 'placental malaria' (PM). Results indicate that PM in this model is associated with suppression of immunoglobulin G, Interferon gamma, and interleukin 6 responses. Tumour necrosis factor alpha and IL 10 responses were upregulated while immunoglobulin M, interleukin 12, interleukin 4 and PBMC proliferation responses did not differ from controls. Haemoglobin and eosinophil levels were significantly reduced in pregnant baboons.

5.2 *Plasmodium knowlesi* Disease Course in Pregnant Baboons

Placental parasitaemia on average was over 29 fold higher than peripheral parasitaemia. This is the first ever clear evidence of sequestration of *P. knowlesi* in the baboon placentas. On the days when PM was characterized, non pregnant control baboons had 6 fold higher peripheral parastaemia than pregnant ones. The difference in peripheral parasitaemia between the two groups is likely to be as a result of sequestration in the placenta of pregnant baboons. The sequestration of *Plasmodium* parasites in placentas has been demonstrated in the murine (Jayekumar and Moore, 2006; Vinayak *et al.*, 1986; Odoula *et al.*, 1986; Desowitz *et al.*, 1989) and rhesus monkey (Das Gupta, 1934) models

before. The relevance of rodent models to malaria in human pregnancy is questionable because of the many differences between rodent and human pregnancies. The presence and sequestration of malaria parasites in the placental tissues, accompanied by low peripheral parasitaemia is one definitive feature of placental malaria in humans (Brabin, 1983). In a typical case of placental malaria, at times there is placental parasitisation even when simultaneous peripheral parasitaemia is negative (Blacklock et al., 1925; Menendez 1995). In the current study, the placenta, an immunologically privileged site (Matteelli et al., 1997), seemed to provide a safe haven where parasites rapidly reproduced while adhering to placental tissue molecules. Pregnant baboons also seemed to be highly susceptible to infection than non pregnant ones since they became parasitaemic two days earlier than non pregnant baboons. Pregnant women are known to have increased susceptibility to malaria infection and are more likely to develop symptomatic malaria than their non pregnant counterparts (Davison et al., 1998). This sequestration finding could suggest that the baboon placenta may contain molecules that mediate parasite adherence to the placental tissue like chondroitin sulphate A which have been reported in humans (Fried et al., 1996; Sartelet et al., 2000).

Placental blood smears contained ring stages as the predominant parasite developmental stage. There is therefore a strong possibility that stages that adhered to the placental tissue were mainly trophozoites and schizonts. These schizonts and trophozoites could have preferentially adhered to placental tissue hence failure of their detection in blood smears prepared from the placenta. *Plasmodium falciparum* can be distinguished from other *Plasmodium* species that infect humans because only immature ring-infected

erythrocytic forms circulate in the peripheral blood (Miller *et al.*, 1998). Mature erythrocytic forms (trophozoites and schizonts) bind to vascular endothelium through 'knobs' (parasite-induced modifications of the red cell surface) that enable them to be sequestered in the venules and to avoid elimination by the spleen (Miller *et al.*, 1998). The mechanisms used by *P. knowlesi* in binding to the baboon placenta are unknown.

Baboons in this experiment developed either chronic or acute malaria regardless of their pregnancy status. Acutely infected baboons had significantly higher parasitaemia than chronically infected baboons. This could mean that chronically infected baboons mounted an immune response that resulted in elimination of most of the parasitized erythrocytes. It was previously reported that chronically infected baboons experienced lower parasitaemia than acutely infected baboons (Ozwara *et al.*, 2005). In that experiment, acutely infected baboons lost appetite and became lethargic while chronically infected baboons developed mild malaria. In the current study similar symptoms were observed in acute and chronic baboons regardless of their pregnancy status.

The *P. knowlesi*-baboon model for pregnancy malaria can therefore be used as a model for further studies of human pregnancy malaria. This model can be exploited in studying other underlying mechanisms of parasite sequestration and abortion. In addition, the model can be used to understand mechanisms underlying increased susceptibility to malaria during pregnancy.

5.3 Clinical and Haematological Profiles

Some baboons developed acute infection (severe symptoms that included apathy, ruffled hair, loss of appetite and vomiting) or chronic infection (mild asymptomatic malaria characterized by low to moderate clinical symptoms as observed in severely infected animals) irrespective of the pregnancy status. Severely infected baboons became lethargic, developed dyspnoea, and produced dark coloured urine suggesting cholestasis. They had reduced skin turgor and ocular tension indicating dehydration. These symptoms observed here were observed in a previous experiment in the baboon system (Ozwarra *et al.*, 2005). Pregnant women, especially those in their first pregnancy (primigravidae) are known to experience severe malaria symptoms (Miller *et al.*, 1998). The current study has been able to reproduce this aspect of human pregnancy malaria in the baboon model.

Two thirds of baboons in this study experienced abortion at about one week post infection. The baboon that did not abort had over 40 fold lower placental parasitaemia compared to the others. Although more animals (a greater sample size) are required to confirm that *P. knowlesi* infection in pregnant baboons causes abortion, this outcome could suggest that high placental parasitaemia in *P. knowlesi* infected baboons results into abortion. This study also shows that the heavier the placental parasitisation, the greater the chances of abortion since the baboon with the lowest placental parasitaemia was the only one that did not abort thus allowing cesarean delivery. Malaria induced abortions have previously been demonstrated in the murine (Jayekumar and Moore, 2006) and rhesus monkey models of malaria (Davison *et al.*, 1998). In the rhesus monkey model study, parasites were not detected in the placenta even though there were *P.*

coatneyi-induced abortions. Malaria causes severe maternal morbidity and the worst infant outcome in areas of low endemicity, where women have little or no immunity to malaria infection (Kern *et al.*, 1989). Malaria during pregnancy leads to many complications in women and their infants like anaemia, hypoglycemia, pulmonary edema, congenital infection and increased foetal and maternal mortality and morbidity, renal failure, intrauterine growth retardation (IUGR), cerebral malaria, abortion and preterm delivery (Menendez *et al.*, 1995; Nathwani *et al.*, 1992). In areas of unstable malaria transmission, pregnant women are highly susceptible to *Plasmodium falciparum* malaria infection and have 2-20 fold higher mortality rates than the non pregnant ones.

Haemoglobin levels decreased following infection in both pregnant and non pregnant baboons. Packed Cell Volume (PCV) levels showed general decrease in all *P. knowlesi* infected baboons by the end of the first week post infection. However, the differences in PCV levels were not significant. This could suggest that even though non pregnant control baboons had higher parasitemia than pregnant ones, they still had a fast replacement rate of ruptured RBC. Pregnant baboons had significantly reduced levels of haemoglobin in the later stages of infection. Since their lower haemoglobin concentration could suggest low RBC density, this could mean that pregnant baboons had slower or poor recovery from infection-mediated RBC reduction. Reduction in PCV and haemoglobin levels is a well characterized occurrence in malaria infection (Davison *et al.*, 1998). In the study in the *P. coatneyi*-rhesus monkey model of malaria pregnant monkeys became anaemic following infection in the first trimester. Reduction of PCV and haemoglobin was also detected in the *P. berghei*-white rat model and also in *P. chabaudi*-

B6 mice model (Jayekumar and Moore, 2006). In the latter study, pregnant mice had accelerated anaemia than non pregnant ones. Malaria parasites (merozoites) invade and develop in RBC and when schizonts rupture the RBC the result is a decline in PCV levels of the infected baboons. In complicated malaria, anaemic conditions do frequently occur. Pregnant baboons had higher baseline eosinophil levels than non pregnant baboons. Following infection, very low eosinophils were observed in the pregnant group henceforth yet they were still detected in non pregnant baboons. Eosinophils have large eosinophilic granules and non segmented or bilobed nucleus. They are few in tissues except in certain types of inflammation and allergies. Eosinophils participate in inflammatory reactions and immunity to some parasites (Roitt *et al.*, 2002). In this study, eosinophils could have infiltrated the placental tissues leading to a drastic reduction (of eosinophils) in the peripheral circulation. This is reported in human placental malaria (Matteelli *et al.*, 1997). Eosinophils could infiltrate the placenta as a result of parasite induced inflammatory reaction.

Acutely infected baboons experienced significantly sharper reduction in Hb levels compared to chronically infected baboons. Probably the reason could be the loss of RBC. Reduction in haemoglobin levels have previously been detected in the baboon model in an experiment in which severely infected baboons had greater reduction in Hb compared to mildly infected baboons (Ozwara *et al.*, 2005).

5.4 Immunological Profiles of Pregnant Baboons Infected with *P. knowlesi*

In this study, the pregnant baboons were tested for the following immunological parameters; antibody (IgG and IgM) responses, PBMC proliferation and cytokine responses. Pregnant baboons had comparable stimulation indices (SI) to non pregnant baboons, showing that PBMC proliferation against the *P. knowlesi* crude antigen was not affected by gravidity. Data from this study suggests that mechanisms involved in antigen processing and presentation in pregnant and non pregnant baboons are similarly effective in terms of their recall proliferation. This may not be surprising since some workers have shown that peripheral immunity normally remains as competent in pregnant women as it is in non pregnant ones against infectious agents (Fievet *et al.*, 2001). In the mouse model for pregnancy malaria, pregnant *P. chabaudi* infected mice were found to have comparable splenocyte proliferation responses compared to non pregnant mice on day 6 post infection (Jayekumar and Moore, 2006). There is no data on proliferation responses during pregnancy malaria in the monkey models. In other studies, workers stipulated that pregnancy exacerbates malaria through a non-specific hormone dependant suppression of the immune system (Brabin *et al.*, 1985). Higher peripheral proliferation could have resulted in reduced infection and or sequestration of *Plasmodium knowlesi* parasites in the placenta.

In this study, the non pregnant group of baboons produced significantly higher titers of IgG than the pregnant group of baboons. This result could mean that, through some mechanism, peripheral production of IgG is suppressed in pregnant *P. knowlesi* infected baboons. In this study high IgG titers were correlated with reduced placental

parasitisation. This could mean that IgG mediates protection from pregnancy/placental malaria in this model. Data from the current study also suggests that IgG is not only important in the protection against placental malaria but also peripheral parasitisation in pregnant baboons and non pregnant ones. This is because the baboons with the highest placental and peripheral parasitaemia had the lowest immunoglobulin G titers of all the animals. Pregnancy causes a number of physiological changes that affect the way the *Plasmodium* parasite invades its host. Down regulation of normal maternal immune response is necessary to prevent rejection of the conceptus. Cell mediated immunity (Th1) is particularly suppressed during late stages of pregnancy, and the mother is increasingly reliant on humoral immunity (Th2) for protection (Samak, 2004). From the current study, results indicate that pregnancy malaria in olive baboons is associated with downregulation of potentially protective IgG.

Generally comparisons done between the pregnant and control non pregnant groups of baboons showed that no significant differences existed between the two groups in terms of IgM synthesis. This means that IgM mediated immunity could be equally effective in both groups. In the pregnant baboons IgM was found to be protective against placental malaria. The first antibodies to be produced in humoral immune response are always IgM, since IgM can be expressed without isotype switching (Roitt *et al.*, 2002). These early IgM antibodies are produced before B cells have undergone somatic hypermutation and tend to be of low affinity (Roitt *et al.*, 2002). Currently, there is no data from murine and monkey experiments on the role of antibody responses in malaria during pregnancy. Previously, maternal antibodies that block/neutralise placental malaria infection have

been found in the sera of pregnant women (Fried *et al.*, 1998). Similarly, this study reports the occurrence of detectable levels of protective IgM during pregnancy malaria in olive baboons.

Non pregnant baboons produced significantly higher IFN- γ levels early during the experimentation than pregnant baboons. This result suggests that IFN γ mediated immunity is depressed during pregnancy malaria. In this experiment production of high levels of IFN γ was associated with reduced placental parasitisation. Therefore peripheral production of high levels of IFN γ could have resulted in protection from high parasitisation of placentas of baboons with high placental parasitaemia. Peripheral IFN- γ production could suggest a similar production of IFN- γ locally in the placenta where it mediates protection. Malaria-infected individuals produce large amounts of proinflammatory cytokines, such as tumor necrosis factor (TNF) and gamma interferon (IFN- γ). These innate cytokine responses are responsible for the high levels of fever that occur within a few days of the onset of blood stage infection in nonimmune individuals (Grau *et al.*, 1997). In a recent human study, IFN- γ was found to be important in the protection against placental malaria. *Plasmodium* antigen-stimulated placental intervillous blood mononuclear cells (IVBMC) from multigravids with less placental infection were found to secrete more interferon gamma than IVBMC from primigravids or secundigravids (Moore *et al.*, 2000).

Tumour necrosis factor alpha synthesis in peripheral blood was significantly increased in pregnant baboons than in non pregnant ones early during the infection. Pregnancy

seemed to confer an environment that resulted in increased TNF α production following *P. knowlesi* infection. In a similar rhesus monkey model study TNF α was detected only in pregnant monkeys that experienced abortion (Davison *et al.*, 2006). In that study, TNF α levels correlated with abortion. Similarly, in this study baboons that aborted had detectable TNF α . Therefore from this study, it is evident that *P. knowlesi* infection during pregnancy in olive baboons is accompanied by upregulation of TNF α levels. Apart from TNF α , no other studies on cytokines have been reported in the mice and monkey models of pregnancy malaria.

Interleukin 12 production was found not to be significantly different between pregnant and non pregnant baboons. This result could mean that pregnancy did not confer a change in terms of IL 12 production. High placental parasitisation was associated with reduced peripheral IL 12 production. This could mean that IL 12 mediates protection from placental malaria. It has been shown in human studies that downregulation of IL 12 levels, is associated with increased parasitaemia/infection in the placenta of *P. falciparum* infected women (Chaisavaneeyakorn *et al.*, 2002). Likewise, the current study has demonstrated that IL 12 has a protective role against *P. knowlesi*-mediated pregnancy malaria in olive baboons.

In this study, pregnant baboons produced higher levels of IL 10 than non pregnant baboons following infection. Baseline analysis was done during pregnancy but before infection. This result could mean that pregnancy (during the last trimester) in baboons is generally associated with a bias to T helper 2 immunity in order to maintain the

conceptus as an immunologically privileged site (Saito, 2000). The presence of malaria infection in the pregnant baboons could have been the factor that provoked increased IL 10 production in these pregnant baboons. Therefore pregnant baboons have higher baseline IL 10 levels than non pregnant baboons and produce higher levels of IL 10 than non pregnant baboons in response to *P. knowlesi* infection. In this study, high levels of IL 10 were associated with reduced placental parasitisation. This definitely shows that the higher the level of peripheral interleukin 10, the lower the extent of placental parasitisation that occurs in a baboon in this model.

Pregnant baboons had higher baseline levels of IL 4 than their non pregnant counterparts. No significant differences existed in terms of IL 4 production between the pregnant baboons and the non pregnant ones following infection. High placental parasitaemia was accompanied by reduced peripheral IL 4. A recent study showed that infected pregnant women tended to produce lower levels of IL 4 than uninfected ones (Moore *et al.*, 1999). In the current study, high levels of IL 4 were associated with reduced placental parasitisation. Another study conducted in Cameroon showed that IL 4 levels were consistently low in women with placental malaria (Fievet *et al.*, 2001). Therefore high placental parasitisation is accompanied by downregulation of IL 4 as supported by the current study.

Non pregnant baboons produced significantly higher levels of IL 6 than pregnant ones through out the period of experimentation. This suggests that IL 6 mediated immunity could be impaired in pregnant baboons. In the pregnant baboons high levels of peripheral

IL 6 correlated with reduced placental parasitisation and vice versa. This could mean that IL 6 plays a protective role against PM. Interleukin 6 has been previously been described as a cytokine marker for complicated *P. falciparum* malaria (Kern *et al.*, 1989). In a recent study, decreased IL 6 mRNA was also observed in cells from malaria infected placentas and while there was increase in T h 1 cytokine production, T h 2 cytokines remained majorly unchanged (Fievet *et al.*, 2001). This study is consistent with this finding in that pregnant baboons were found to produce reduced levels of IL 6. Pregnancy malaria in this model involves reduced IFN gamma, IL 6 and IgG mediated responses and upregulation of TNF alpha and IL 10 responses. Recall proliferation, IL 12, IL 4 and IgM responses were largely unchanged in this model.

In the current study chronically infected baboons had significantly higher proliferation than acutely infected baboons through all the time points. Antigen processing and presentation pathways in acutely infected baboons could have been interfered with or suppressed in some way making elimination of parasites difficult. Since antigen induced proliferative responses are pre-dominantly mediated by T cells, these results suggest the occurrence of T cells reactivity in *P. knowlesi* infected baboons. These results are in agreement with other studies in humans and chimpanzees where lymphocyte specific proliferative response was demonstrated (Pombo *et al.*, 2000; Taylor *et al.*, 1985). Increased proliferation of peripheral blood mononuclear cells could be an effective factor in the reduction of parasitaemia in *P. knowlesi* infected baboons.

Chronically infected baboons produced higher levels of IgG and IgM than acute ones during the infection. Low production of these antibody isotypes could have been the reason why acutely infected baboons were not able to keep their parasitaemia levels low. There is growing evidence for the protective role of IgG in *P. falciparum* infections (Christophe *et al.*, 2000). Passive transfer of IgG has provided protection against *P. falciparum* blood stage in South American monkeys (Fandeur *et al.*, 1984; Groux and Gysin, 1990), and in humans (Cohen *et al.*, 1961). Furthermore, human antibodies efficiently inhibit *in vitro* *P. falciparum* merozoite proliferation, and mediate opsonisation of infected erythrocytes (Groux and Gysin, 1990). It is possible that increased levels of IgG and IgM have a protective role in immunity against *P. knowlesi* in baboons.

The roles of cytokines in the regulation of immune responses against *Plasmodium* infections and pathogenesis of malaria has been extensively studied in rodent malaria models but there are few studies on cytokine studies in primates especially after experimental malaria (Chunfu *et al.*, 1999). In the current study acutely infected baboons produced higher levels of IFN γ than chronically infected baboons. Production of high levels of IFN γ could have resulted in pathological changes that resulted in increased parasitaemia in acutely infected baboons. In one study in which human volunteers were infected experimentally with *Plasmodium falciparum*, there was increased production of IFN γ (Harpaz *et al.*, 1992). In another study *P. coatneyi* in infected Rhesus monkeys demonstrated the increase of IFN γ production occurring in the early stage of infection, and is thought to have been associated with the pathologic changes that led to death of the

infected animals (Chunfu *et al.*, 1999). This observation is supported by Riley (1990) who suggests that low amounts of IFN γ may be associated with resolution of infection. Other data available are consistent with a requirement for early production of IFN γ to give resistance against infection. In support of this view, analysis of IFN γ R^{-/-} mice infected with *P. chabaudi chabaudi* reveals a critical role of IFN γ in immunity against this pathogen (Favre *et al.*, 1997). From the current study it is evident that high levels of IFN γ exacerbate *P. knowlesi* infection in baboon.

Acutely infected baboons produced higher level of IL 6 and TNF α than chronically infected baboons. It is possible that the production of high levels of IL 6 and TNF α could be the factor that causes increased parasitisation and development of acute malaria symptoms in acutely infected baboons. These cytokines have previously been found to be markers of complicated *Plasmodium falciparum* malaria (Kern *et al.*, 1989). Data gathered from the current study show that the induction of high levels of IL 6 and TNF α are factors that result in increased severity of malaria and high parasitaemia in baboons infected with *P. knowlesi*.

Chronically infected baboons produced higher levels of IL 4, IL 10 and IL 12 than acutely infected baboons. Production of high levels of these cytokines could have resulted in an effective immune response that resulted in maintenance of low levels of parasitaemia. Data gathered from cytokine administration and neutralization with antibodies shows that these cytokines are important in reduction of parasitaemia (Angulo

and Fresno, 2002). Results we have gathered here show that these cytokines (IL 4, IL 10 and IL 12) have a protective role against *P. knowlesi* in baboons.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

- a) This study has demonstrated that *P. knowlesi* sequesters in the baboon placenta and sequestration is mediated by mature parasite stages, mainly schizonts.
- b) This study has shown that placental malaria in the baboon model is associated with abortion, severe malaria, reduction in peripheral haemoglobin and eosinophil levels.
- c) Antibody responses determined in this study show that IgG responses are reduced while IgM responses remain comparable to controls during pregnancy malaria in this model of pregnancy malaria.
- d) Placental malaria in this model is associated with suppression of Interferon gamma, and interleukin 6 responses. Tumour necrosis factor alpha responses were upregulated while interleukin 10, interleukin 12, interleukin 4 and PBMC proliferation responses remained unchanged.

6.2 Recommendations

6.2.1 Application

- a) This model can be used to study the mechanisms underlying the sequestration of *P. knowlesi* in the baboon placenta.

6.2.2 Future Studies

- a) Future studies in this model could involve large animal group studies to conclusively understand the role of antibody and cytokine responses.
- b) Other studies could be carried out to determine the molecules involved in sequestration

in the placenta.

c) Studies can be done do ascertain the cell types involved in placental infiltration.

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APPENDICES

Appendix I: Buffers and Reagents**Carbonate-Bicarbonate buffer (pH 9.6)**

Na_2CO_3	1.59g
NaHCO_3	2.93g
NaN_3	0.2g

Make up to 1 litre with distilled water, store at 4°C for not more than 2 weeks.

Phosphate Buffered Saline (pH 7.2-7.4)

NaCl	8.0g
KH_2PO_4	0.2g
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	2.9g
KCl	0.2g
NaN_3	0.2g

Make up to 1 litre with distilled water, store at 4°C

0.05% Tween 20 in PBS (pH 7.2-7.4)

NaCl	8.0g
KH_2PO_4	0.2g
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	2.9g
KCl	0.2g
NaN_3	0.2g
Tween 20	0.5 ml

Make up to 1 litre with distilled water, store at room temperature.

Giemsa Stain

Giemsa stain 4 ml

Methanol 4 ml

Double distilled water 92 ml

Store at room temperature

Alsevers Solution

Dextrose 10.25 g

NaCl 2.1g

Trisodium citrate 4.0 g

Double distilled water 500 ml

Sterilise by filtering through 0.2 μm pore size filter, store at 4°C

Appendix II: Project Pictures

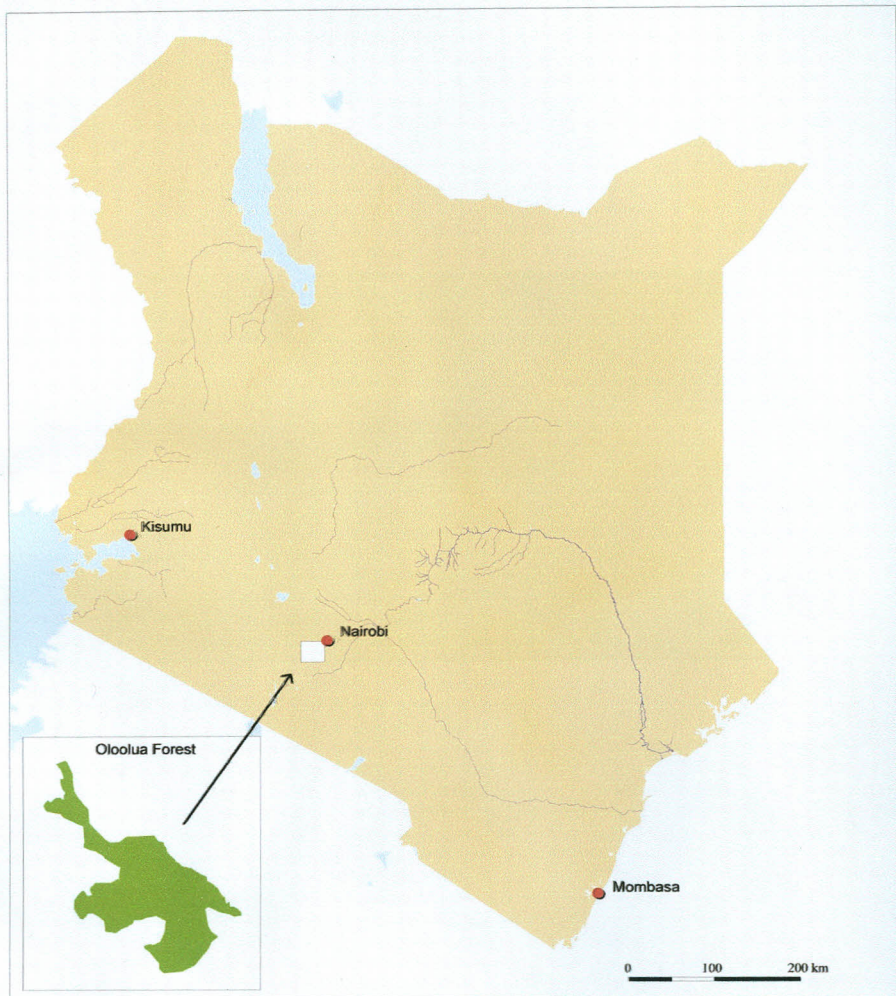
Plate 4: Veterinary surgeons performing cesarean surgery. The IPR (Institute of Primate Research) ARD (Animal Resource Department) facility was used for cesarean operations.



Plate 5: Placenta from PAN 2809. This placenta was pricked using sterile forceps and smears were made from the blood that oozed out.



Plate 6: An infant delivered by cesarean section.

Appendix III: A Map of Kenya Showing Ooloolua Forest, Where IPR is Located

The Institute of Primate Research (IPR) is a semi-autonomous constituent institute of the National Museums of Kenya. It is located in the serene setting of the Ooloolua forest in the outskirts of the city of Nairobi, Kenya (Figure 1). IPR is an internationally recognized center of excellence for biomedical research and is designated a World Health Organization collaborating center for tropical diseases and reproductive health research.