EFFECT OF ABO/Rhesus BLOOD GROUP PHENOTYPES ON IMMUNOSURVEILLANCE TO *PLASMODIUM FALCIPARUM* MALARIA IN CHILDREN IN KISUMU TOWN, KENYA

NG'ONG'A GABRIEL OTIENO
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DECLARATION

This thesis is my original work and has not been presented for a degree or any other award in any other University

Ng'ong'a Gabriel Otieno

Department of Zoological Sciences, Kenyatta University

Signature _____ Date 17/4/9

We confirm that the work reported in this thesis was carried out by the candidate under our supervision as the University supervisors.

Dr. Michael M. Gicheru

Department of Zoological Sciences, Kenyatta University

Signature _____ Date 4-05-2009

Dr. Margaret Oduor

Director Regional Blood Transfusion Center- Kisumu and the Provincial Pathologist New Nyanza General Hospital, Kisumu

Signature _____ Date 20-04-09

Dr. John Vulule

Director Center for Vector Biology and Control Research, Kenya Medical Research Institute (KEMRI), Kisumu

Signature _____ Date 20/04/09

DEDICATION

To my late father Hesborn Ng'ong'a and my elder brother George Ogutu may the Almighty God rest their souls in eternal peace. To my mother Brigitter Ng'ong'a for the motherly passion, love, encouragement and support she accorded me. My wife Eunice for the love, patience and understanding she offered me during the entire study without which this work could not have seen the light of the day.

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ACRONYMS AND ABBREVIATIONS

CD-Cluster of differentiation

CVBCR-Center for Vector Biology and Control Reseach

GNP-Gross National Product

Hb-Haemoglobin

HPE-High power field

ICF- Guava Instrument Cleaning Fluid

ICMA-1-Immunoglobin Superfamily-Intercellular Adhesion Molecules

IFNγ-Interferon-gamma

IL-Interleukin

KMRI-Kenya Medical Research Institute

NK-cell-Natural Killer Cell

PBMC-Peripheral blood mononuclear cell

PE -Phycoerythrin

PfEMP-1-*Plasmodium falciparum* Erythrocyte Membrane Protein-1

PHG-Provincial General Hospital

RBC-Red Blood Cell

TH-T-helper cell

SE-Standard Error

SPSS-Statistical Package for Social Sciences

TNF-Tumor Necrosis Factor

Var-Variant

WHO-World Health Organization

ABSTRACT

Malaria is the world leading cause of deaths among the tropical infectious diseases particularly in children and pregnant women. A safe and effective vaccine would have been the easiest way to control this disease, but the development of vaccine has not been successful despite decades of research. The periodic mutation and production of parasite variants has not only posed a challenge to the vaccine production but also drug resistance. Also important is host-related factors that contribute to disease susceptibility and resistance. Although ABO Blood group phenotypes have been correlated with protection against malaria infection especially in Sir Lanka and Gabon, little study has been done in Africa and Kenya in particular. This study aimed at investigating the effect of ABO/Rh blood group phenotypes on immunosurveillance to *P. falciparum* infection in children 1-10 years in Kisumu town. The study design adopted was simple cross – sectional study, while population sampling technique used was probability proportion to size. The study was done with 78 malaria symptomatic children both males and females who presented at Nyanza Provincial General Hospital and living within Kisumu town. Children age between 1-10 years whose parents or guardians consented to the study were enrolled. The study population exhibited a predominance of group O+, in the order of O+ >A+ >B+ > AB+ and O-. Blood group O was negatively correlated with malaria infection. Parasitaemia levels were significantly higher in blood group A+ and B+ than in group O+. It was further established that blood group A+ registered the highest percentage malaria severity (45.8%) while group O+ recorded the least severity (8 .6%). Helper CD_4^+ and cytotoxic CD_8^+ subsets of T-lymphocytes cells were both inversely correlated with parasitaemia levels ($r = -162$, $p = 0.04$; $r = -0.348$, $p = 0.002$) respectively. Nevertheless, no significant association was observed between parasitaemia levels and differential leukocyte counts ($P = 0.05$). ANOVA revealed significant difference in malaria infection between and females with males having higher infection ($p = 0.04$). However age was inversely correlated with parasitaemia levels ($r = -0.0278$, $p = 0.007$) with younger children having higher parasitaemia levels than the older ones. Parasitaemia levels were significantly higher in males than female children, sex was positively correlated with malaria parasite infection ($r = 0.199$, $p = 0.040$). The results of this study therefore implicate blood group O in protection against malaria while group A and B are likely to predispose the victims to the infection. Since blood group A correlated with malaria infection and disease severity, its advisable that blood group A individuals traveling to malaria endemic areas be given prophylaxis. T-helper and cytotoxic T- lymphocyte cell levels decrease in severe malaria cases hence malaria infected individuals have reduced immunosurveillance. Since Helper T-cell (CD4+) and cytotoxic (CD8+) subsets of T-lymphocytes cells were reduced in malaria cases it is therefore imperative for the clinicians to monitor the levels of such cells in severe malaria cases and if possible immunopotentiaters be given since reduced levels of Helper T-cells and cytotoxic T-lymphocytes cells may subject the victim to prolonged healing and coinfections. Higher parasitaemia levels in males than in females living in the same malaria holoendemic areas are an indicator that males are more vulnerable to malaria infection than females.

CHAPTER ONE: INTRODUCTION

1.1 Epidemiology of Malaria

Despite intensive worldwide attempts to control malaria, it remains the most fatal and widespread protozoan infection of humans (Swash, 2002). Malaria in humans is caused by four species of Protozoan parasites belonging to Kingdom Protoctista, Family Plasmoiidae and Genus *Plasmodium*: *P. falciparum*, *P. vivax, P. ovale, and P. malariae*. It is transmitted by the bite of female anopheline mosquitoes and occurs throughout the tropics and subtropics (Goodman, 1999). Of the four species, *P. falciparum* accounts for most of the infection in sub-Saharan Africa and over one third of the infections in the rest of the world (Phillips, 1993). Malaria is a public health problem in more than 90 countries, inhabited by a total of some 2.4 billion people representing about 40% of the world's population (WHO, 2002). The disease occurs throughout the tropic and subtropics especially where the temperature exceeds the 60.8° F (16 ° C) isotherm. Best estimates currently describe the annual global burden of malaria as: 1.1 million deaths, 300-500 million cases, 44 million disability adjusted life every year (Sachs and Malaney, 2002). It has been estimated that economical burden is also extremely high, accounting for a reduction of 1.3% in the annual economic growth rate of malaria endemic countries, and the long-term impact in these countries is the reduction in Gross National Product (GNP) of more than a half (WHO, 2002).

Malaria has many clinical manifestations and its impact varies depending on the epidemiological setting (Goodman, 1999). Over 90% of the disease burden is in the Sub-Saharan Africa, and almost all deaths due to *P. falciparum* occur in Africa. Most of the

remaining burden is distributed between the Indian sub-continent, South-East Asia, and the South Americas. Second to *P. falciparum* in significance is *P. vivax*. It causes up to 80 million cases per year approximately 15% in Africa (Sachs and Malaney, 2002)

1.2 Impact of Malaria in Kenya

In Kenya, malaria is the leading course of mortality among all infectious diseases accounting for up to 30% of the deaths. More than 4 million cases are reported annually, resulting to a mortality rate of 5.1% in those admitted to health facilities (Republic of Kenya, 2002). About 8.5 million Kenyans are at risk of infection, mainly in the highlands while approximately 20 million people (more than half the entire population) are exposed to stable transmissions including 3.5 million children age below five years (Rapuoda *et al.*, 1997). The cumulative human suffering caused by malaria is immense with children and pregnant women being the most vulnerable (Republic of Kenya, 2003). Malaria account for 19% of all admissions to the health facilities and each year an estimated 26,000 children die from the direct consequences of its infection translating into 72 deaths each day (Republic of Kenya, 2003). Malaria endemic areas in Kenya include Western, Nyanza, Coast, and Eastern Rift Valley provinces. Regions that lie at altitudes above 1600m such as Nairobi, Mt. Kenya and the surrounding areas are considered malaria free zones (Republic of Kenya, 1992).

Unstable malaria also occurs in several areas in Kenya including Garrisa, Isiolo, Narok, Kajiado, Turkana and Wajir (Republic of Kenya, 2003). Areas with seasonal malaria include parts of Eastern Province (Machakos, Embu and Kitui) and Rift Valley (Marigat and Ngurumani). This eco-zone has been extended in the recent years by population

movement and small scale irrigation projects (Republic of Kenya, 2003). Since 1988, epidemics have been reported frequently in the highlands. The malaria outbreak of 1994 affected more than 12 districts of varying climatic conditions ranging from the highlands (Kisii, Nyamira and Kericho) to semi-arid lands (Turkana and Narok). Under certain circumstances, migrating populations such as refugees, settlers in new schemes may "import" the disease in areas considered free from malaria (Republic of Kenya, 2003).

1.3 Problem Statement

Malaria has remained the world-leading cause of deaths among the infectious diseases with special reference to Africa. In such malaria victims, it has not been understood the role played by sex differences in disease exposure, prevalence, severity as well as susceptibility to the parasite. It has been noted with concern that some individuals living in the same malaria holoendemic areas may remain uninfected while others develop servere disease and even succumb to it. It is therefore important to understand host related factors that predispose and those that confer resistance to malaria such as ABO/Rh-blood groups, sex and age for the proper management of the disease

1.4 Justification

Much attention has been paid to immunological research especially related to cytokines in the fight against malaria. Despite being the primary avenue for the parasite invasion, less studies has been directed to erythrocytes. ABO- blood types in the protection against *P. falciparum* malaria paradigm has remained an area of concern. Although the ABO-blood group phenotypes of human host have been reported to influence malaria infection, there have been few clinical observations in Kenya and other parts of the world. For

proper management of malaria, it is vital to understand the relationship between blood group and immunity to malaria in a malaria holoendemic area. A hospital -based comparative study is therefore necessary to investigate the relationship between blood group types and severity of the disease in *P. falciparum* infection. The current study focused on children aged 1-10 years since they are more vulnerable to malaria infection. Kisumu town was chosen because it is malaria holoendemic area in Kenya.

1.5 Research Questions

(i) What is the relationship between ABO-Blood group phenotypes and resistance to malaria infection in the study population?

(ii) What is the effect of age and sex on malaria susceptibility in the study population?

(iii) What is the relationship between T-helper cells and cytotoxic T-lymphocytes levels and *P. falciparum* parasitaemia levels in the study population?

(iv) What is the effect of malaria infection on the expression of differential leukocyte count in the study population?

1.6 Null Hypotheses

(i) There is no relationship between ABO-Blood group phenotypes and resistance to malaria infection in the study population.

(ii) There is no effect of age and sex on malaria susceptibility in the study population

(iii) No relationship exists between T-helper cells and cytotoxic T-lymphocytes levels and *P. falciparum* parasitaemia levels in the study population.

(iv) There is no effect of malaria infection on the expression of differential leukocyte count in the study population.

1.7 Objectives

1.7.1 General Objective

To study the effect of ABO/Rh blood group phenotypes on immunosurveillance to *Plasmodium falciparum* infection in children 1-10 years in Kisumu town.

1.7.2 Specific Objectives

(i) To determine the relationship between ABO/Rh-Blood group phenotypes and resistance to malaria infection in the study population.

(ii) To determine the effect of age and sex on malaria susceptibility in the study population

(ii) To determine the relationship between T-helper cells and cytotoxic T-lymphocytes levels and *P. falciparum* in the study population.

(iii) To determine the effect of malaria infection on the expression of differential leukocyte counts in the study population.

CHAPTER TWO: LITERATURE REVIEW

2.1 Epidemiology of Malaria in Kenya

Malaria affects 20 million Kenyans annually, the cumulative human suffering and economic loss caused by malaria is immense (Snow *et al.*, 1998). It estimated that 26,000 children under five years of age (72 per day) die from the direct consequences of malaria infection annually (Republic of Kenya, 2003), and pregnant women suffer severe anaemia and have a high likelihood of delivering infants with low birth weight (Menendez, 1999). All Kenyan households are affected by financial hardship caused by malaria. It is estimated that 170 million working hours are lost each year because of malarial illness, which intern affect the country's economy, leading to increased poverty (Republic of Kenya, 2003).

The distribution of malaria is not uniform because of geographical differences in altitude, rainfall and humidity (Kenya Demographic Health Survey, 2003). These factors influence the transmission pattern, as they determine vector densities and intensity of biting (Republic of Kenya, 2003). The country may be divided into four malaria zones (Kenya Demographic Health Survey, 2003): stable malaria transmission (Nyanza, Coastal and Western Provinces), Seasonal malaria zones (Central, Eastern and North Eastern Provinces), High land malaria (Rift valley Province and some parts of Nyanza especially Kisii) and Malaria free zone (Nairobi, and some parts of Central Province (Kenya Demographic Health Survey, 2003; Fig 2.1).

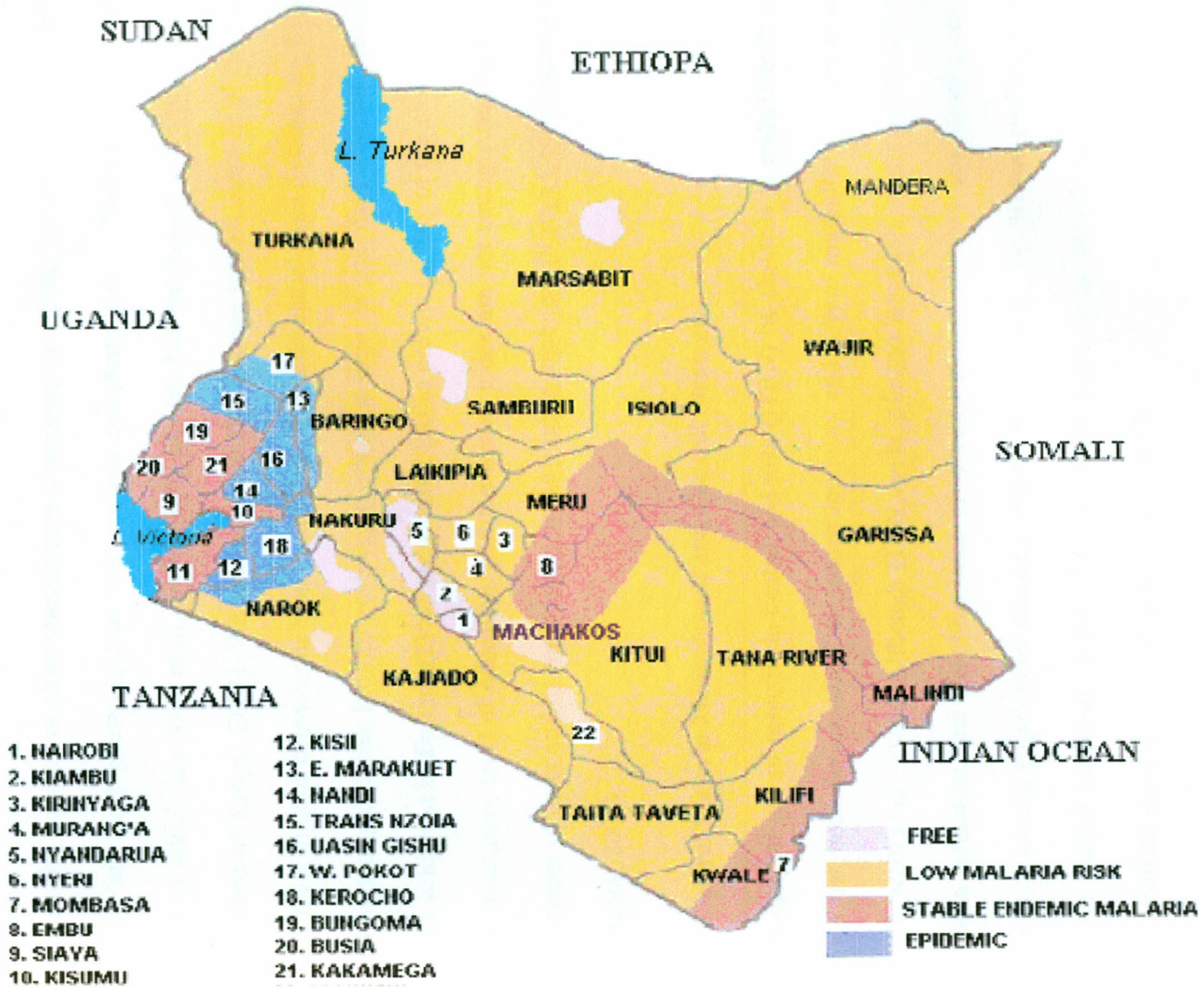

Fig. 2.1: The Endemicity of Malaria in Kenya (Republic of Kenya, 2003)

2.2 Malaria Cases among Kenyan Children

The prevalence of fever and/ or convulsions is higher among children age 6-23 months than among younger or older ones (Kenya Demographic Health Survey, 2003). Western Kenya has the highest children with malaria symptoms (60%), followed by Nyanza province which has 49% each, while North Eastern has the lowest prevalence (23%) (Republic of Kenya, 2003). Western province also has the highest number of children with fever and/ or convulsions who are treated with antimalarial drug followed by Coast and Eastern provinces. Prompt treatment (within the same or next day) with antimalarial drugs is highest in Eastern (21%) and Coastal (20%) provinces. Sex of the child, urban-rural resistance, level of education, and the wealth index are not strongly related to the prevalence of fever and/or convulsions or the prompt treatment (Kenya Central Bureau of Statistics, 2003).

2.3 General Malaria Management and Control Strategies

The optimal use of insecticide-treated bed nets (ITBN) by children in malaria endemic areas has been demonstrated to reduce mortality and severe morbidity (Alonso *et al.*, 1991; Neill *et al.*, 1996). Although ITBN use is safe and inexpensive strategy to prevent malaria infections, the scientific issue of whether or not effective control of parasite exposure among populations naturally expose to intense *P. falciparum* challenge will reduce natural immunity to malaria, need to be taken in to consideration when such interventions are implemented (Snow *et al.*, 1997).

The spraying of mosquito habitats with insecticides has been one of the traditional methods of killing adult mosquitoes. However, 1,1-bis (4-chlorophenyl-2,2,2)

trichloroethane (DDT) insecticides, which were widely used, previously demonstrated side effects such as reduction in milk and living tissues of animals including humans (Ogonda *et al.*, 2001). The organophosphates and carbamate insecticides that are currently in use are considerably more expensive (Curtis, 1994). Therefore majority of the population cannot afford them.

2.3.1 Malaria Control and Prevention Strategies in Kenya

The Kenya government in its commitment to the control of malaria has developed a strategy document outlining several intervention measures (Ministry of Health Kenya, 1999). The four intervention measures outlined in national malaria strategy (NMS) document are; management of malaria illness, vector control by the use of insecticide treated mosquito nets (ITNS) and other methods such as indoor house spraying, control of malaria in pregnancy and control of malaria epidemic (Ministry of Health Kenya, 1999).

Untreated nets window screening has long been considered useful protection methods against mosquitoes and other insects (Lindsay and Gibson, 1988). Over the past two decades, significant advances have been made in the privation of malaria using ITNS and curtains by the use of synthetic pyrethroids (Kenya Demographic Health survey, 2003).

2.4 Nomenclature of Malaria Parasite

Malaria is an infection caused by a parasite in the Kingdom Protocstista; Phylum Protozoa and Subphylum Sporozoa (Marsh, 1992). According to the classification by the society of protozoologists (Honigberg *et al.*, 1964), malaria parasite belongs to class

Telosporea and subclass Coccidia. More recently, (Cox, 1992) included malaria parasite in the phylum of Apicomplexa, Class Sporoasida and Subclass Coccidiasina which is subdivided into the orders Protococcida and Eucoccidia. The latter is subdivided into three suborders including Haemosporina. Haemosporina is classified into three families, which include Plasmoiidae. The genus *Plasmodium* is within this family (Pasvol, 1995).

The zoological family of the Plasmodiidae includes parasites, which undergo two distinct life cycles of multiplication by sexual (sporogony) in the vertebrate host and asexual (schizogony) in the insect vector. *Plasmodium* is a characteristic of this family. *Plasmodium* that infects humans can be further classified into four different species: *P. falciparum, P. vivax, P.ovale* and *P. malariae* (Cohen, 2004). There are other species of *Plasmodium* that infect other vertebrates such as *P. yoeeli, P.berghi* and *P.chabaudi* (Roitt, 2002). Different species have major influence on all aspect of the diseases, including epidemiology, pathogenesis, clinical features and management (Phillips, 1992).

2.5 The Life Cycle of Malaria Parasites

The female mosquito becomes infected after taking a blood meal containing gametocytes the sexual form of malaria parasite. The developmental cycle in the mosquito usually takes 7-20 days depending on temperature dominating. The infective sporozoites migrate to the insect salivary glands (Kumar and Clark, 2002). After the bite by mosquito, those parasites that are not destroyed by the immune system are rapidly taken up by the liver (Cohen, 2004). Inside the liver they multiply within the hepatocytes as merozoites; this is the pre-erythrocytic or exo-erythrocytic sporogeny. After a few days the infected hepatocytes rapture, releasing merozoites into blood where they are rapidly taken up by

the erythrocytes. In the case of *P.vivax* and *P.ovale*, a few parasites remain dominant in the liver as hyprozoites. These may reactivate at any time relapsing infection (Kumar and Clark, 2002). Inside the red blood cells (RBC) the parasites again multiply changing from merozoites to trophozoites to schizont and finally appearing at 8-24 hours as now merozoites. The erythrocyte raptures releasing the merozoites to infect other cells. Each cycle of this process, which is called erythrocytic schizogeny takes 48 hours in *P. falciparum, P. vivax* and *P.ovale* and about 72 hours in *P. malariae*. *P.* vivax and *P. ovale* mainly attack reticulocytes and young erythrocytes while *P. malaria* tends to attack older cells while *P. falciparum* will parasitize any stage of erythrocyte (Phillips and Warrell, 1986). Schizogony refers to a replicative process in which the parasite undergoes multiple rounds of nuclear division without cytoplasmic division followed by a budding, or segmentation, to form progeny. The progeny, called merozoites, are released into the circulatory system following rupture of the host hepatocyte (Kochar *et al.*, 2005; Fig. 2.2)

Generally the parasites lifecycle stages are highly synchronised, such that at any one time all the parasites are at the trophozoite stage or all are at the schizont stage. Erythrocytes infected with these stages adhere to endothelial cells and sequester in the microvasculature of vital organs, especially brain, heart and lungs. Sequestration in the brain is a contributing factor in cerebral malaria (Billker *et al.*, 1998; Billker *et al.*, 2004). The episodes of fever paroxysms often associated with malaria are due to the synchronous rupture of infected erythrocytes and release of merozoites and toxic breakdown of product of the malarial metabolism. In addition, on infection of new blood cells, instead of forming trophozoites the parasites may grow into the immature

gametocytes (Cohen, 2004; Fig 2.2). For these to develop further, they must be taken up in the blood meal of a mosquito, to initiate the stages within the intermediate host. Trophozoite enlargement is accompanied by an active metabolism including the ingestion of host cytoplasm and the proteolysis of hemoglobin into amino acids.

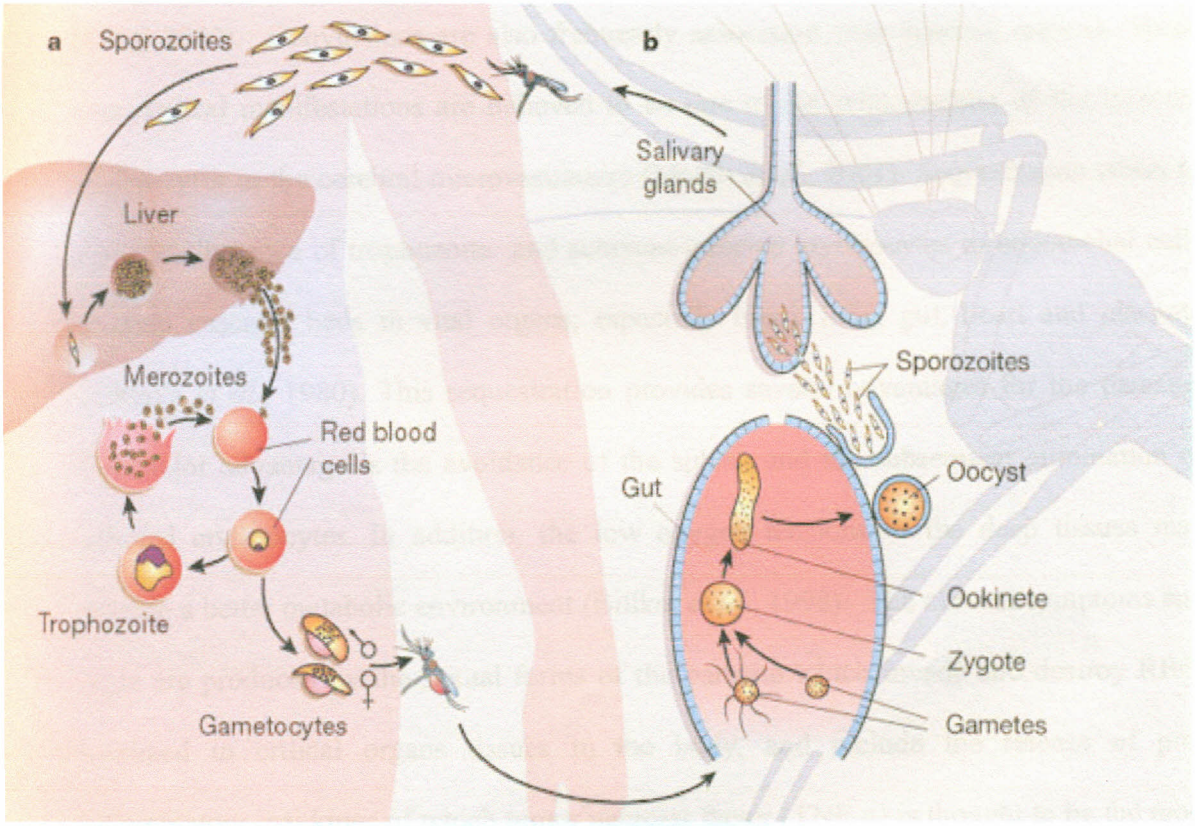

Fig. 2.2: *Plasmodium falciparum* Life Cycle (www.sanger.ac.uk)

characteristic feature of malaria (Kumar and Clark, 2002). There is an increased activity of the reticuloendothelial system, particularly in the liver and spleen and thus their enlargement, as evidenced by macrophages with ingested infected and normal erythrocytes and hemozoin (Billker *et al.*, 1998; Billker *et al.*, 2004). Except for *P.*

falciparum, the pathology associated with malaria tends to be rather benign. Several severe complications can be associated with *falciparum* malaria with cerebral malaria being the most notable and a frequent cause of death (Billker *et al.*, 2004).

Cerebral malaria is characterized by an impaired consciousness. The presenting symptoms are severe headache followed by drowsiness, confusion, and ultimately coma (Roitt, 2002). Convulsions are also frequently associated with cerebral malaria. These neurological manifestations are believed to be due to the sequestration of the infected erythrocytes in the cerebral microvasulature (Marsh *et al.*, 2001). Sequestration refers to the cytoadherence of trophozoite- and schizont-infected erythrocytes to endothelial cells of deep vascular beds in vital organs, especially brain, lung, gut, heart and placenta (Abdalla *et al.*, 1980). This sequestration provides several advantages for the parasite. The major advantage is the avoidance of the spleen and the subsequent elimination of infected erythrocytes. In addition, the low oxygen tensions in the deep tissues may provide a better metabolic environment (Billker *et al.*, 1998). The clinical symptoms and signs are produced by the sexual forms of the parasite which invade and destroy RBC, localized in critical organs tissues in the body, and include the release of pro-inflammatory cytokines of which tumor necrosis factor (TNF-α) is thought to be the most important (Roitt, 2002).

The sporozoites injected by the bite of the infected mosquito, the exo-erythrocytic parasite, which subsequently develop in the hepatocytes and the sexual forms of the parasite (macro-and microgametocytes), which arise from sexual form do not cause clinical disease (Abdalla *et al.*, 1980). The pathology of malaria is related to anaemia,

cytokine released and in the case of *P. falciparum*, widespread organ damage due to impaired microcirculation (Kumar and Clark, 2002).

In *P. falciparum* malaria, the RBC containing schizonts adhere to the lining of capillaries in the brain, kidney, gut, liver and other critical organs (Warhurt, 2001). This exacerbates the development of severe disease. Immune complexes have been reported in malaria-infected individuals who are associated with antibodies reacting with many self tissues (Roitt, 2002). These complexes may be deposited in the blood vessels leading to vasculitis, glomerulonephritis and nephritic syndrome.

2.6 Clinical Manifestations

The pathology and clinical manifestations associated with malaria are almost exclusively due to the asexual erythrocytic stage parasites (Roitt, 2002). Tissue schizonts and gametocytes cause little, if any, pathology. *Plasmodium* infection causes an acute febrile illness which is most notable for its periodic fever paroxysms occurring at either 48 or 72 hour intervals (Abdalla *et al.*, 1980). The severity of the attack depends on the *Plasmodium* species as well as other circumstances such as the state of immunity and the general health and nutritional status of the infected individual. The disease has a tendency to relapse or recrudesce over months or even year (Billker *et al.*, 1998).

The typical prevalent and incubation periods following sporozoite inoculation vary slightly according to species. The prevalent period is defined as the time between sporozoite inoculation and the appearance of parasites in the blood and represents the duration of the liver stage (Marsh *et al.*, 2001). Incubation periods tend to be a little

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longer and are defined as the time between sporozoite inoculation and the onset of symptoms. Sometimes the incubation periods can be prolonged for several months in *P. vivax, P. ovale,* and *P. malariae* (Abdalla *et al.,* 1980; Billker *et al.,* 1998). All four species can exhibit non-specific prodromal symptoms a few days before the first febril attack. These prodromal symptoms are generally described as 'flu-like' and include: headache, slight fever, muscle pain, anorexia, nausea and lassitude (Warhurt, 2001). The symptoms tend to correlate with increasing numbers of parasites.

These prodromal symptoms will be followed by febrile attacks also known as the malarial paroxysms (Swash, 2002). These paroxysms will exhibit periodicities of 48 hours for *P. vivax, P. ovale*, and *P. falciparum*, and a 72-hour periodicity for *P. malariae.* Initially the periodicity of these paroxyms may be irregular as the broods of merozoites from different exoerythrocytic schizonts synchronize (Warhurt, 2001). This is especially true in *P. falciparum*, which may not exhibit distinct paroxysms, but exhibit a continuous fever, daily attacks or irregular attacks (36-48 hour periodicity). Patients may also exhibit splenomegaly, hepatomegaly (slight jaundice), and hemolytic anemia during the period in which the malaria paroxysms occur.

The malarial paroxysm will usually last 4-8 hours and begins with a sudden onset of chills in which the patient experiences an intense feeling of cold despite having an elevated temperature (Warhurt, 2001). This is often referred to as the cold stage and is characterized by a vigorous shivering. Immediately following this cold stage is the hot stage (Swash, 2002). The patient feels an intense heat accompanined by severe headache. Fatigue, dizziness, anorexia, myalgia, and nausea will often be associated with the hot

stage. Next a period of profuse sweating will ensue and the fever will start to decline (Kumar and Clark, 2002; Swash, 2002). The patient is exhausted and weak and will usually fall asleep. Upon awakening the patient usually feels well, other than being tired, and does not exhibit symptoms until the onset of the next paroxysm.

2.7 Malaria Chemotherapy

Several anti-malarial drugs are available. Many factors are involved in deciding the best treatment for malaria. (Wellem, 2001). These factors include the parasite species, the severity of disease, the patient's age and immune status, the parasite's susceptibility to the drugs (drug resistance), and the cost and availability of drugs (Warhust, 2001). Therefore, the exact recommendations will often vary according to geographical region. In addition, the various drugs act differentially on the different life cycle stages.

Fast-acting blood schizontocides, which act upon the parasite within erythrocytes, are used to treat acute infections and to quickly relieve the clinical symptoms (Warhust, 2001). Chloroquine is generally the recommended treatment for patients with *P. vivax*, *P. ovale*, *P. malariae*, and uncomplicated chloroquine-sensitive *P. falciparum* infections. Chloroquine is safe and usually well tolerated (White, 1996; Wongsrichanalia, 2002). Side effects may include pruritus (itching), nausea, or irritation. Patients infected with either *P. vivax* or *P. ovale*, and that are not at a high risk for reinfection, should also be treated with primaquine (a tissue schizontocide). Primaquine is effective against the liver stage of the parasite, including hypnozoites and will prevent future relapses. The combination of chloroquine and primaquine is often called 'radical cure' (Warhust, 2001; Wongsrichanalia, 2002).

Severe, or complicated, falciparum malaria is a serious disease with a high mortality rate and requires urgent treatment (Phillips, 2001). Typically treatment requires parenteral drug administration (injections) since patients are often comatose or cannot take the drugs orally. Parenteral formulations are available for chloroquine, quinine, quinidine and artemisinin derivatives (Swash, 2002). Artemisinin suppositories have also been developed. Patients need to be continuously monitored for hematocrit, parasitemia, hydration levels, and signs of drug toxicity and other complications. A switch to oral administration should be made as soon as the patient is ableto ingest the drug (Wellem, 2001).

The efficacy of chloroquine is greatly diminished by the wide spread chloroquine resistance of *P. falciparum* and the emergence of chloroquine-resistant *P. vivax* (Warhust, 2001; Wellem, 2001). If chloroquine therapy is not effective, or if in an area with chloroquine-resistant malaria, common alternative treatments include: mefloquine, quinine in combination with doxycycline, or Fansidar. (Wongsrichanalia, 2002). Derivatives of artemisinin (artesunate and artemether) are increasingly used in Asia and Africa (Wellem, 2001).These drugs were originally derived from the wormwood plant (*Artemesia annua*) and have been used for a long time in China as a herbal tea called quinhaosu to treat febrile illnesses (Warhust, 2001; Wellem, 2001). To prevent the high recrudescence rates associated with artemisinin derivatives and to slow the development of drug resistance it is recommended that treatment be combined with an unrelated anti-malarial.

2.8 Drug Resistance in Malaria Infection

Drug resistance, and in particular, chloroquine resistance is a major public health problem in the control of malaria. (White, 1996) Drug resistance is defined by a treatment failure and can be graded into different levels depending on the timing of the recrudescence following treatment (White, 1996). Traditionally these levels of drug resistance have been defined as sensitive (no recrudescence), RI (delayed recrudescence), RII (early recrudescence), and RIII (minimal or no anti-parasite effect) (Warhust, 2001).

Drug resistance develops when parasites with decreased sensitivities to antimalarial drugs are selected under drug pressure (Wellems and Plowe, 2001) Decreased drug sensitivity can be conferred by several mechanisms and reflects genetic mutation (s) or polymorphisms in the parasite population. The drug-resistance parasites will have a selective advantage over the drug-sensitive parasites in the presence of drug and will be preferentially transmitted (Wongsrichanalai, 2002). Major factors in the development of drug resistance are the use of subtherapeutic doses of drugs or not completing the treatment regimen (White, 1996). The lower drug levels will eliminate the most susceptible parasites, but those which can tolerate the drug will recover and reproduce (Wellems and Plowe, 2001; Warhust, 2001). Over time this will lead to a continued selection for parasites which can tolerate even higher doses of the drug. It is crucial to maintain an adequate concentration of the drug for a sufficient time to completely eliminate the parasites from any given individual.

2.9 Malaria Vaccine

Several studies have supported the feasibility of developing effective malaria vaccines. Repeated long-term exposure to malaria infection to malaria infection induces partial protection immunity characterized by significant reduction in disease severity and death (McGregor, 1987; Trape *et al.*, 1994; WHO, 2005). Sterile immune response has been achieved in human and animal models through the use of irradiated sporozoites (Nussenzweig, 1989; Good and Doolan, 1999). Furthermore, massive immunization with IgG antibodies confers immunity to naïve individuals (Cohen *et al.*, 1961; Fandeur *et al.*, 1984; Bouharoun-Tayoun *et al.*, 1990; WHO, 2006). Vaccines have benn demonstrated to represent the most feasible cost-effective strategy for reducing the burden of the infectious disease (Targett, 1995).

2.9.1 Vaccine Development

The complex life cycle of the malaria parasite provide a number of potential targets for vaccines (Targett, 1995 and WHO, 2006). Extensive experimental evidence has shown that sporozoite antigen may play a role in introducing immune protection against and various pre-erythrocytic stage *P. falciparum* antigens. This antigen has been targeted in human vaccine trials (Good and Doolan, 1999).

2.9.2 Exo-erythrocytic Stage Vaccine

One of the pre-erythrocytic stage vaccine candidates a recombinant vaccine which consists of repeat and non-repeat against the circumsporozoite protein (CSP) fused to hepatitis B surface antigen, formulated in adjuvant (Stoute *et al.*, 1998; Good and Doolan, 1999; WHO, 2006). Protection against challenge in six out of seven volunteers

immunized in the initial clinical trial using a formulation of an oil in water emulsion containing adjuvants monophosphoryl lipid A and QS21 (Stoute *et al.*, 1998 and WHO, 2005).

2.9.3 Erythrocytic Stage Vaccine

The merozoite surface proiten-1 (MSP-1) and apical membrane protein antigen-1 (AMA-1) are two antigens of the sexual erythrocytic stages of *P. falciparum*. The MSP-1, a precursor to the major vaccine candidate against asexual stages of the parasite (Good and Doolan, 1999). Several studies suggest a protective role for anti-MPS-1 antibodoes in anti-malarial immunity (Riley *et al.*, 1992). Anti-MPS-1 antibodies responses and protection from malaria has been found in human (Tolle *et al.*, 1993; Branch *et al.*, 1998; Good and doolan, 1999; WHO, 2005).

2.9.4 Transmission Blocking Vaccines

Transmission blocking vaccines are being developed to inhibit developmental stage of parasites that are found in the mid-gut of mosquitoes (Kalson, 1993). Although such vaccines provide no protection for an immunized individual, they reduce or even eliminate parasite infectivity in the mosquito vector and therefore limit transmission of malaria in a community (Allano and Cater, 1990). Several proteins expressed in the asexual stage of *P. falciparum* malaria parasite have been identified as candidate antigens for the development of malaria transmission blocking vaccines (Quakyi *et al.*, 1987; Kalsow, 1993; Kalsow *et al.*, 1994; Kochen *et al.*, 1994). The Pfs 230, Pfs 45/48 and Pfs 27, classified as early sexual stage antigens are synthesized predominantly in the gametocyte (vertebrate host) and expressed briefly after gametogenesis and fertilization,

have limited immunogenicity (Vermeulen *et al.*, 1986; Lobo *et al.*, 1994 WHO, 2005). Despite the advancement in the identification and characterization of potential vaccine candidate antigens, there is a need to fully understand the immunological role of ABO/Rh blood group phenotypes and protection against malaria

2.10 Cytoadherence of *Plasmodium falciparum*

Cytoadherence is the process by which mature cells of malaria parasite specifically bind to endothelial cells in post capillary venules, which appears to play a pivotal role in the pathogenesis of the *falciparum* malaria, possibly by localizing mature forms of the parasite in critical organs such as the brain (Cohen, 2004). Despite these effects many studies have failed to find an association between cytoadherence and disease severity (Marsh *et al.*, 2001). During parasite maturation a number of regular, symmetrically arranged 'Knobs' appear on the surface of the infected cell. The 'Knob' is thought to be the site to which the parasite attaches to the endothelial cell (Cohen, 2004). A number of high-molecular weight proteins protrude from these 'knobs' of which the best known is the *P. falciparum* erythrocyte membrane protein (PfEMP-1) coded for by the variant (var) genes. A single parasite may be capable of expressing up to 50 var of PfEMP-1 (Cohen, 2004). This antigenic variation may also account for different disease outcomes. For example, intercellular adhesion molecule-1 (ICAM-1) is usually implicated in cerebral pathology (Marsh *et al.*, 2001).

The sequestration hypothesis and cytokine theory for the pathophysiology of cerebral malaria are not mutually exclusive, and both phenomenon are likely to be involved. For example, parasite exo-antigens, which are released at erythrocyte rupture, are known to

stimulate macrophages to secrete tumor necrosis factor alpha (TNF-α) (Marsh *et al.*, 2001).TNF-α is known to upregulate the expression of adhesion molecules such as ICAM-1 on the surface of brain endothelial cells. This would lead to increase binding of infected erythrocytes and amplify the effects through vascular blockage, soluble mediators, metabolic effects, or a combination (Pasloske and Howard, 1994; Turnor, 2000; Fig 2.3).

Intern PfEMP-1 may bind to a number of protein receptors on the surface of the endothelial cell, which include among others; adhesion molecule CD36, binding molecule thrombospondin, two members of the immunoglobulin superfamily-intercellular adhesion molecules-1 (ICAM-1) and vascular cell adhesion molecule (VCAM), E-selectin and recently described glycosaminoglycan, chondroitin sulfate A (Turnor, 2000).

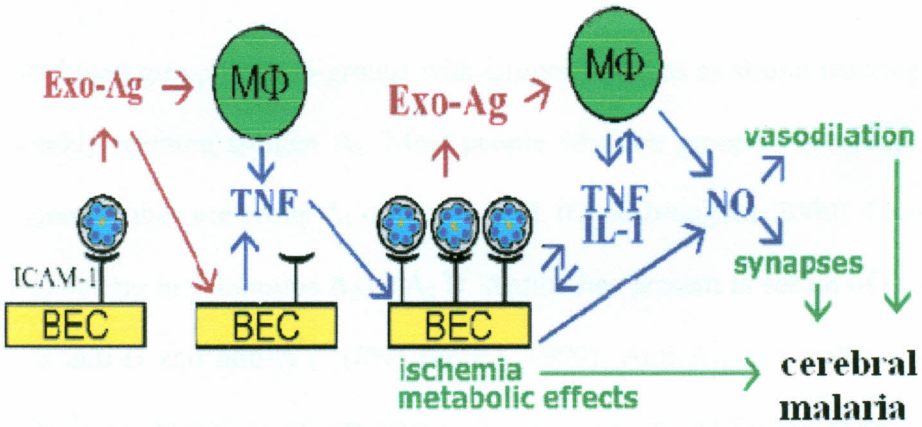


Fig. 2.3: A schematic model depicting some possible mediators of cerebral malaria

Adapted from Pasloske and Howard (1994)

BEC = cytoadherence of infected erythrocytes to brain endothelial cells, Exo-Ag = exorythrocytic antigens, MΦ = macrophages, Tumor necrosis factor-α = TNF-α), NO = Nitric oxide, IL-1 = Interleukin-1 and Intercelluar adhesion molecule-1 (ICAM-1).

2.11 ABO Blood Group System

In the ABO blood groups, naturally occurring IgM anti-A and anti-B antibodies are present in the serum in the absence of the corresponding antigen (Hill, 2000). Although described as naturally occurring (allo-) antibodies, anti-A and anti-B are not detectable in the blood of newborn infants (WHO, 1990). The antibodies become detectable at about 3 months of age following exposure to A and B like substances in the environment e.g. bacteria and some foods (Cheesbroughht, 2000; Hill, 2000). As a person gets older the concentration of naturally occurring anti-A and anti-B in the antibodies may be difficult to detect in the serum of some elderly patients (Cheesbroughht, 2000).

ABO blood group has sub-groups with antigen A exists as strong reacting antigen A_1 and a weakly reacting antigen A_2. Most people who are group A or group AB possess A_1 antigen i.e. they are group A_1 or group A_1 B. (Cheesbroughht, 2000). Fewer people (up to 20%) belong to subgroups A_2 or A_2 B. Antibodies present in serum of those with antigen A_1 is anti-B and anti-A^*_1 (2%) (WHO, 1990). Anti-A^*_1 is usually a weakly reacting antibody and therefore not important when selecting blood for transfusion.

2.11.1 Rhesus Blood Group System

The Rhesus (Rh) blood group system is next in importance to ABO blood group system in blood transfusion practice but it's not of equal significance in every country because of the frequency of the most important of the Rhesus antigen; D antigen, varies in different population (WHO, 1999; Cheesboroughht, 2000;Tab. 2.1).

Tab. 2.1 Frequencies of Rhesus Blood Group System

Population	Rhesus D Positive (%)
Asian	90-95
Africa	94-95
Nepalese	99-100
South American	91-97
Causian	About 85

The Rhesus blood group consists of six genes: Cc, Dd, and Ee. A single chromosome can carry C but not c, D but not d and E but not e (McFarland *et al.*, 1997). A person inherits from each parent a set of three closely positioned Rhesus genes e.g. CDE/cde. At least 36 Rhesus genotypes are possible based on the combination of genes that can be inherited; $cDe^{R^1}, cDE^{R^2}, cDe^{R^O}, CDE^{R^{2.}}, Cde^{r^1}, cdE^{r''}, CdE^{ry}, cde^{r}$. (Cheesborought, 2000). These genes are only expressed on erythrocytes but not body fluids. Medically, antigen D is the most important Rhesus antigen because it's the most immunogenic, capable of producing immune (IgG) anti-D antibody that can cause haemolytic reactions (McFarland *et al.*, 1997).

2.11.2 ABO/Rh-Blood Group Types and Protection against Malaria

The ABO-blood group phenotypes have been exemplified in the influence of malarial transmission and parasitaemia levels. Blood group O has been shown to protect against cerebral malaria as compared to A and B (Hill, 1992). The O-phenotype has been proposed to protect against cerebral malaria via impaired rosette formation (Udomsangpetch *et al.*, 1993). It was established that the impairment of rosetting hampers parasite proliferation and enhances parasite immunosurveillance through non-specific immune responses such as phagocytosis (Hill, 1992). Therefore sequestration is prevented and the victim is protected against pathogenesis of cerebral malaria (Pathirana *et al.*, 2005). *Plasmodium falciparum* utilizes molecules on the surface of the uninfected RBC for rossetting, and a dependency on ABO antigens have been previously shown (Telen, 1995). Mounting evidence suggests that the *P. falciparum* membrane protein 1 (PfEMP1), has been identified as a rossetting ligand (Chen *et al.*, 2000; Rowe *et al.*, 1995). Various host receptors on the surface of uninfected RBC have been proposed, including ABO blood group antigens (Carlson *et al.*, 1992). CD_{36} (Handunnetti *et al.*, 1992). CD_{35} (Rowe *et al.*, 1995). And heparan sulfate (HS) or HS-like molecules (Baragan *et al.*, 1998; Chen *et al.*, 1998). Nevertheless, little has been shown about the relative contribution of different receptors in rossetting and their prevalence in nature.

In *P. falciparum* parasite, a preference for specific blood group phenotype has been shown in both laboratory strains and wild isolates (Thaitong *et al.*, 1984). In clinical isolates, preference has shown less clear-cut, there has been simultaneous preference for blood group A and B. *Plasmodium falciparum* has shown minimal preference to blood

group O. Significantly, the sickle cell trait has been associated with mild malaria and blood group A with severe malaria (Lell *et al.*, 1999). Despite strong evidence on the role of ABO-blood group phenotypes in malaria protection in some countries (Sri Lanka and Gabon), less study has been directed on the effect of ABO-blood groups on the immune status during *P. falciparum* infection in Kenya particularly malaria holoendemic areas.

2.11.3 Rosetting

During rosetting process RBC containing more mature stages of parasite binds uninfected RBC to their surface. The mechanism by which rosetting leads to disease remain obscure and dogmatic, but may involve microcirculatory obstruction (Carlson *et al.*, 1990). Rosetting *falciparum* parasite have been associated with severe disease, but both *P.vivax* and *P.ovale* are capable of rosetting without causing severe disease (Rowe *et al.*, 1995) The specific molecule involved in rosetting on the infected and uninfected RBC is yet to be described, although PfEMP-1 and small molecular weight 20-40KDa rosetting protein on the parasitized cell and CD36 molecule and the ABO blood molecules on the uninfected cells have been implicated (Cohen, 2004). Most recently the complement receptor-1 (CR1), which is present at low levels (250 copies per cell) on uninfected cells, has been exemplified as binding to PfEMP-1 and playing a role in rosetting (Rowe *et al.*, 1997). Genes for two lethal diseases, thalassemia and sickle cell anemia, are favored by evolution because, in their heterozygous form, they protect against cerebral malaria (Carlson *et al.*, 1990; Fig. 2.4).

In rosetting *P. falciparum* parasites, specific blood group preferences have been seen in all laboratory strains and so far in all wild isolates tested (Udomsangpetch *et al.*, 1993),

In clinical isolates the preference has sometimes less clear-cut, for example, simultaneous preference for blood group A and B has seen. This may be due to clonal diversity in isolates (Thaitong *el al.*, 1984), although simultaneous preference for A and B in the same strain cannot be excluded. Importantly, in no case has preference for blood group O observed (Al-Yaman *et al.*, 1995).

Experimental findings support the interpretation that the receptor site is contained within the distal sugars of the ABO antigens and coexists with another receptor(s), for instance, the observation that the sensitivity of the cell-cell binding to heparin sulfa or heparin drastically decreases when parasites are cultivated in their preferred blood group (A or B) compared to when they are cultivated in blood group O (Fischer *et al.*, 1998; Lell *et al.*, 1999). Soluble A-trisaccharide and B-trisaccharide strongly inhibited formation of rosettes when the parasites are grown in the preferred blood group, but the rosettes are never abolished.

There are solid field data showing the influence of ABO blood group type on rosetting (Al-Yaman *et al.*, 1995; Udomsangpetch *et al.*, 1993), and RBC polymorphisms also affect rosette formation (Carlson *et al.*, 1994; Rowe *et al.*, 1997). Interestingly, blood group A has been reported to be a risk factor for the development of severe malaria (Fischer *et al.*, 1998; Lell *et al.*,1999) and blood group O seems to confer a relative protection against cerebral malaria compared to blood groups A and B (Hill *et al.*, 1992). Why the blood group O gene, although common in Africa, has not attained a high fixation rate may be explained by diversity in the rosetting receptor preferences and/or the simultaneous importance of other ABO blood group-independent mechanisms of

protection against severe malaria, for instance, complement receptor 1 polymorphisms submitted and CD36 (Handunnetti *et al.*, 1992; Fig. 2.4).

In relation to the pathology of severe malaria, it is tempting to speculate whether the present findings could relate to excessive sequestration due to enhanced rosetting. Could the blood group of the host in combination with a parasitic preference for that same blood group (A, B, or A and B) constitute an enhanced risk factor? (Carlson *et al.*, 1990).

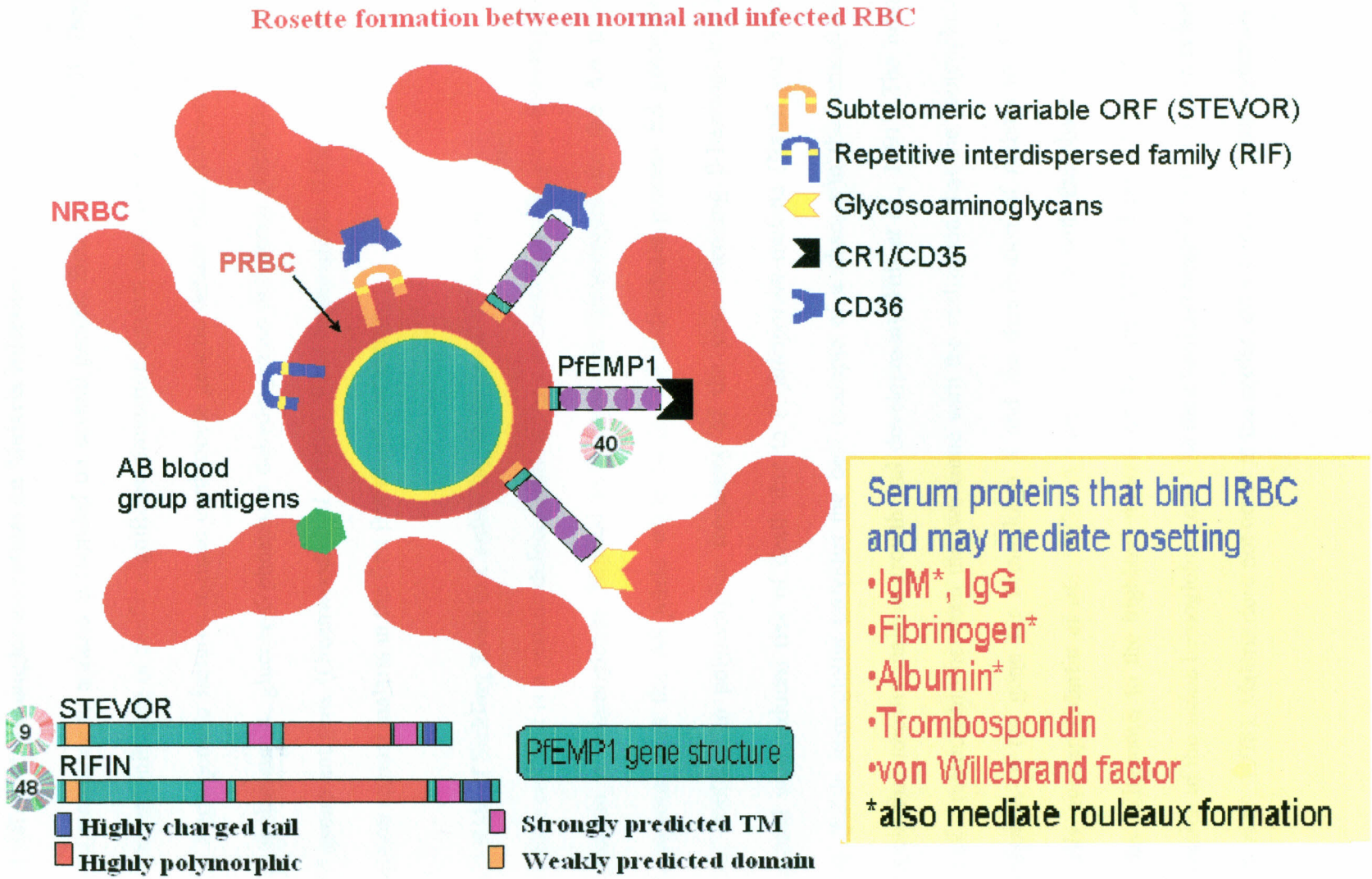


Fig. 2.4 Rosetting in normal erythrocytes and parasitized cells (www.pubmed.gov.)

2.11.4 Effect of Haemoglobinopathies on Malaria Infections

Natural resistance to malaria is founded on several genetic factors (Were, 2000). They may prevent infection or confer significant protection against malaria (Greenwood et al., 1991). Such genetic features include erythrocyte genetic defects like sickle-cell trait, α and β thelassaemia, glucose-6-phosphate dehydrogenase deficiency (G6PD) and Duffy blood group antigens (Greenwood *et al.*, 1991). Fetal haemoglobin (HbF) also accords protection against malaria in the first few months of infancy.

2.11.5 Role of Sickling Trait in Conferring Resistance in Malaria

The sickle cell trait is a genetic defect resulting from a mutation on the gene encoding for the β-chain of haemoglobin. Normal individuals are homozygous for the AA gene, heterozygous have the AS allele while sickle cell individuals possess SS genotypes (Were, 2000). The polymorphic frequency of the gene encoding β-heamoglobin is associated with reduced risk of death due to *P. falciparum* malaria (Nagel and Roth, 1989). Sickle hemoglobin provides the best example of a change in the hemoglobin molecule that impairs malaria growth and development (Hill *et al.*, 1991). The initial hints of a relationship between the two came with the realization that the geographical distribution of the gene for hemoglobin S and the distribution of malaria in Africa virtually overlap (Martin *et al.*, 1989). A further hint came with the observation that peoples indigenous to the highland regions of the continent did not display the high expression of the sickle hemoglobin gene like their lowland neighbors in the malaria belts (Roth *et al.*, 1978). Malaria does not occur in the cooler, drier climates of the highlands in

the tropical and subtropical regions of the world. Neither does the gene for sickle hemoglobin (Anastasi, 1984).

Sickle trait provides a survival advantage over people with normal hemoglobin in regions where malaria is endemic. Sickle cell trait provides neither absolute protection nor invulnerability to the disease (Luzzatto *et al.*, 1970). Rather, people (and particularly children) infected with *P. falciparum* are more likely to survive the acute illness if they have sickle cell trait (Roth *et al.*, 1978). When these people with sickle cell trait procreate, both the gene for normal hemoglobin and that for sickle hemoglobin are transmitted into the next generation.

The genetic selective scenario in which a heterozygote for two alleles of a gene has an advantage over either of the homozyous states is called "balanced polymorphism". A key concept to keep in mind is that the selection is for sickle cell trait (Luzzatto *et al.*, 1970). A common misstatement is that malaria selects for sickle cell disease. This is not true. A person with sickle cell disease is at an extreme survival disadvantage because of the ravages of the disease process (Anastasi, 1984). This means that a negative selection exists for sickle cell disease. Sickle cell trait is the genetic condition selected for in regions of endemic malaria. Sickle cell disease is a necessary consequence of the existence of the trait condition because of the genetics of reproduction (Luzzatto *et al.*, 1970).

The precise mechanism by which sickle cell trait imparts resistance to malaria is unknown. A number of factors likely are involved and contribute in varying degrees to

the defense against malaria (Martin *et al.*, 1989). Red cells from people with sickle trait do not sickle to any significant degree at normal venous oxygen tension. Very low oxygen tensions will cause the cells to sickle, however. For example, extreme exercise at high altitude increases the number of sickled erythrocytes in venous blood samples from people with sickle cell trait (Martin *et al.*, 1989). Sickle trait red cells infected with the *P. falciparum* parasite deform, presumably because the parasite reduces the oxygen tension within the erythrocytes to very low levels as it carries out its metabolism. Deformation of sickle trait erythrocytes would mark these cells as abnormal and target them for destruction by phagocytes (Luzzatto *et al.*, 1970).

Experiments carried out *in vitro* with sickle trait red cells showed that under low oxygen tension, cells infected with *P. falciparum* parasites sickle much more readily than do uninfected cells (Roth *et al.*, 1978). Since sickle cells are removed from the circulation and destroyed in the reticuloendothelial system, selective sickling of infected sickle trait red cells would reduce the parasite burden in people with sickle trait. These people would be more likely to survive acute malarial infections.

Other investigations suggest that malaria parasites could be damaged or killed directly in sickle trait red cells. *Plasmodium falciparum* parasites cultured in sickle trait red cells died when the cells were incubated at low oxygen tension (Friedman, 1978). In contrast, parasite health and growth were unimpeded in cells maintained at normal atmospheric oxygen tensions. The sickling process that occurs at low oxygen tensions was presumed to harm the parasite in some fashion. Ultrastructural studies showed extensive vacuole formation in *P. falciparum* parasites inhabiting sickle trait red cells that were incubated at

low oxygen tension, suggesting metabolic damage to the parasites (Friedman, 1979). Prolonged states of hypoxia are not physiological, raising questions about degree to which these data can be extrapolated to human beings. However, they do suggest mechanisms by which sickle hemoglobin at the concentrations seen with sickle cell trait red cells could impair parasite proliferation.

Other investigations suggest that oxygen radical formation in sickle trait erythrocytes retards growth and even kills the *P. falciparum* parasite (Anastasi, 1984). Sickle trait red cells produce higher levels of the superoxide anion (O_2-) and hydrogen peroxide (H_2O_2) than do normal erythrocytes. Each compound is toxic to a number of pathogens, including malarial parasites. Homozygous hemoglobin S red cells produce membrane associated hemin secondary to repeated formation of sickle hemoglobin polymers. This membrane-associated hemin can oxidize membrane lipids and proteins (Rank *et al.*, 1985). Sickle trait red cells normally produce little in the way of such products. If the infected sickle trait red cells form sickle polymer due to the low oxygen tension produced by parasite metabolism, the cells might generate enough hemin to damage the parasites (Orjih *et al.*, 1985).

2.11.6 Thelassaemia Trait

Alph-thelassaemia is associated with protection against malaria although the mechanisms involved are unclear (Hill, 1989). Studies have indicated that erythrocytes from individuals with α and β-thelassaemia as well as sickle cell trait or HbE, form smaller and weaker rosettes than normal (HbAA) red blood cells (Carlson, 1992). This suggests that, impaired rosette formation may be one of the genetic features that constitute the

innate resistance to severe *P. falciparum* malaria. Recent studies done to investigate the role of oxidant stress in thelassaemic trait erythrocytes (α andβ) have shown that antioxidants improves parasite growth while pro-oxidants have a parasiticidal effect effect (Senok *et al.*, 1997). This suggests that oxidant stress play a role in conferring protection against malaria in thelassaemic erythrocyte.

The inbalance in globin chain production characteristic of thalassemia produces membrane oxidation by hemichromes and other molecules that generate reactive oxygen species (Grinberg *et al.*, 1995; Sorensen *et al.*, 1990). Reactive oxygen species also injure and kill malaria parasites (Clark *et al.*, 1989).

In vitro malaria toxicity of thalassemic red cells is most easily seen in cells containing hemoglobin H (ß-globin tetramers) (Ifediba *et al.*, 1985; Yuthavong *et al.*, 1988). Hemoglobin H occurs most often in people with three-gene deletion alpha-thalassemia (Zhu *et al.*, 1993). The compound heterozygous condition of two-gene deletion alpha thalassemia and hemoglobin Constant Spring also produces erythrocytes that contain hemoglobin H (Derry *et al.*, 1988). Two gene deletion alpha thalassemia also protects the host from malaria, however. The process is difficult to demonstrate with *in vitro* cultures of malaria parasites. Alpha thalassemia may protect against malaria in part by altering the immunue response to parasitized red cells (Luzzi *et al.*, 1991) In any event, epidemiological studies show clear evidence of protection provided by two-gene deletion alpha thalassemia (Flint *et al.*, 1986; Modiano *et al.*, 1991).

One of the key reasons for the high fatality rate in *P. falciparum* malaria is the occurence of so-called cerebral malaria (Carlson *et al.*, 1994). Patients become confused,

disoriented and often lapse into a terminal coma. Clumps of malaria-infested red cells adhere to the endothelium and occlude the microcirculation of the brain with deadly consequences (Luzzi *et al.*, 1991). The *P. falciparum* parasite alters the characteristics of the red cell membrane, making them more "sticky". Clusters of parasitized red cells exceed the size of the capillary circulation blocking blood flow and producing cerebral hypoxia. Thalassemic erythrocytes adhere to parasitized red cells much less readily than do their normal counterparts (Carlson *et al.*, 1994). This alteration would lessen the chance of developing cerebral malaria. The rise to high frequency of alleles that produce red cells deficient in glucose-6-phosphate dehydrogenase activity is one of the most dramatic examples of the selective pressure of malaria on humankind (Ruwende *et al.*, 1995; Tishkoff *et al.*, 2001).

2.11.7 Duffy Blood Group Antigens

Studies have shown that individual deficient of Duffy blood group antigens are protected against *P. vivax* infection (Bruce-chwatt, 1985). This antigen serves as an anchor for invasion of the erythrocyte with the parasite and its absence is associated with protection against *P. vivax* infection especially among the West African population (Miller *et al.*, 1976). At the red cell membrane, the Duffy antigen is the molecule used by the parasite *P. vivax* to enter the red cell. The high association of Duffy antigen null red cells in some groups of people with sickle cell trait suggested that the Duffy antigen might provide some protection against malaria (Gelpi and King, 1976). Later investigations showed the Duffy antigen to be the receptor by which the merozoites of *P. vivax* enter red cells. People who lack the Duffy antigen are resistant to *P. vivax* (Hamblin and Di Rienzo,

2000). The Duffy null phenotype is most common in people whose ancestors derive from regions in Africa where vivax malaria is endemic.

2.11.8 Glucose-6-Phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PD4) deficiency is associated with protection against severe effect of *P. falciparum* infection. It confers selective advantage in the G6PD enzyme deficient individuals who tend to have lower parasitaemia than the normal individuals infected with *P. falciparum* (Allison and Clycle, 1961; Gilles *et al.*, 1967). The rise to high frequency of alleles that produce red cells deficient in glucose-6-phosphate dehydrogenase activity is one of the most dramatic examples of the selective pressure of malaria on humankind (Ruwende *et al.*, 1995; Tishkoff *et al.*, 2001). Reactive oxygen species are formed continually as erythrocytes take up oxygen from the lungs and release it to the preriperal tissues. As noted above, malaria parasites are easily damaged by these reactive oxygen species (Friedman, 1979). Glucose-6-phosphate dehydrogenase prevents oxidation of the heme group. In its absence, hemichromes and other species that generate reactive oxygen species accumulate in erythrocytes (Janney *et al.*, 1986). *Plasmodium falciparum* grow poorly in erythrocytes that are deficient in G-6-PD (Roth *et al.*, 1983). Malaria continues to battle back in this struggle, however. The advent of *P. falciparum* parasites that produce their own G-6-PD provides ample evidence of the continuing moves and counter-moves in the battle between man and malaria (Usanga *et al*, 1985).

2.12 Role of CD_3, CD_4 and CD_8 in Conferring Immunity in Malaria

Intracellar parasite like *Plasmodium* typically elicit a number of immunological responses both antibody and cell-mediated. The Adaptive immunity to malaria includes antibodies that block the invasion of sporozoites into liver cells. Interferon- gamma (INFγ) and CD_8^+ T cells inhibit parasite development in the hepatocytes (Stevenson *et al.*, 2004). The infection is controlled by cell types especially the CD_4 cell which differentiate into the TH1 and TH2 phenotypes. This does not work particularly well in determining the control of the effector mechanisms against malaria as different mechanisms are required against different life stages (Roitt, 2002). Infection of the hepatocytes by sporozoites can be prevented by cytokines such as IFN-γ and IL-12 produced by activated CD_4 of TH1 phenotype cell. IFN-γ and CD_4^+ T cells activate macrophages to phagocytose intra-erythrocytic via Fc and C_3 receptors on parasites and free merozoites. Macrophages play an important role in adaptive immunity, by serving as antigen presenting cells (Stevenson *et al.*, 2004).

Resent studies have shown that the level of spontaneous apoptosis in peripheral blood mononuclear cells is increased during acute malaria attack (Lisse *et al*, 1994). Malaria parasite is also known to perturb the normal profile of immune cells in peripheral blood. For instance total leukocyte, total lymphocytes. NK cells and T-cell proportion has been reported in *P. falciparum* and *P. vivax* infection (Lisse *et al.*, 1994 ; Worku *et al.*, 1994) in contrast, others have reported no significance difference in the WBC count, the percentages of CD_4^+ and CD_8^+ cell or CD_4:CD_8 ratio in *P. falciparum* patients (Lisse *et al.*, 1994). Both the CD_3^+ and CD_{14}^+ populations are required for early TNF response to *Plasmodium*-infected erythrocyte (Pfe) (Lisse *et al.*, 1994; Worku *et al.*, 1994). In

addition both CD_3-CD_{16}^+ and CD_3-CD_{56}^+ NK cells from *P. falciparum* infected individuals lysed schizonts. The cytotoxicity was enhanced by the addition of IFNγ and/or IL-2 notably the CD_3-CD_{56}^+ subset (Lisse *et al.*, 1994).

2.13 Sex Differences in Parasitic Infections

Among human and other mammals, the prevalence and severity of parasitic infections is higher in males than females. Sex differences in exposure as well as susceptibility to parasites probably contribute to sex-based differences in the severity and prevalence of parasites. For example, males are more likely to engage in behaviours, such as aggression, dispersal, and grouping, that increase the likelihood of contact with parasites; both ecto- and endoparasites (Zuk and McKean, 1996; Klein, 2000). Despite differences in the likelihood of exposure, several studies illustrate that immunological differences exist between the sexes that may underlie increased parasitism in males. Females typically have higher immune responses than males (Zuk and McKean, 1996; Klein, 2000). However much studies have been directed to rodents than human beings.

2.13.1 Immuno-endocrine Interaction and Sexual Dimorphism in Response to Parasitic Infections

Elevated immunity among females creates a double-edged sword, in which it is beneficial against infectious diseases, but is detrimental in terms of increased development of autoimmune diseases (Wizemann *et al.*, 2001). Several field and laboratory studies link sex differences in immune function with circulating steroid hormones (Zuk and McKean, 1996; Klein, 2000 and Robert *et al.*, 2001). The prevailing hypothesis for immunological differences between the sexes is that sex hormones, in particular, testosterone, oestrogen, and progesterone, influence the immune system

(Benten *et al.*, 1997). The localization of sex hormone receptors in immune cells, including lymphocytes, macrophages, granulocytes, and mast cells, illustrates that there are direct connections between the endocrine and immune systems and that endocrine factors can directly modulate the expression of target genes in immune cells (Benten *et al.*, 1997).

Although males are more susceptible than females to many parasites, there are parasites for which males are more resistant than females (Morels-Montor *et al.*, 2004). For example, male mice (*Mus musculus*) are less susceptible than females to several parasites, including *Babesia microti*, *Toxoplasma gondii*, *Schistosoma mansoni*, and *Taenia crassiceps* (Aguilar-Delfin *et al.*, 2001).

Field studies of vertebrates illustrate that the prevalence and severity of parasite infections are often higher in males than females (Walker *et al.*, 1997). The prevalence and serverity of infection with *Leishmania*, *Plasmodium*, *Entamoeba*, *Necator*, and *Schistosoma* parasites, for example, is higher among men than women (Eloi-Santos *et al.*, 1992). Although clinical studies of humans and field studies of non-human are suggestive, several factors, including exposure rates, social behaviour, habitat, and diet cannot be held constant and could contribute to the observed differences in parasite infection (Larralde *et al.*, 1995). Studies of rodents in a controlled laboratory setting, however, reveal that sex differences are present and may be mediated by endocrine–immune interactions. Although males are more susceptible to a majority of parasite infections, exceptions are noted and should be further characterized (Morels-Montor *et al.*, 2004).

One genus of protozoan parasites that causes a pronounced sexual dimorphism in vertebrate hosts is *Plasmodium*. Among humans, although the incidence of infection is generally similar between the sexes (Weise, 1979; Wildling *et al.*, 2001). Sex differences in the intensity of infection are reported in which men have higher parasitaemia than women (Landgraf *et al.*, 1994; Wildling *et al.*, 2001). Studies of rodent malarias illustrate that mortality rates are higher in males than females and may involve immunological differences between the sexes. Response to *P. chabaudi* may be mediated by sex differences in the expression of genes that modulate pro-inflammatory and helper T-cell type 1 (Th1) responses (IFN-α) against infection (Cernetich *et al.*, 2004). The immunomodulatory effects of testosterone may underlie increased susceptibility to *Plasmodium* infections in males compared with females. Exposure of adult female mice to testosterone reduces antibody production, decreases major histocompatibility complex (MHC) class II cells in the spleen, and increases CD8+ T-cells in the spleen (Benten *et el.*, 1997). Th1 and Th2 responses are important for protection against infection with Plasmodium (Stevenson and Riley, 2004). Studies utilizing IFN-α (Th1) and IL-4 (Th2) knock-out (KO) mice reveal that the effects of IFN-α and IL-4 on survival are more pronounced in males than females. Male IFN-α and IL-4 KO mice have shorter survival times than their male wild-type (WT) counterparts; in contrast, no differences are observed between female KO and WT mice (Zhang *et al.*, 2000). Sex differences are apparent in both IFN-α and IL-4 KO mice in which mortality rates are still higher in males than females (Zhang *et al.*, 2000).

Humoral immune responses (antibody production by B-cells) are typically greater in females than males (Falter *et al.*, 1991; Gomez *et al.*, 1993). Cell-mediated immune

responses also differ between males and females. T-cells, in particular CD4 + helper T-cells, are functionally and phenotypically heterogeneous and can be differentiated based on the cytokines they release. Reliance on subsets of Th cells (Th1 or Th2 cells) to overcome infection differs between males and females, with females reportedly exhibiting higher Th2 responses (i.e. higher IL-4, IL-5, IL-6, and IL-10 production) than males (Bijlsma *et al.*, 1999; Klein, 2000). There are reports of females having higher Th1 responses (higher concentrations of IFN-α) than males (; Barrat *et al.*, 1997).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study area

This study was conducted at Nyanza Provincial General Hospital and Center for Vector Biology and Control Research (CVBCR) of KEMRI, Kisumu. The city covers an area of approximately 417km^2 of which 297km^2 is dry land and 120km^2 is under water. The area lies at an altitude of 1100m above the sea level situated approximately 00° 06′ south of the equator and 34° 45′ East of Greenwich and boarders the eastern shows of Lake Victoria (Othoro, 1996). Kisumu has semi-humid tropical climate with a mean temperature of 23°c. The highest temperature recorded after 39 years is 37°c and lowest temperature of 11°c. The mean annual rainfall is approximately 1300mm with marked peaks between March and May and short rains between November and December. Residents experience between 100 -300 infective mosquito bites per year (Hoffman *et al.*, 1989). The area is holoendemic for malaria in children below 10 years. *Plasmodium falciparum* account for approximately 97% of the malarial infections with 1.6% of the infections being caused by *P. malariae* and 0.4% due to *P. ovale* (Beier *et al.*, 1990).

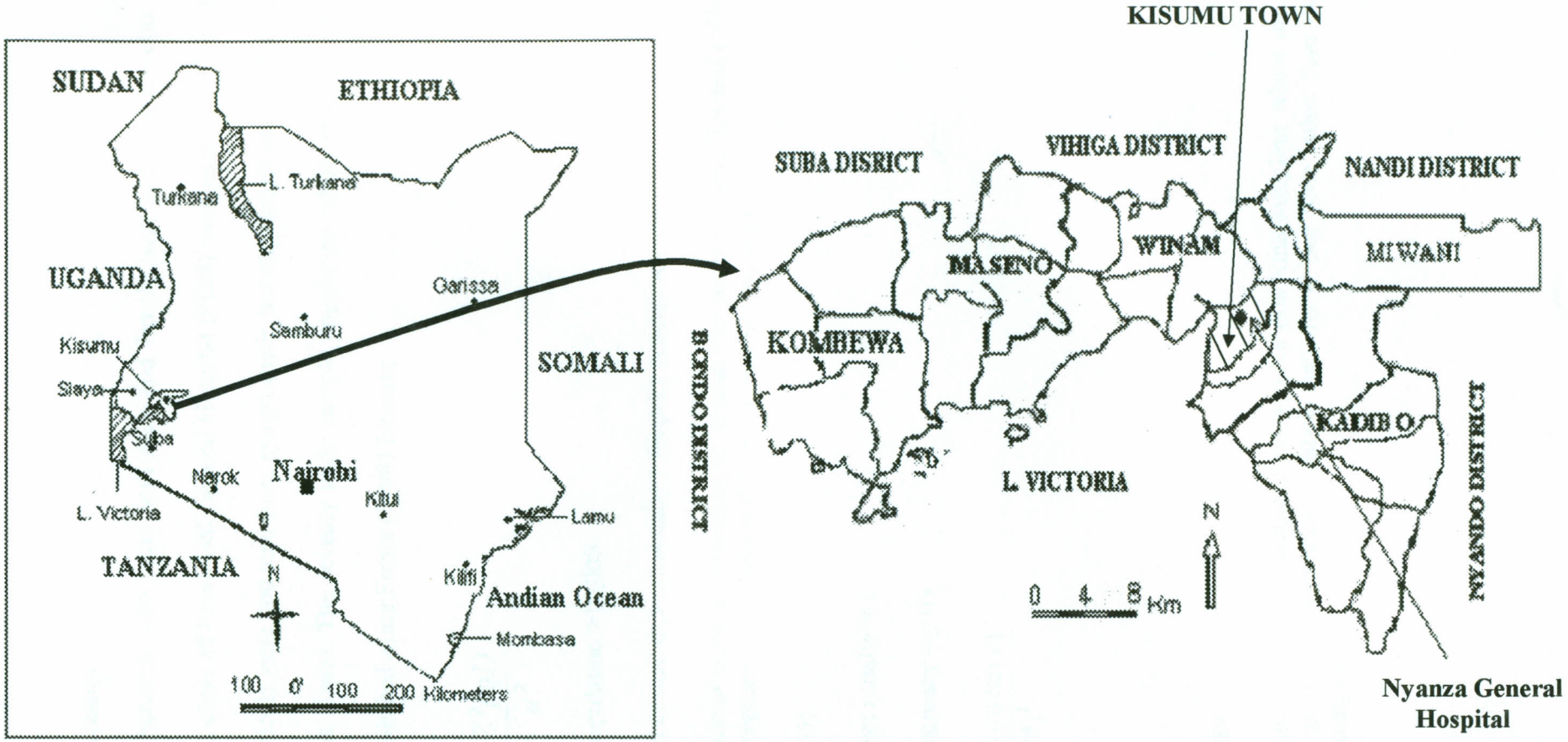


Fig. 3.1: Position of Kisumu City on the Eastern Shows of Lake Victoria, Kenya (Adopted from Republic of Kenya Ministry of Finance and Planning, Kisumu District Development Plan 2002-2008)

3.2 Study Population

Children aged between 1-10 years both male and female with malaria symptoms who presented to the clinic of Provincial General Hospital (PGH) were enrolled for the study. In addition, controls were obtained from symptomatic but apparasiteamic children living within the same region. The seventy eight sample population which was used for this study was estabilished using Fisher (1998) formula:

$$N = \frac{Z^2 pqD}{d^2}$$

N = the target population >10,000

Z = the standard normal deviate usually 1.96 (≈2), confidence level 95%.

p = the proportion of children presenting to Nyanza Provincial General Hospital (NPGH) with malaria symptom = 0.05 (adopted from the hospital records)

q = 1-p = (1-0.05)

D = Design effect usually = 1

d = degree of accuracy = 0.05

$$N = \frac{2^2 (0.05)(1-0.05) \times 1}{(0.05)^2}$$

$N \approx 80$

3.3 Study Design

The study design was cross – sectional study, with one time sampling where the data collected on a group of symptomatic malaria patients at one time rather than over a period of time analyzed.

3.4 Informed consent

Parental informed consent was obtained for all participants. Those who consented to participate filled and signed consent forms in (Appendix I).

3.5 Ethical Consideration

Participation in this study presented no life threatening risks to the subjects other than those related to the disease. Nevertheless, there was the discomfort when the blood was being drawn which resulted in no more than slight pain and little bruising, if any that disappeared within a day. Five milliliters blood sample was collection aseptically and all safety measures observed under the care of trained clinicians and laboratory technologists. The scientific steering committee of KEMRI and ethical review committee approved this study.

3.6 Confidentiality

All the participants in the study were assigned codes. Any reference to be made to the study subjects in this report was by codes only. The medical records related to this study were kept under confidentiality.

3.7 Study Benefits

The parents were made aware of the malaria status of their children and referred to the doctors for medical prescription. They were also made aware of their children's blood group and Rh factor. To the health providers, relationship between blood group

phenotypes and susceptibility to malaria was given leading to better management of malaria.

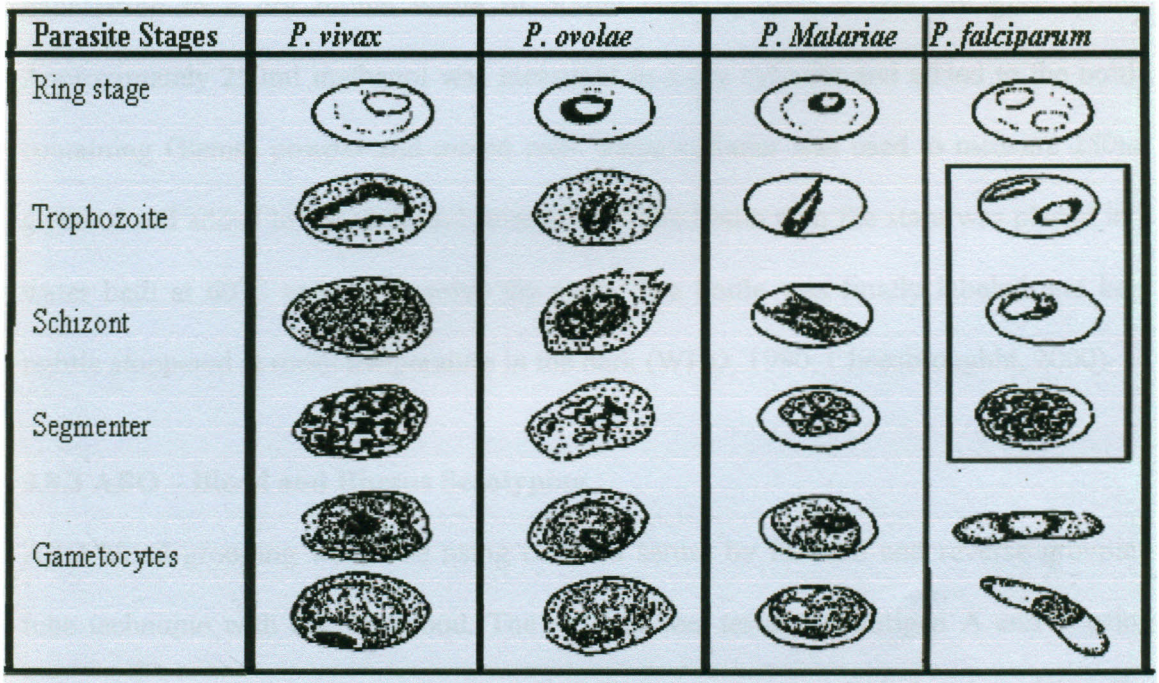
3.8 Specimen Collection and Processing

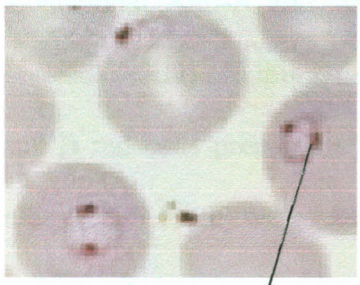
3.8.1 Blood Sample Collection and Parasiteamia Determination

The disease status of the individual was determined by clinical examination of promodal symptoms; headache, fever, jaundice, muscle pain, anorexia, nausea, fatigue dizziness. Of the five milliliters of blood collected, two milliliters was used for CD4 and CD8 count. Two milliliters was used for ABO/Rh serotyping, while out of the remaining one milliliters, thick and thin blood smears were made for absolute leukocyte count and parasitaemia level determination. Thick and thin blood smears were made at the time of sample collection. Thick smears were air dried for 30 minutes and stained with 5% Geimsa solution for 30 minutes. Thin smears were air dried and fixed with 70% v/v methanol prior to staining as above. Slides were then washed, dried and examined for parasites according to Bench AIDS for parasitaemia level determination protocol, (WHO, 1995). *Plasmodium faciparum* were distinguished from the other species especially *P. vivax* with the help of trained clinitian using bench AIDS protocol (WHO, 1995; Fig.3.2).

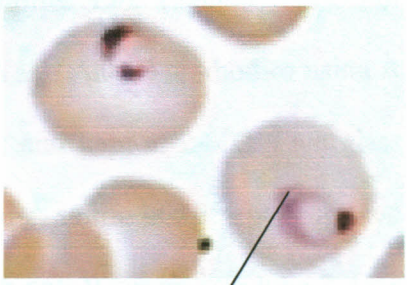
The number of parasites was counted against 200 WBCs. The parasite density per microliter of blood was calculated by multiplying the number of parasites counted by the number of WBCs divided by 200. At least 200 red blood cells per microscopic fields were scanned before a smear was regarded as negative. Malaria severity was determined in terms of parasitaemia level. Patients with 500 $\geq$ parasites per µl of blood were considered to be having severe malaria, below which were recorded to have mild malaria.

Those without any parasite were recorded as apparasitaemic (Ogonda *et al.*, 2001). Once the status was determined and the subject enrolled for the study, 5ml of peripheral blood was drawn from the client into sterile citrated anti-coagulated tubes (Swash, 2002). The total WBC count, differential count, ABO- blood group phenotypes and Rh determination were done in PGH while lymphocyte count (Helper CD_4^+ and cytotoxic CD_8^+ subsets of T-lymphocytes cells) was done in KEMRI – Kisian laboratory.





Ring stage of *P. falciparum*



Ring stage of *P. vivax*

Fig. 3.2: Parasite Morphological Identification (WHO, 1995)

3.8.2 Absolute Differential Leukocyte Count

Thin blood smears were prepared from blood collected after finger pricking with lancets at the time of blood sample collection. White Blood Cells were determined on thin films microscopically using a hand tally counter. The smears were fixed with 70% v/v methanol prior to staining with 5% Giemsa solution. The slides were washed, dried and examined. Giemsa solution was made by weighing 3.8g of Giemsa powder and transferring to a dry brown bottle of 500ml capacity with a few dry glass beads. Approximately 250ml methanol was measured in a dry cylinder and added to the bottle containing Giemsa powder and mixed well. Same cylinder was used to measure 250ml glycerol and added to the stain and mixed well. The bottle with the stain was placed in a water bath at 60°C to help dissolve the stain. The bottle was finally labeled and kept tightly stoppered at room temperature in the dark (WHO, 1990; Cheesbroughht, 2000).

3.8.3 ABO – Blood and Rhesus Serotyping

ABO blood grouping was done using cell and serum by forward and reverse grouping tube technique with client's blood. The red cell was tested for antigen A and B using monoclonal Anti-A and Anti-B sera, (BIOTEC Laboratories Ltd, London, UK). For reverse (serum) grouping, the serum was tested for Anti-A and Anti-B antibodies using A and B red cells prepared at Kisumu regional blood bank and kept at 2-8°C. Five glass tubes (63x9.5mm) were labeled 1 to 5. One volume of anti-A serum and 3% patient's red cells was pipetted into tube 1. The same was repeated with anti-B serum into tube 2. One volume of patient's serum and 3% antigen A cell was pipetted into tube 3 and the same was repeated with antigen B cell into tube 4. One volume of patient's serum and 3% patient's red cell was pipetted into tube 5 which acted as control. Contents of each tube

were mixed by gently tapping the base with the finger after which the tubes were left at room temperature for five minutes. (WHO, 1995; Cheesbroughht, 2000 and Dacie and Lewis, 2001; Tab. 3.1).

Tab. 3.1 Result Interpretation

Tube 1 anti-A	Tube 2 anti-B	Tube 3 A cells	Tube 4 B cells	Tube 5 Control	Blood group
+	-	-	+	-	A
-	+	+	-	-	B
+	+	-	-	-	AB
-	-	+	+	-	O

Key

(+) = Agglutination/ Haemolysis, (-) = No agglutination/ Haemolysis

The Rhesus (Rh) factor was determined using Coomb's method of monoclonal IgM anti - D serum. (Cheesbroughht, 2000; Dacie and Lewis, 2001). The results were read by observing agglutination or haemolysis where 1 volume of anti-D serum was pipetted into a tube containing red cells and gently mixed. Agglutination of red cells meant Rhesus positive whereas absence of agglutination meant Rhesus negative.

3.8.4 Enumeration of Helper CD_4^+ and cytotoxic CD_8^+ subsets of T-lymphocyte cells in blood

The procedure for Guava Easy CD_4/CD_8 was adopted from the manual of Guava Technologies for the enumeration of mature CD_4^+ T lymphocytes and cytotoxic CD_8^+ subsets of T-lymphocytes cells (Guava Technologies, Hayward, USA). For the CD_4^+ T lymphocytes analysis, Guava Easy CD_4 ™ Ab reagent working solution (1µL of anti-CD_3-PECy5, 1µL of anti-CD_4-PE, 8µL of Antibody Diluents per tube) was prepared and

ran. While for the CD_8 analysis, Guava Easy CD_8^{TM} Ab reagent working solution was used. The samples acquisition and reading was done on the Guava PCA system using the Easy CD_4^{TM} Software following the instruction on the manual. Guava EasyCD4 or CD8 Antibody working solution consisting of Guava anti-human CD4 or CD8-PE reagent, anti-human CD3-PE-Cy5 and Guava Antibody dilution buffer were prepared according to number of samples to be analyzed. Ten µl of Guava EasyCD4 or CD8 Antibody working solution was then pipetted to each labeled sample tubes. Ten µl of whole blood (from a well mixed EDTA lavender top tube) was pipetted into the bottom of a 1.5 mL microcentrifuge tube. After which the tube was capped and then vortexed at medium intensity. Incubation was done for 15 minutes at room temperature (25°C) before the sampled were ready for the acquisition and analysis on the Guava PCA system using the EasyCD4 software (Guava Technologies, Hayward, USA).

3.9 Data Analysis

Data was analyzed using SPSS programme. The comparison of indices, lymphocyte levels and other parameters were made using the non-parametric Kruskal Wallis test to study relationship among variables and also to test whether the samples came from the same population. The relationship between and within two independent variables were determined using Correlations. The analysis of variance was also done.

CHAPTER FOUR: RESULTS

4.1 Description of the Study Subjects

Seventy eight children both male and female age between 1-10 years who presented at the Nyanza Provincial General Hospital (NPGH) with symptoms of malaria and whose parent or guardian consented to the study were enrolled for the study. The study was done between the month of April to June 2006 since it is the period of high rainfall and malaria is at its peak in Kisumu town, which is holoendemic for malaria (Were *et al.*, 2000). Out of the 78 samples, 2 were destroyed hence their T-lymphocyte phenotypes could not be established. The ages of 4 individuals were incidentally not recorded during sample collection. It was worth determining whether age, sex, ABO/Rh-blood group system and CD_4/CD_8 count level had an effect on susceptibility and severity of malaria in children living in malaria holoendemic area.

4.1.1 Inclusion and Exclusion Criteria of the Study Subjects

The parents or guardians were interviewed to ascertain that only children coming from Kisumu town, were included in the study. It was also mandatory for the children to be examined by the clinicians and only those found to have malaria symptoms were included in the study. Patients who were referred to the hospital for CD_4/CD_8 counts and those who were recommended by the clinicians for the diagnosis of other diseases other than malaria were excluded from the study. Children with ages above 10 years were also excluded from this study. The individual whose blood smears were observed to have sickle cells (sickle cell anaemic) were not considered subjects for this study.

4.2 Relationship between ABO/Rh –Blood Group Phenotypes on Malaria Infection

There were a total of 5 ABO/Rh-blood group phenotypes serotyped in the sampled population namely A+, B+, AB+, O+ and O-. Blood group O+ was found to be the most abundant and blood group O- found to be the least abundant in Kisumu (Fig. 4.1).

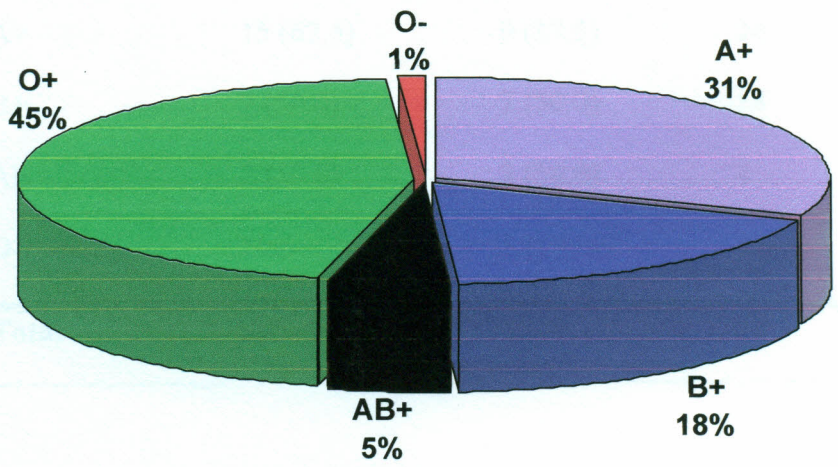


Fig. 4.1: Percentage ABO/Rh Blood Group Phenotypes in the Study Population

Although blood group O+ was found in majority of the children, only 37.1% of malaria positive cases were reported to be O+, while negative cases formed the bulk of the children (62.9 %.) with this blood group. The highest malaria positive cases were observed in blood group A+ (62.5%) , suggesting that children with blood group A+ were the most vulnerable to malaria infection of all the 5 ABO/Rh-blood groups reported in children in Kisumu town (Tab.4.1).

Tab. 4.1: Relationship between ABO/Rh-Blood Group Phenotype and Malaria Infection

	MPS		
Blood group	Positive (%)	Negative (%)	**Total**
O+	13 (37.1)	22 (62.9)	**35**
A+	15 (62.5)	9 (37.5)	**24**
B+	7 (50.0)	7 (50.0)	**14**
AB+	2 (50.0)	2 (50.0)	**4**
O-	None	1 (100.0)	**1**
Total	**37 (48.9)**	**41 (51.9)**	**78**

The fact that A+ was the most susceptible to *P. fulciparam* was further confirmed by mean parasitaemia levels which were higher in A+ (4187 parasites μl^{-1} of blood) than any other blood group. Among the blood groups which were positive for malaria parasite, O+ registered the least mean parasitaemia level of 314 parasites μl^{-1} of blood`, suggesting that it was the blood group that was least susceptible to *P. falciparum* among all the blood groups sampled for the study (Fig. 4.2)

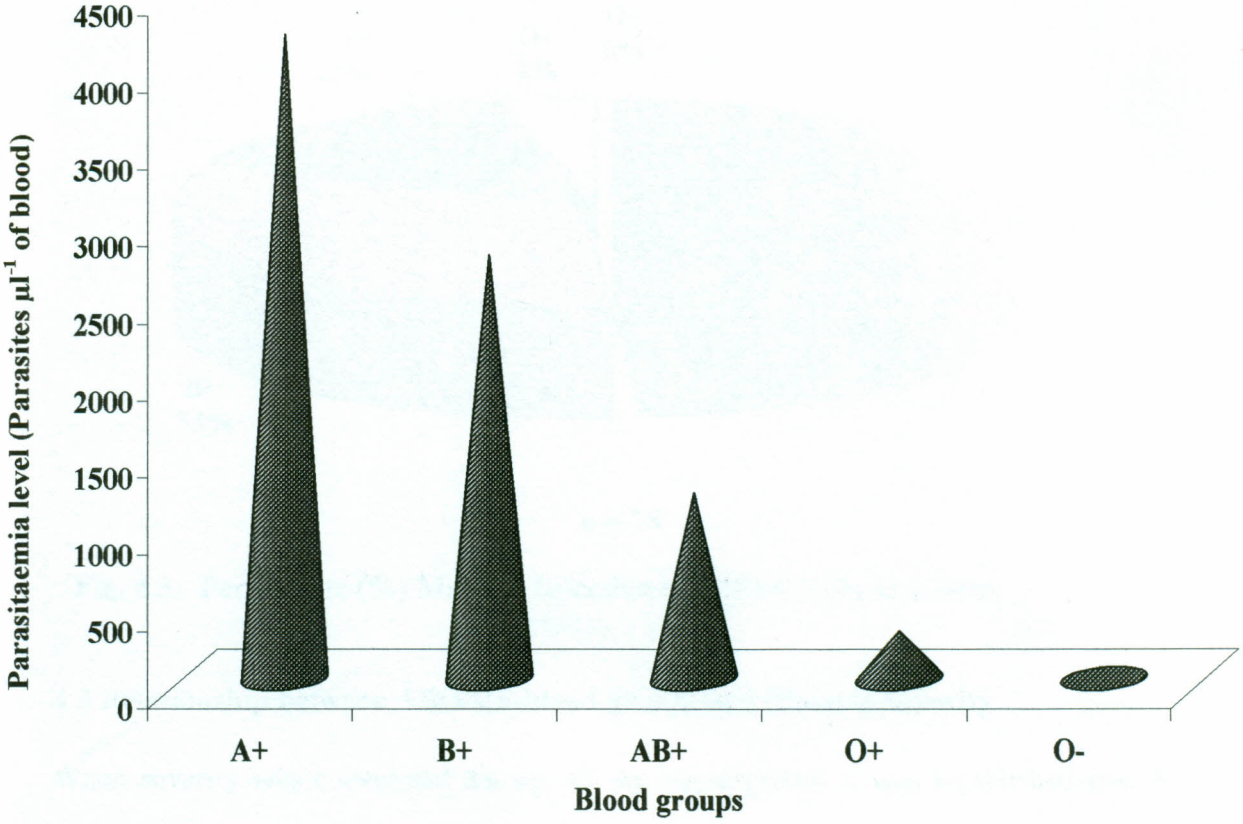
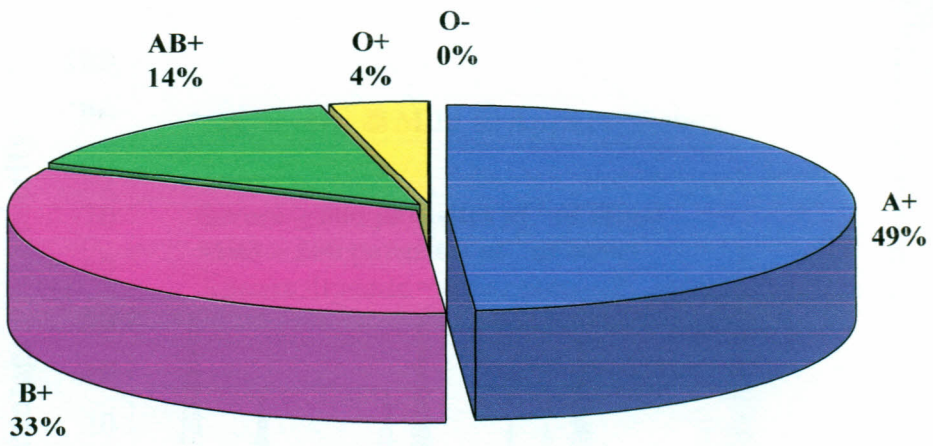


Fig. 4.2: ABO/Rh-blood Group Phenotypes and Parasitaemia level

Blood groups could therefore be arranged sequentially in order of susceptibility from the most to the least vulnerable as follows; A+, B+, AB+, O+ and O- (Fig. 4.2) This arrangement was also observed with the percentage susceptibility of each blood group, A+ had the highest percentage (Fig. 4.3) while O+ had the least percentage susceptibility (4%). Blood group O- was insignificant since it was only one in the sampled population and was apparasitaemic giving O% vulnerability, therefore little could be deduced from only one sample. It was also noted that Rhesus negative blood group phenotype was rare in sample population with the only one case reported in blood group O.



n = 78

Fig. 4.3: Percentage (%) Malaria Infection in ABO/Rh-blood Group

4.3 Relationship between ABO/Rh-blood groups and Malaria Severity

When severity was considered among all the blood groups it was established that A+ registered the highest percentage severity (45.8%) with mild cases making 16.7%. The least percentage severity was registered in blood group O+ (8.6%) with mild cases recording 28.6%. This was the group with the highest percentage of apparasitaemic case s (62.9%). Blood group B+ recorded the highest mild malaria cases of all the 5 blood groups sampled for the study (Fig. 4.4). These observations further confirmed the fact that blood group A+ was implicated in malaria susceptibility while group O+ showed resistance to malaria infection.

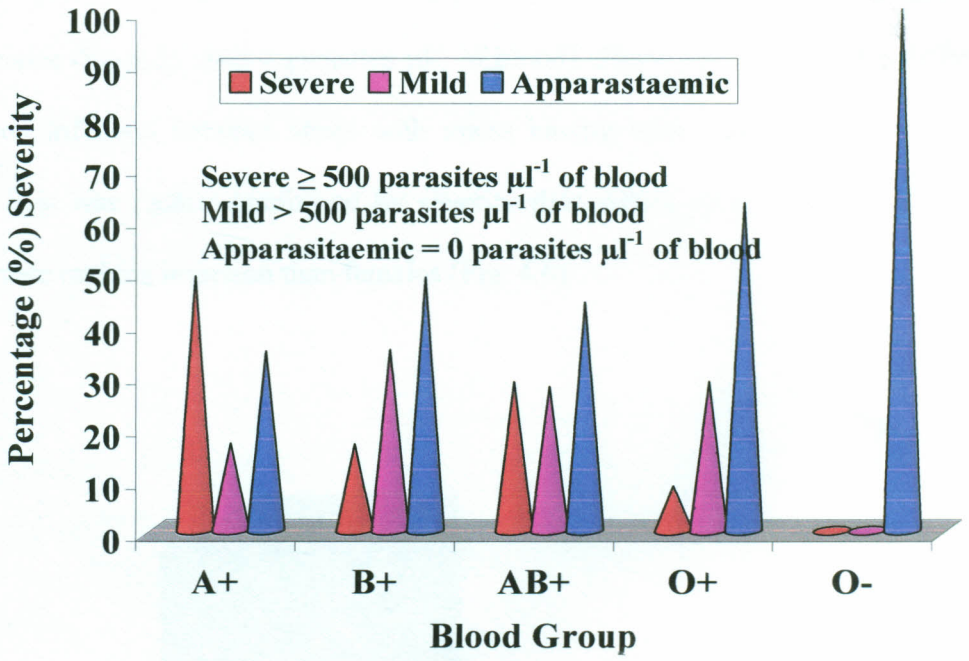


Fig. 4.4: Relationship between ABO/Rh- blood groups and Percentage of Malaria Severity

4.4 Sex on Malaria Susceptibility

When males were considered 60.4% were positive for malaria. This contrasted with female that had infection of 44.0%. Based on this data it was evident that males were more susceptible than females (Tab. 4.2).

Tab. 4.2 Effect of Sex on Malaria Susceptibility

	MPS		TOTAL
	Positive (%)	Negative (%)	
Male	29 (60.4)	19 (39.6)	**48 (100.0)**
Female	12 (40.0)	18 (60.0)	**30 (100.0)**

This observation was further confirmed by parasitaemia level, which was higher in male than females (Fig.4.5; 1902.7 parasites μl^{-1} of blood). There was significantly difference in malaria infection between sexes with males having higher infection (Fig. 4.5; $p = 0.040$). This was further confirmed by severity data where males were found to have more severe malaria infection than females (Fig. 4.6)

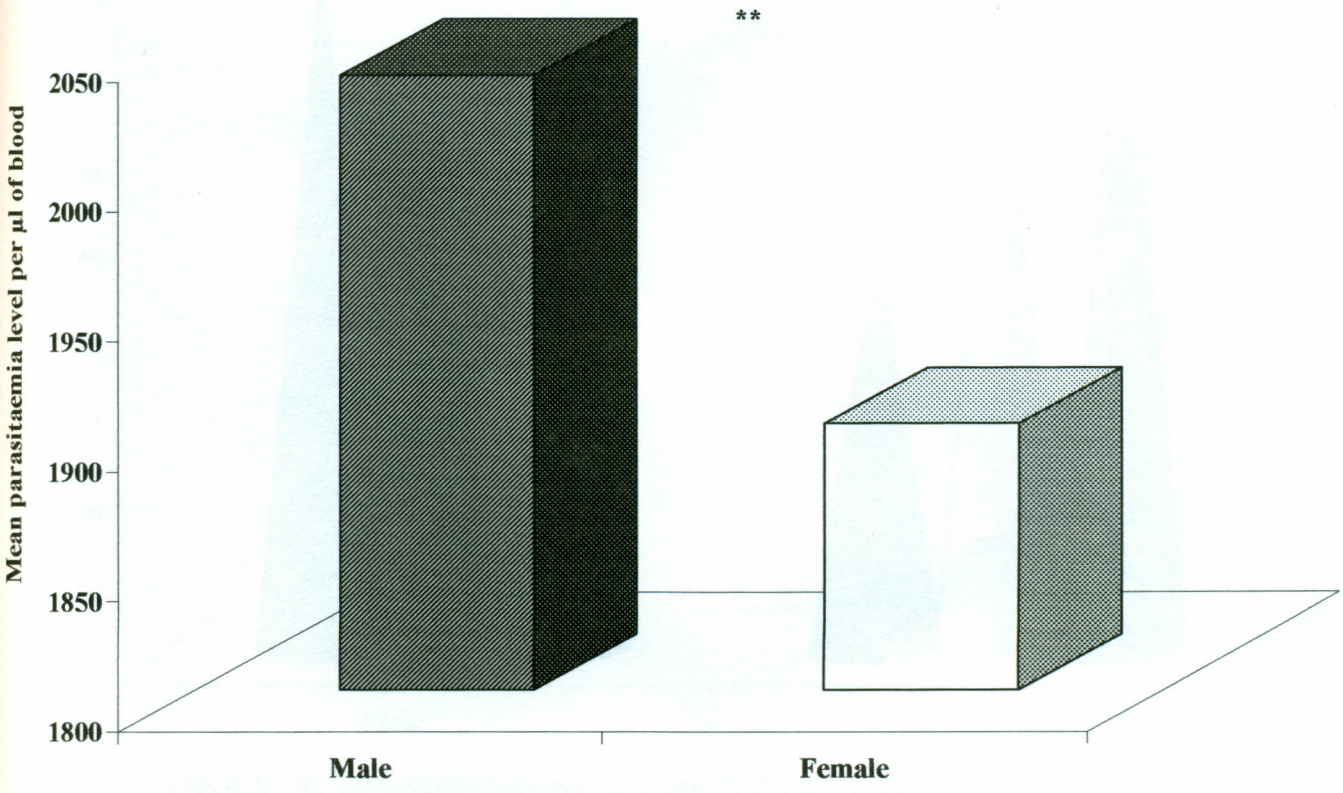


Fig 4.5: Effect of sex on Parasitaemia level (** mean significant difference between males and female; $p = 0.04$; $n = 78$; male = 48, female = 30)

It was observed that 29 of male patients were malaria positive out of which 39.6% had severe malaria, which was higher than that of the female (23.3%; Fig. 4.6). This was appointer towards suggesting that severe malaria was more common in males than in females; males are more infected with malaria parasite and develop severe infection than the females at the age of 1-10 years.

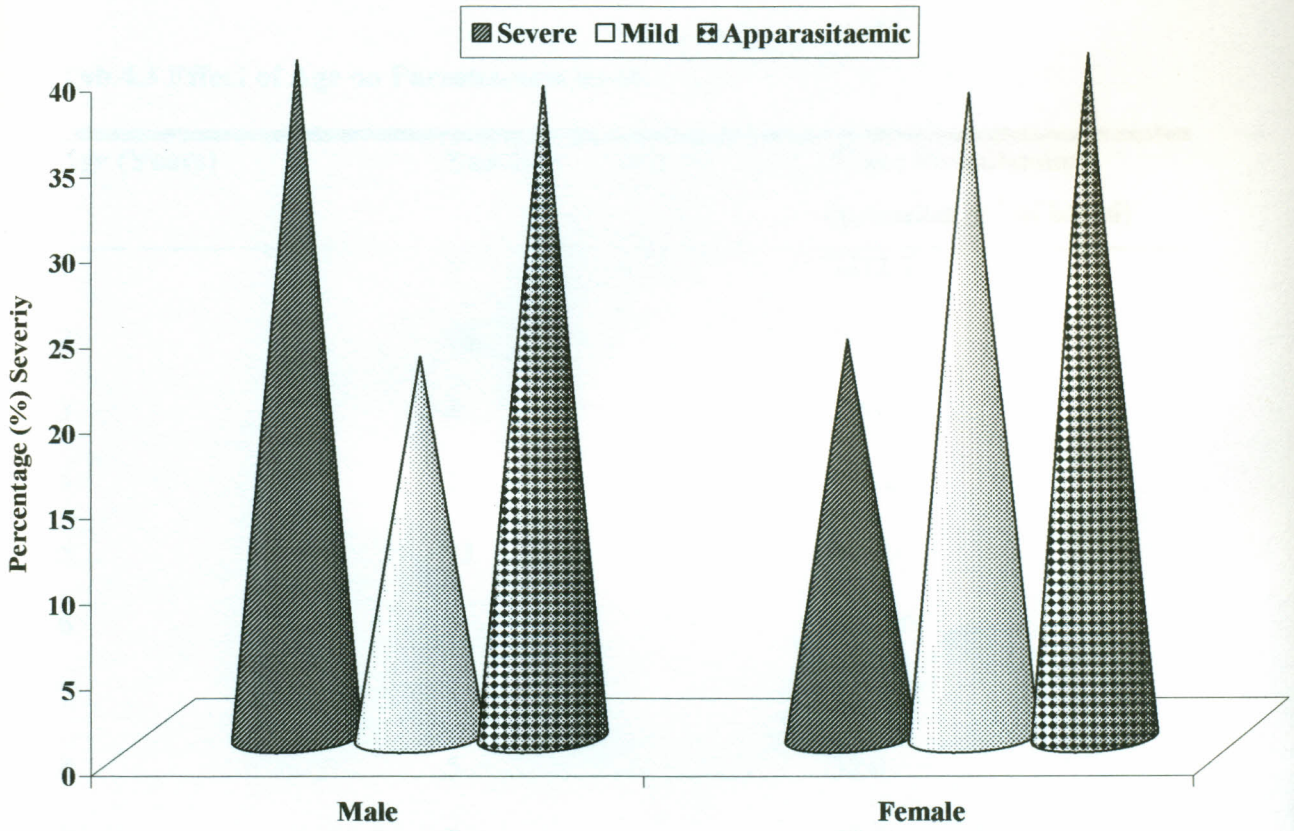


Fig. 4.6: Relationships between Sex and Malaria Severity

4.5Influence of Age on Malaria Infection

Children ages between 1-10 years were sampled for the study. Out of 78 samples the ages of 4 individuals were incidentally missing. It was observed parasitaemia levels reduced tremendously with increase in age from 1-10 years (Tab. 4.3). Young children had higher

mean parasitaemia level than the older ones. The highest mean parasitaemia level was observed in 1-year-old children (7212.3 parasites μl^{-1} of blood; Tab. 4.3) while the least parasitaemia was observed in 8-year-old children (32.0 parasites μl^{-1} of blood; Tab. 4.5). Age was observed to be inversely correlated with parasitaemia (Tab.4.3; r = -0.0278, p = 007) indicating that young children were more susceptible to malaria infection than old ones.

Tab.4.3 Effect of Age on Parasitaemia levels

Age (Years)	**Number**	**Mean Parasitaemia (parasites μl^{-1} of blood)**
1	5	7212.3
2	16	2660.0
3	4	1190.0
4	15	112.0
5	13	784.0
6	5	2043.0
7	5	168.0
8	5	32.0
9	2	40.0
10	4	60.0

4.6: Age and Malaria Severity

When severity and age was considered, it was revealed that younger children had higher percentages of severe malaria cases than the older ones. The highest percentage severe case was observed in 1 year old children (60%; Tab. 4.4), while most of the older children (6-10 years) had no severe malaria infection with exception of one 8-year-old child. Mild malaria cases were sporadic in most ages (Tab. 4.4)

Tab. 4.4: Effect of Age on Malaria Severity

Age (Years)	Severe (%)	Mild (%)	Apparasitaemic (%)	Total
1	3 (60.0)	0 (0.0)	40 (0.0)	5 (100.0)
2	4 (25.0)	4 (25.0)	8 (50.0)	16 (100.0)
3	1 (25.0)	1 (25.0)	2 (50.0)	4 (100.0)
4	1 (6.7)	3 (20.0)	11 (73.3)	15 (100.0)
5	5 (38.5)	4 (30.8)	4 (30.8)	13 (100.0)
6	0 (0.0)	1 (20.0)	4 (80.0)	5 (100.0)
7	0 (0.0)	3 (60.0)	2 (40.0)	5 (100.0)
8	1 (20.0)	1 (20.0)	3 (60.0)	5 (100.0)
9	0 (0.0)	1 (50.0)	I (50.0)	2 (100.0)
10	0 (0.0)	2 (50.0)	2 (50.0)	4 (100.0)
Total	**15**	**20**	**39**	**74**

4.7 Relationship between T-helper cells/Cytotoxic T-lymphocytes levels and Malaria Infection

The mean T-helper cells/Cytotoxic T-lymphocytes (CD_4 and CD_8) count level was found to be lowest in severe malaria cases; 429.37; SE (64.73) and 311.72; SE (37.13) respectively. This was contrasted with apparasitaemic cases which registered the highest mean for both CD4 (651.60; SE 64.90) and CD8 (526.92; SE 70.46) respectively. Mean CD4 for mild malaria cases was 595.6 while mean CD8 for mild malaria was found to be 330.28 (Fig. 4.7). This suggested that severity in malaria had a negative effect on both CD_4 and CD_8 count. When the data was subjected to correlation analysis, it was revealed that there was a weak negative correlation between CD_4 and parasitaemia (Fig. 4.7; $r = -162$, $p = 0.04$), When CD_8 was considered, it was also found to correlate negatively with parasitaemia (Fig. 4.7; $r = -0.348$, $P = 0.002$). It should be noted that the normal of CD4 in children has not been established while for the adults is between 800-1200 μl^{-1} of blood.

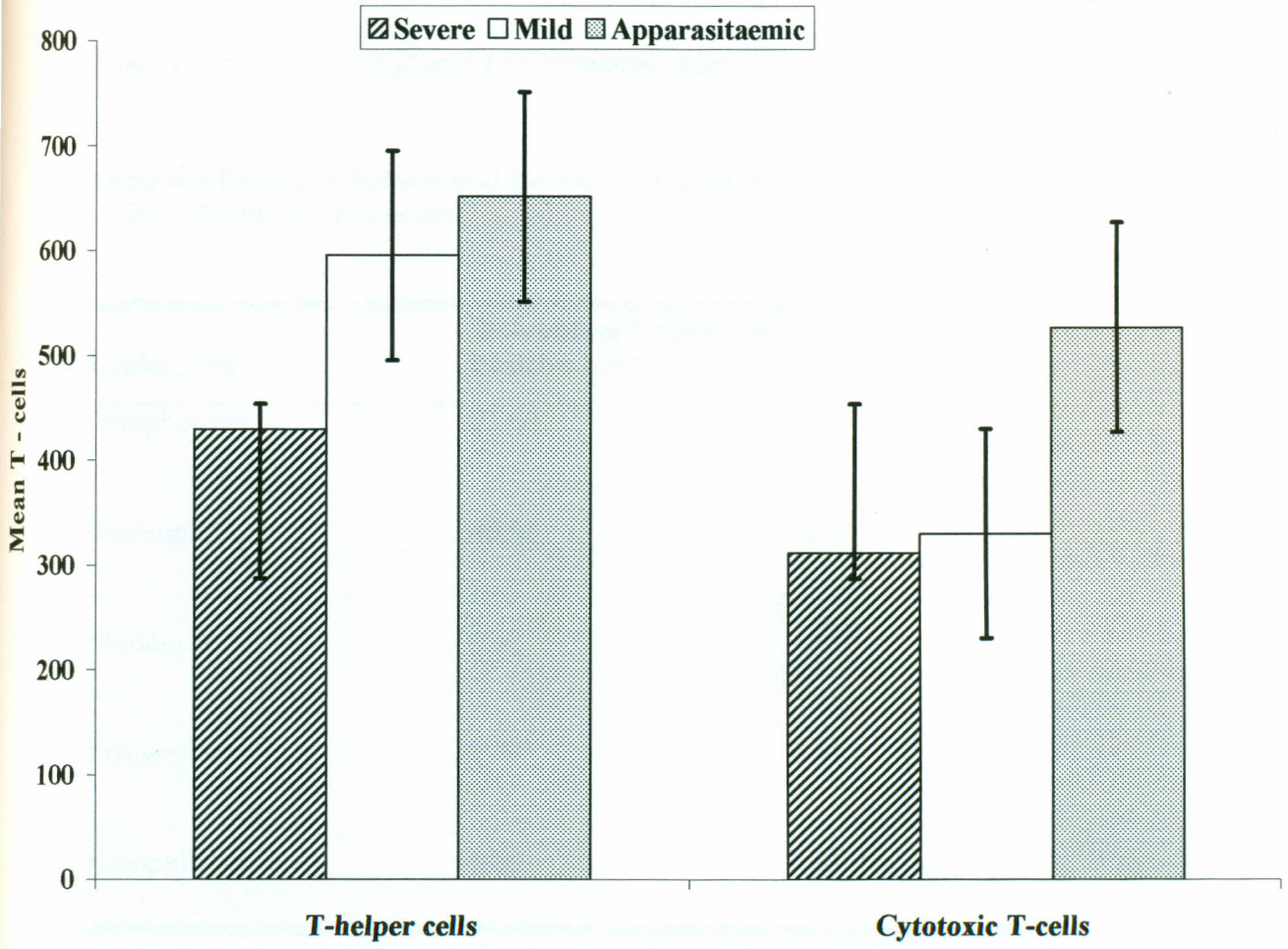


Fig 4.7: Relationship between CD4 and CD8 and Malaria Severity

4.8: Effect of Differential Leukocyte count on Malaria Infection

Out of 67 samples analysed for differential count, 35 (52.2%) were negative for *P. falciparum* while 32 (47.8%) were positive. Malaria adversely affected lymphocytes, monocytes and eosinophil levels in circulation (Tab.4.5), while malaria had positive influence on neutrophil and basophil which indicated that lymphocytes, monocytes and eosinophils were less in positive malaria case than apparasitaemic one (Tab.4.6). This was further confirmed by effect of severity of malaria on differential leukocyte count. However, there was no significant correlation between malaria severity and all the

differential leukocyte count (Tab.4.5; $p>0.05$), although severe cases had relatively lower leukocyte counts than mild and a parasitaemic cases (Tab. 4.6)

Table 4.5 Effects of Differential Leukocyte Count on Malaria infection (MPS = Malaria Parasites)

Leukocytes	Percentage Leukocyte count Positive MPS	Negative MPS
Lymphocyte	53.00	57.55
Neutrophil	40.56	37.91
Monocyte	1.50	1.57
Eosinophil	1.97	2.54
Basophil	0.66	0.43

4.9: Effects of differential Leukocyte Count on Malaria Severity

Leukocytes	(%) Severe	(%) Mild	(%) Apparasitaemic
Lymphocyte	38.4	46.3	48.9
Neutrophil	47.1	49.1	53.1
Monocyte	1.5	1.3	1.7
Eosinophil	1.8	2.1	2.5
Basophil	0.6	0.7	0.5

CHAPTER FIVE: DISCUSSION

5.1 Overview of the Discussion

Studies done in different parts of the world have indicated that ABO/Rh blood groups phenotypes may influence malaria infection (Thaitong *et al.*, 1984; Hill, 1992), however little has been done in malaria holoendemic areas of Africa and Kenya in particular. It was therefore imperative to investigate the effect of ABO/Rh blood group phenotypes and immunosurveillance to *P. falciparum* infection in a holoendemic area for malaria. The study was conducted using blood samples obtained from young children aged between one to ten years since they are the most vulnerable to the severe effects of malaria infection. To fulfill this aim, blood samples were drawn intravenously from malaria symptomatic children and used for the analysis of malaria positive cases, parasitaemia levels, ABO/Rh blood groups phenotypes, differential leukocyte counts, and helper CD_4^+ and cytotoxic CD_8^+ subsets of T-lymphocyte cell leves in blood.

5.2 Frequency of ABO/Rh Blood Group Phenotypes in the Study Population

The frequencies of ABO and rhesus blood groups vary from one population to another (Pramanik and Pramanik, 2000). In this study in Kisumu town of Western Kenya, blood group O+ was revealed to be the most frequent (45%) followed by A+ (31%), B+ (18%), AB (5%) and the least frequent blood group was O- registering only 1%. These results were almost comparable to the results of unrelated study carried out in Aga Khan Hospital, Nairobi that showed that Blood group O was the most frequent (49%) among the indigenous African donors and 45% in the general donor population (Mwangi, 1999). The result of blood group AB in the current study was also comparable to the above

mentioned study: Group AB was 5% in the general donor population and 4% in the African donors. These findings are also comparable to those established in Nepal among 120 Nepalese medical students where; 34% were blood group A, 29% group B, 4% group AB and 32.5% group O (Pramanik and Pramanik, 2000). The frequency of Rh-negative blood was 3.33% and Rh-positive 96.66% (Pramanik and Pramanik, 2000).These results contrasted sharply with the results of this study ; O+(45%), A+ (31%), B+ (18%), AB (5%) and O- (1%). While in a study conducted in Pakistan by Mohammad *et al.* (2004), revealed that the frequencies of ABO/ Rh blood were as follows: 31.03% (A), 36.23% (B), 7.67% (AB), and 25.07% (O). However, Rh positive and negative distribution in the studied population was 89.23% and 10.77% respectively which was different from the result of this study with 99% Rh positive and 1% Rh negative. In addition a study carried out in Central Province of Kenya on blood groups antigens ABO and Rh by Mwangi (1999), established the following frequencies: A 26.20%, B 22.00%, AB 4.40%, O 47.48%, which is comparable with the data reported in this study. Nevertheless Mwangi (1999), revealed that Kikuyu, a tribe in Central Kenya had the following frequencies of blood groups: O 60%, A 19%, B 20% and AB 1%.

Pathirana *et al.* (2005) agued that although blood types are 100% genetically inherited, the environment potentially can determine which blood types in a population will be passed on more frequently to the next generation. ABO blood types are inherited through genes on chromosome 9, an individual's ABO type is determined by the inheritance of 3 alleles (A, B or O) from each parent. It is imperative that the dominant blood group in this study population of malaria holoendemic area is blood group O which was found to be less susceptible to malaria infection. It can be postulated that there is selection

advantage for group O which may offer protection against severe malaria and hence it survival strategy.

5.3 Relationship between ABO/Rh Blood Group Phenotypes on Malaria Infection

The highest parasitaemia levels was observed in blood group A+ (49%) and this group had also the highest percentage malaria severity (45%), whereas the least parasitaemia level was recorded in blood group O+ (4%) it also had the least malaria severity (8.6%). This study therefore suggests that the individuals of blood group O are relatively resistant to severe effects of malaria infection.

These findings are comparable to the results of a similar study conducted in Colombo, Sri Lanka which revealed that the cases of severe malaria were significantly less likely to be of blood group O and it appeared that individuals who were of blood-group O were relatively resistant to the severe disease caused by *P. falciparum* infection (Pathirana *et al.*, 2005; Uneke, 2007). Similar results was reported in Gabon which shows that in clinical isolates, *P. falciparum* has shown less clear-cut to blood group phenotypes preference due to clonal diversity in isolates (Antonio *et al.*, 2000). *Plasmodium falciparum* has shown minimal preference to blood group O (Antonio *et al.*, 2000). Studies in German by Lell *et al.* (1999) suggested that blood group A and B are associated with severe malaria. This suggests that blood group A and B predispose individuals to malaria infection and severity in malaria endemic areas while O confers resistance to both malaria infection and where infection occurs it reduces severity. This can be explained in part by the fact that malaria parasite (*P. falciparum*) utilizes molecules present on the surface of uninfected red blood cells (RBC) for rosette

formation, and a dependency on ABO antigens has been previously shown (Antonio *et al.*, 2000). Rosetting is the spontaneous binding of uninfected erythrocytes to erythrocytes infected with mature asexual parasites (David *et al.*, 1988). Study in Madagascar showed that the median rosette frequencies were higher in cases of cerebral and other severe malaria than in cases of uncomplicated malaria (Ringwood *et at.*, 1993). Two subsequent studies in Gambia, indicating that rosette-disrupting antibodies were commonly found in the plasma of children with uncomplicated malaria but not in the plasma of children with cerebral malaria (Carlson *et at.*, 1990; Treutiger *et al.*, 1999).

Although erythrocytes have traditionally been considered relatively inert containers of hemoglobin, recent studies have mounting evidence suggesting that they in fact bear numerous surface molecules that are active in microbial attachment and processes (Telen *et al.*, 1995). Asexual stages of *P. falciparum* utilize molecules on the surface of uninfected red blood cells (RBC) for rosette formation i.e. binding of parasite-infected RBC (pRBC) to uninfected RBC. Rosetting has been associated with the occurrence of severe malaria; cerebral malaria and anemia (Carlson *et al.*, 1990; Rowe *et al.*, 1995; Newbold *et al.*, 1997; Kun *et al.*, 1998). The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a member of a family of high-molecular-weight polypeptides encoded by the *vars* genes (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995) has been identified as a rosetting ligand (Rowe *et al.*, 1997)

An *in vivo* preference for blood group A and B by *P. falciparum* was further revealed in Germany where it was reported that Blood group A-preferring FCR3S1 strain and blood group B-preferring TM 284 strain rosetting parasites strains retain their blood group

preferences after being propagated for more than 100 cycles in blood group O RBC. This indicates that a single ligand or genetically linked ligands are expressed on the surface of the pRBC, accounting for binding to the blood group A antigen or B antigen, respectively, and to one or more other receptors on the RBC. One such parasite-derived ligand is PfEMP1 Rowe *et al.* (1997)

Similar study by Udomsangpetch *et al.* (1993) also reported O-phenotypes to protect against cerebral malaria via impaired rosette formation. It was established that impairment of rosetting hampers parasite proliferation and enhances parasite immunosurveillance through non-specific immune response such as phagocytosis (Hill, 1992). Therefore sequestration is prevented and a victim is protected against pathogenesis of cerebral malaria (Pathirana *et al.*, 2005). These findings were consistent with the studies carried out in Kilifi, Kenya which examined the ABO blood groups of the patients from whom isolates were obtained and found that parasitized RBC from group O patients rosette less well than those from group A (Rowe *et al.*, 1995). It was similarly revealed in this study that blood group A and B are high likely to predispose individuals to malaria infection and eventually to the development of severe effect of the disease more than group O. It is therefore suggestive that parasitized blood group A and B rosette well more than O which seems to impair rosette formation. Individuals of group A and B who live in or visit malaria endemic areas should seek agent diagnosis and treatment in case of suspected malaria symptoms since they are likely to develop severe malaria infection than individuals with other blood groups.

5.4 Immune Responses to Malaria Infection

The results of this study showed that *Plasmodium falciparum* impacted negatively on lymphocytes, monocytes and eosinophils levels in circulation. While the percentage of neutrophil and basophil of the parasitaemic individual were more than those of apparasitaemic ones. The same trend was observed in effect of severity on differential leukocyte count where less percentages of lymphocytes, monocytes and eosinophils were observed in severe cases than in apparasitaemic one. This study also established no correlation between differential leukocytes and parasitaemia level. These results were comparable to the findings in India, which noted no statistically significant correlation in the white blood cell (WBC) count and *P. falciparum* malaria in age between 20 to 65 years old (Jadhav *et al.*, 2003). However a study done in Malawi revealed contrasting findings where it was reported that the proportion of both neutrophils and monocytes were significantly higher in patient with severe malaria (Kirsten *et al.*, 2003). The decrease in Leukocyte counts in *P. falciparum* malaria reported in this study was in agreement with earlier reports from other geographical locations (Worku *et al.*, 1997; Ordi *et al.*, 2001), while Lisse *et al.* (1994) established that malaria parasites perturb the normal profiles of peripheral blood leukocytes, total lymphocytes, NK cell, αβ and γδ T-cell, B-cell counts and T-cell proportions. However little has been done on differential leukocyte counts in relation to malaria infection as prognostic marker for prediction of the course and implication on malaria.

In this study it was established that T-helper and cytotoxic T-lymphocyte cells were down regulated in both mild and severe malaria cases as compared to those in healthy apparasitaemic samples. This postulates that *Plasmodium falciparum* levels and malaria

severity had a negative effect on CD_4^+ and CD_8^+ count levels. The significant reduction in CD_4 helper cells and cytotoxic CD_8 cells in this study is not surprising since these findings are consistent with the result of a study carried out in Addis Ababa, Ethiopia which characterized peripheral blood lymphocytes subsets in patients with acute *P. falciparum* and *P. vivax* malaria infections and reported a significant decrease in absolute counts of CD_4^+, CD_8^+, B, $CD3^+$ cells and total lymphocytes during both *P. falciparum* and *P. vivax* malaria infections (Desta *et al.*, 2006). These tenets and the results of the present investigations are in general agreement with the findings of Hviid *et al.* (1997), Lee *et al.* (2001) and Lisse *et al.* (1994) who reported that lower absolute counts of CD_4^+, CD_8^+, $CD3^+$, NK cells and total lymphocytes during acute *P. falciparum* malaria. The results of the current study are more less consistent with the findings in Paris France by Pied *et al.*, (2000) who suggested that up-regulation of $CD4^-CD8^-$ $NK1.1^+$ $\alpha\beta$ T cells and down-regulation of $CD4^+$ $NK1.1^+$ $TCR\alpha\beta^{int}$ cells may contribute to the early immune response induced by the *Plasmodium* during the prime infection. In the current study decrease in monocytes, eosinophils, CD_4^+, CD_8^+ and lymphocytes associated with severe malaria infection were observed.

It is important to take into account the fact that even though children who were enrolled for this study were only those referred to the laboratory by the clinicians for the diagnosis of malaria and no any other disease, it cannot be precluded that they did not have any other infection which could lead to significant decrease in absolute monocytes, eosinophils, CD_4^+, CD_8^+ and lymphocytes counts. It is also generally known that malaria parasite negatively affect the normal profile of immune cells in peripheral blood (Desta *et al.*, 2006). Nevertheless, there are probably two main potential mechanisms that could

explain the depletion of lymphocyte subsets from the peripheral blood in *P. falciparum* malaria patients: sequestration of cells into the lymph nodes or other body parts and/or abnormal death of the cells through apoptosis (Grossman *et al.*, 1997).

Resent studies have shown that the level of spontaneous apoptosis in peripheral blood mononuclear cells is increased during acute malaria attack (Lisse *et al.*, 1994). However, the exact mechanism of apoptotic cell death and its impact on the decrease in the lymphocyte population in *P. falciparum* malaria is still obscure and will require further investigation.

In support of the first hypothesis, sequestration, several pieces of physiological and immunological evidence have been suggested. The levels of cytokines (TNF-α and IFN-γ) that are known to induce the expression of the adhesion molecules (selectins, integrins) and chemoattractants chemokines have been observed to correlate with the severity of malaria coursed by *P. falciparum* infections (Rosenberg *et al.*, 1998). Moreover, Elhassan *et al.* (1994) and Hviid *et al.* (1997) reported the levels of these adhesion molecules; Intercellular Adhesion Molecules-1 (ICAM-1) and Vascular Adhesion Molecules-1 (VCAM-1) in plasma and expression of ECAM-1 on the surfaces of endothelial cells have also been reported during malaria infection (Rosenberg *et al.*, 1998). Therefore, the emergence and disappearance of these adhesion molecules during acute malaria infections might prompt different movements of the cells from blood to lymphoid organs. Grossman and Herberman. (1997), further established that this movement can result in alterations in the proportions and absolute counts of immune cells in peripheral blood. In agreement with these findings, Langhorne *et al.* (1991), asserts that, while reactive T

cells will be detected in a splenic cell population during and after infection, these cells are not be detectable within the peripheral blood T cells during acute malaria infection indicating the withdrawal of T cells away from the peripheral blood to other body tissues.

Although the results of this study revealed pronounced correlation between parasitaemia levels and CD_8^+ cells, a study in South Korea established sharply contrasting findings and reported no significance correlation in the percentages of CD_4^+ and CD_8^+, or the CD_4/CD_8 ratio in *P. falciparum* patients (Lisse *et al.*, 1994). A study done by Worku *et al.* (1997) showed significant decrease in CD_8^+-T cell, CD_3^+ T cells and total lymphocytes count in acute *P. falciparum* patients which is in agreement with this study. However, Worku *et al.* (1997) found no change in the absolute count of CD_4^+ cells. This can be agued that although there is ample evidence showing the potential of malaria infection to affect the counts of lymphocyte subpopulations in the peripheral blood, there may be variations when infections is considered in different geographical locations undergoing different parasite challenge.

The same sentiments were echoed in Senegal by Trape *et al.* (1994), and added that the difference in findings could be due to differences in the immune status of the study subjects related to the malaria endemicity or pathogenesis, host genetics (Allen *et al.*, 1997; Hill, *et al.*, 1991), and parasite factors (Chotvanich *et al.*, 2000). Similarly Chotvanich *et al.* (2000) posits that the difference could be possibly due to difference in parasite strains, which may course difference in the activation of the immune system. However, Tsegaye *et al.* (1999) while reporting similar results asserted that the difference

could also be due to differences in the baseline values of the absolute counts of the immune cells of the study subjects.

It is important to note that there could have been other infections in the study population that could influence CD_4 and CD_8 profiles. The findings of this study that malaria infection cause a depletion of lymphocyte populations in the peripheral blood is a very important development since enumeration of peripheral lymphocyte cells can not only be used for research purposes but also for the diagnostic evaluation of the immune status of patients and monitoring malaria severity.

More importantly, is in the regions where HIV infections is high and patients need to be put on antiretroviral treatment (ART) of which the criteria for commencement of treatment are based on the CD_4^+ cells (< 200/µl of blood) (WHO, 2001) among other criteria. Therefore, a reduction in the number of CD_4^+ cells due to *P. falciparum* malaria in patients coinfected with HIV could mislead the physicians to prescribe ART for HIV-positive individuals who actually could not start antiretroviral drugs. Reductions in the number of CD_4^+ cells due to malaria infection can also lead to exaggerated estimates of the total number of HIV-positive people who should start ART in regions where there are overlapping infections with HIV and malaria (Desta *et al.*, 2006).

5.5 Sex and Malaria Susceptibility

The data presented in this study provides the evidence that male children are more vulnerable to *P. falciparum* malaria infection as compared to their female counterparts living in the same malaria holoendemic area. This is attributed to the fact that males had

higher percentage positive malaria parasite cases (60.4%) and higher parasitaemia levels than the females. It was also established that males are more likely to develop severe effect of malaria more than the females. Similar sentiments were given in USA by Klein (2004) while carrying out his studies on hormonal and immunological mechanisms mediating sex differences in parasite infection and noted that the prevalence and intensity of infection with *Plasmodium, Leishmania, Entamoeba* and *Schistosoma* parasites, for example, is higher among males than females.

The correlation between sex and malaria infection revealed in this study was also apparent in German where it was reported that sex correlates with *P. falciparum* infection among the human and that men have higher parasitaemia levels than the women (Weise *et al.*, 1979). These results corroborates the observations made in Gabon by Widling *et al.* (1995) while carrying out a study on malaria epidemology in the province of Moyen Ogoov, observed that studies of rodents malaria illustrate that mortality rates are higher in males than females. These tenets and the results of the present investigations are in general agreement with the findings in Ghana by Landgraf *et al.* (1994) who investigated the density of *P. falciparum* malaria in Ghana school children and evidence for influence of sex hormones. However, Schwartz *et al.* (2001) had contrasting findings and reported that male subjects did not differ from female subjects with regard to severity of the disease.

Although males are more susceptible than females to many parasites, there are parasites for which males are more resistant than females for example; male mice (*Musmusculus*) are less susceptible than females to several parasites, including *Babesia microti*,

Toxoplasma gondii, Schistosoma mansoni and *Taenia crassiceps* (Morales-Montor *et al.*, 2004). Sex difference in response to *P. falciparum* infection may possibly be due to the previous reports that in the vicinity of humans, when exposed to a blend of physical and olfactory signals from more than one host, *Anopheles gambiae* can effectively and consistently express host-selection behaviour that results in non-random biting on males hosts compared to females (Mukabana *et al.*, 2002). Host-specific cues cause differential attractiveness of men to the Africa malaria vector *Anopheles gambiae* (Mukabana *et al.*, 2002). In some other related studies by Schuurs *et al.* (1990) while investigating the effect of gender and sex steroids on the immune response agued that males generally exhibits lower immune responses than female specifically, innate responses, antibody-mediated responses and cellular responses typically are higher in females than males. Immunological differences between the sexes may explain why males and females differ in their responses to parasites (Mukabana *et al.*, 2002).

A study by Mondal and Rai (1999) established that among humans and lizards, the phagocytic activity of neutrophils and macrophages is higher in females than males. However, Barna et al. (1996) agued that following parasitic or antigenic stimulation, the production and release of prostaglandin E2, thromboxane B2 and nitric oxide is reportedly higher in females than males. They also noted that antigen-presenting cells (APC) from females are more efficient at presenting peptides than are APC from males.

Humoral and cell-mediated immune responses also differ between males and females; antibodies produced by B-cells are observed to be typically greater in females than males (Falter *et al.*, 1991). In addition Bijlsma *et al.* (1999) asserts that T-cells, in particular

CD_4^+ helper T-cells, are functionally and phenotypically heterogeneous with females reportedly exhibiting higher Th2 associated cytokines (IL-4, IL-5, IL-6 and IL-10) than males. Previous studies have also hypothesed the critical role of immunoendocrine interaction, genetical responses and behavioral variation mediated by hormone in different responses of sex to parasitic infection (Olsen and Kovacs, 1996). It is therefore evident that in males are less immunocompetent than females living under the same malaria holoendemic area.

The findings of this study implies that male children should be highly protected from malaria and parents or guardians should be made aware of this fact so that incase of suspected malaria symptoms, immediate diagnosis and treatment should be sort. Nevertheless, further research should be done on male and female mortality rate due to malaria to justify the findings of this study.

5.6 Effect of Age on Malaria Infection

This study revealed that age inversely correlates with parasitaemia level such that the yonger the child the higher the parasitaemia level. This could be probably due to immunocompetance of the older children. This same sentiment was also echoed in London by Richard (2005) who asserted that from an early age the immune system is introduced to a variety of infectious agents. Aspinal *et al.* (2003) concurred with the argument and also observed that the immune responses are deliberately exposed either to attenuated organisms or to components of infectious pathogens in order to provoke a response. People living in malaria endemic areas tend to be infected repeatedly and over time, they gradually acquire immunity to malaria (Pawelec *et al.*, 2004)

A study carried out in Burkina Faso by Calissano *et al.* (2003) revealed that neonates begin to synthesize antibodies of the IgM class at an increased rate very soon after birth in response to immense antigenic stimulation of their new environment. Premature infants appear to be as capable of doing this as do full-term infants. At about 6 days after birth, the serum concentration of IgM rises sharply (Pawelec *et al.*, 2002). This rise continues until adult levels are achieved by approximately 1 year of age. Maternal IgG gradually disappears during the first 6-8 months of life, while the rate of infant IgG synthesis increases until adult concentrations of total IgG are reached and maintained by 7-8 yr of age. However, IgG1 and IgG4 reach adult levels first, followed by IgG3 at 10 yr and IgG2 at 12 yr of age (Aspinal *et al.*, 2000; Pawelec *et al.*, 2002; Calissano *et al.*, 2003; Pawelec *et al.*, 2004).

Moreover older children might have developed immunological memory to parasite leading to reduced parasitaemia while the younger one still had limited immune responses. The results of this study is comparable to findings in an area of Burkina Faso with hyperendemic malaria, which showed that in malaria-endemic regions, the levels of antibodies against malaria increase with age as a reflection of exposure to parasite antigens. The increase in antibody (IgG) levels is usually accompanied by a decreased risk to develop severe clinical symptoms of malaria (Calissano *et al.*, 2003). Whereas Desowitz (1989) while studying *Plasmodium*-specific immunoglobin E in sera from an area of holoendemic malaria revealed that IgE antibodies increased with age, becoming most significantly elevated in children more than four years of age probably explain the

reason why older children are relatively more resistant to malaria infection than the younger ones.

The findings of this study that younger children had higher percentage severe cases than the older ones has been supported in Tanzania where it was reported that age appears to influence not only the acquisition of clinical immunity to malaria but also the susceptibility to and clinical manifestations of severe malaria (Nicholas *et al.*, 1999). Another related study by Schwart *et al.* (2001) while evaluating the influence of age and other factors that affect clinical outcome of *P. falciparum* malaria in nonimmune patients established contrasting results that only 5% of the age below 40 years developed severe malaria as compared with 18% of the subjects who were 40 years of age. Moreover, all deaths occurred in the latter group. These findings could be possibly due to the fact that immune responses reduce with old age hence people above 40 years may be more prone than those below 40 years to malaria infection. However, this study is different from the above study since it focused on young children age 1-10 years. The younger children indicated clear higher malaria susceptibility than the older ones.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

(i) The data generated in this study suggest that individuals with blood group O are relatively resistant to severe effect of malaria infection compared to the ones with blood group A or B who are more susceptible and prone to developing severe malaria infection.

(ii) The severe malaria was observed to reduce the number of almost all the WBC except for the basophils which were increased.

(iii) Helper T-cell (CD4+) and cytotoxic (CD8+) subsets of T-lymphocytes cells were redused in malaria cases.

(iv) Male children were more vulnerable to malaria than the females in the study population and parasitaemia levels reduced with increase in age, indicating that younger children were more susceptible to severe malaria infection.

6.2 Recommendations

6. 2. 1 Application of the results

(i) Since Helper T-cell (CD4+) and cytotoxic (CD8+) subsets of T-lymphocytes cells were reduced in malaria cases it is therefore imparative for the clinicians to monitor the level of such cells in severe malaria cases and if possible immunopotentiaters be given since reduced levels of Helper T-cells and cytotoxic T-lymphocytes cells may subject the victim to prolonged healing and coinfections.

(ii) The fact that male children were more vulnerable to malaria than the females is a very important pointer towards disease management in sexes. Boys are likely to suffer severe malaria bouts than the girls.

(iii) Since blood group A correlated with malaria infection and disease severity, its advisable that blood group A individuals traveling to malaria endemic areas be given prophylaxis

6.2.2 Recommendation for future study

(i) It was noted that blood group O confers resistance to malaria infection, further studies should be carried out to establish the mechanism.

(ii) Despite no statistically significant difference in absolute leukocyte count and malaria infection, there was decrease and increase of some differential leukocytes, further investigations should be directed on leukocytes to establish their implications on malaria out come.

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APPENDICES

APPENDIX I: CONSENT FORM

(i) Title of Project Proposal: **Effect of ABO/Rh-Blood Group Phenotypes on Immmunosurveillance to *Plasmodium falciparum* Malaria in Young Children in Kisumu Town, Kenya.**

(ii) **Name of the guardian/parent**.....Sex.....Age.....

Resident.....Address.....

Name of the study subject.....Sex.....Age.....

(iii) **Purpose of the study:** Despite being the primary avenue for the parasite invasion, less study has been directed on erythrocytes. ABO- blood types and protection against *Plasmodium falciparum* malaria paradigm has remained an area of concern. Although the ABO- blood group phenotypes of human host have been reported to influence malaria infection, there have been few clinical observations on this effect and none has been carried out in Kenya. For proper management of malaria, it is vital to understand the relationship between blood group and immunity to malaria. A hospital -based comparative study is therefore necessary to investigate the relationship between blood group types and severity of the disease in *falciparum* infection, which shall be the priority of this project. The study is under the direction of Dr. J Vulule (Director CVBCR), Mr. I.O Genga (Chief Technologist CVBCR), Dr. M Oduor (Director Nyanza Regional Blood Transfusion Center and Provincial Pathologist) and Dr. M.M Gicheru (Lecturer Kenyatta University)

(iv) **Procedure to be followed:** One time point sampling for patient presented with malaria symptoms.

(v) **Risks:** There is no life threatening risk involved in this study.

(vi) **Benefits:** The parents/guardians shall be made aware of the malaria status of their children and referred to the doctor for medication. They will also be aware of ABO / Rh blood groups of their children.

(vii) **Confidentiality of the Records:** Your medical records related to this study will be kept under confidentiality.

(viii) **Obtaining Additional Information:** Your are encouraged to ask questions to get more information.

(ix) Sample collected will **not be used for HIV testing**.

(x) **Signature:** I have read the above information and have had an opportunity to ask questions and all of my questions have been answered. I consent to participate in this study. I have been given a copy of this consent.

Signature (parent/Legal guardian).....Date.....

I, the undersigned. Have fully explained the relevant details of this study to the guardian/ parent to consent for the patient. I am qualified to perform this job.

Signature (Investigator).....Name.....Date.....

APPENDIX II: Guava Easy CD4™ Reagent Kit (For the Enumeration of $CD3^+CD4^+$ T Lymphocytes in Blood)

1. Product Description and Intended use

The Guava® EasyCD4™ Kit is a two-color direct immunofluorescence reagent kit for enumeration of mature CD4+ T Lymphocytes in human blood. The Kit consists of a monoclonal anti-human CD3 antibody conjugated to the tandem dye phycoerythrin (PE)-Cy5 (PECy5), a monoclonal anti-human CD4 anti-human CD4 antibody conjugated to PE and Guava 1X Lysing Solution to lyse erythrocytes. The CD3 antibody uniquely identifes T cells and recognizes an epitope that is expressed on the epsilon chain of the CD3/T cell antigen receptor (TcR) complex. The CD4 antibody allows for the identification of human helper/inducer $CD4^+$ T cell (HLA Class II reactive) and recognizes a 60,000 Da MW surface antigen. CD4 is also present on monocytes but at much lower density and lack co-expression of the CD3 molecules.

2. Guava EasyCD4 Materials and Equipment

- Anti-human CD4-PE Reagent
- Anti-human CD3-PECy5 Reagent
- Guava Antibody Dilution Buffer
- Guava 1X Lysing Solution
- Guava Technologies PCA instrument with EasyCD4 Cytosoft™ software
- Guava Check Beads
- Anticoagulated Blood Sample for primary antibody labeling
- Pipettes
- Sample acquisition tubes, 1.5mL microcentrifuge tubes with screw caps
- Vortex mixer

- Disposable pipette tips
- Disposable Gloves
- Guava Instrument Cleaning Fluid (ICF)
- Deionized, distilled or RO water
- 10% Bleach Solution

APPENDIX III: DIFFERENTIAL BLOOD COUNT

Client No.	Lymphocyte No. Per µl of blood (%)	Neutrophil No.Per µl of blood (%)	Monocyte No. Per µl of blood (%)	Eosinophil No. Per µl of blood (%)	Basophil No. Per µl of blood (%)
001	22	74	2	1	1
002	16	79	3	0	1
003	34	64	0	1	1
004	65	27	1	7	0
005	55	41	2	2	0
006	45	52	2	1	0
007	78	21	0	0	1
008	48	49	1	1	0
009	34	63	3	0	0
010	28	71	1	9	0
011	24	74	1	1	0
012	35	49	1	14	0
013	20	75	7	2	1
014	64	32	2	0	0
015	41	55	2	1	1
016	39	56	1	4	0
017	44	51	1	3	1
018	31	62	1	6	0
019	46	48	5	0	0
020	38	59	0	2	1
021	33	64	2	0	1
022	40	57	1	2	0
023	15	14	1	0	0
024	31	64	3	2	0
025	34	61	0	1	0
026	58	39	1	2	0
027	29	62	1	2	0
028	68	28	1	3	0
029	67	26	2	4	1
030	39	47	2	4	1
031	49	48	1	1	1
032	43	52	1	1	1
033	63	32	2	2	1
034	42	55	2	1	0
035	40	57	2	1	0
036	52	44	2	1	0
037	43	50	1	1	1
038	41	48	2	8	1

Client NO.	Lymphocyte **No. Per µl of Blood (%)**	Neutrophil No. Per µl of Blood (%)	Monocyte **No. Per µl of Blood (%)**	Eosinophil No. Per µl of Blood (%)	Basophil **No. Per µl of Blood (%)**
039	54	40	1	0	0
040	37	54	1	2	1
041	32	60	0	1	1
042	67	31	2	1	0
043	62	34	2	2	0
044	57	38	1	2	0
045	56	41	3	2	0
046	62	31	3	4	0
047	31	61	1	3	1
048	34	63	1	1	1
049	36	58	3	3	0
050	52	46	2	0	0
060	67	30	2	4	1
061	42	55	1	2	1
062	43	46	1	2	1
063	68	29	3	0	1
064	63	31	1	0	2
065	51	46	2	1	2
066	13	85	1	1	0
067	78	22	0	1	0
068	62	26	1	4	1
069	20	76	1	7	0
070	31	61	1	3	0
071	68	31	1	3	0
072	41	56	1	2	0
073	34	61	0	1	1
074	45	41	1	8	0
075	24	72	2	1	1
076	44	50	3	0	1

APPENDIX IV: BLOOD GROUP/Rh PHENOTYPE

Client No.	Sex	Anti-A	Cell-A	Anti-B	Cell-B	Anti-D	Blood group
001	M	-	+	-	+	+	O+
002	M	+	-	-	-		A+
003	F	+	-	-	+	+	A+
004	M	-	+	-	+	+	O+
005	F	-	+	+	-	+	B+
006	M	+	-	-	+	+	A+
007	M	-	+	-	+	+	O+
008	M	-	+	+	-	+	B+
099	M	-	+	+	-	+	B+
010	M	-	+	-	+	+	O+
011	M	-	+	-	+	+	O+
012	M	+	-	-	+	+	A+
013	M	-	+	-	+	+	O+
014	F	-	+	-	+	+	AB+
015	M	+	-	-	+	+	A+
016	F	-	+	+	-	+	B+
017	M	-	+	-	+	+	O+
018	F	-	+	+	-	+	AB+
019	F	+	-	-	+	+	A+
020	M	+	-	-	+	+	A+
021	M	-	+	-	+	+	O+
022	F	-	+	-	-	+	AB+
023	F	-	+	-	+	+	O+
024	M	+	-	-	+	+	A+
025	M	-	+	-	+	+	O+
026	F	+	-	-	+	+	A+
027	F	-	+	-	+	+	O+
028	M	-	+	-	+	+	O+
029	M	-	+	-	+	+	O+
030	M	-	+	-	+	+	O+
031	F	-	+	+	-	+	B+
032	F	-	+	-	+	+	O+
033	M	+	-	-	+	+	A+
034	M	+	-	-	+	+	A+
035	F	-	+	+	-	+	B+
036	F	-	+	-	+	+	O+
037	M	+	-	-	+	+	A+
038	M	-	+	-	+	+	O+
039	F	-	+	-	+	+	O+

Client No.	Sex	Anti-A	Cell-A	**Anti-B**	Cell-B	Anti-D	Blood group
040	F	-	+	-	+	+	O+
041	M	+	-	+	-	+	AB+
042	F	-	+	-	+	+	O+
043	F	+	-	-	+	+	A+
044	F	+	-	-	+	+	A+
045	M	-	+	-	+	+	O+
046	M	-	+	+	-	+	B+
047	F	-	+	+	-	+	B+
048	F	-	+	-	+	+	O+
049	F	-	+	-	+	+	O+
050	M	+	-	-	+	+	A+
051	M	-	+	-	+	+	O+
052	F	-	+	-	+	+	O+
053	M	+	-	-	+	+	A+
054	M	+	-	-	+	+	A+
055	M	+	-	-	+	+	A+
056	M	-	+	-	+	+	O+
057	M	-	+	-	+	+	O+
058	F	-	+	-	+	+	O+
059	M	-	+	+	-	+	B+
060	F	+	-	-	+	+	A+
061	M	-	+	+	-	+	B+
063	M	-	+	+	-	+	B+
064	F	+	-	-	+	+	A+
065	M	+	-	-	+	+	A+
066	M	-	+	-	+	+	O+
067	M	-	+	+	-	+	B+
068	F	-	+	+	-	+	B+
069	F	-	+	+	-	+	B+
070	M	-	+	-	+	-	O-
071	M	+	-	-	+	+	A+
072	M	+	-	-	+	+	A+
073	F	-	+	+	-	+	B+
074	M	-	+	-	+	+	O+
075	M	-	+	-	+	+	O+
076	F	+	-	-	+	+	A+
077	F	-	+	-	+	+	O+
078	M	+	-	-	+	+	A+

APPENDIX V: SUMMARY OF MPS/PARASITAEMIA/CD4/CD8

Client No.	Age Years	Sex	MPS	Parasitaemia Level per µl	Blood Group	CD_4 Count/µL	% CD4	CD_8 Count/µl	% CD_8
001	5	M	-	-	O+	423.95	5.1	279.11	8.6
002	4	M	+	280	A+	311.98	2.1	169.02	7.1
003	4	F	+	360	A+	714.86	8.0	445.16	8.1
004	2	M	-	-	O+	624.62	11.2	407.0	8.1
005	2	F	+	4320	B+	373.93	2.7	265.0	3.2
006	2	M	+	17600	A+	396.29	1.3	167.12	1.2
007	2	M	+	293	O+	759.64	6.6	467.0	5.4
008	2	M	-	-	B+	340.90	4.4	286.0	3.8
009	9	M	-	-	B+	842.47	10.2	767.07	10.0
010	10	M	+	120	O+	340.90	4.4	163.31	1.9
011	9	M	+	80	O+	706.92	8.2	444.63	5.2
012	10	M	-	-	A+	*58.92	0.7	523.58	8.2
013	5	M	+	200	O+	162.84	2.3	241.22	3.2
014	3	F	-	-	AB+	1268.42	20.8	589.73	11.6
015	6	M	+	160	A+	318.79	16.5	161.57	3.8
016	5	F	+	360	B+	483.83	8.7	284.09	4.6
017	5	M	-	-	O+	219.85	11.7	188.11	3.9
018	2	F	-	-	AB+	311.02	7.9	278.0	6.9
019	8	F	+	1280	A+	737.60	10.6	500.04	6.4
020	5	M	+	17480	A+	495.94	10.9	409.0	8.3
021	1	M	-	-	O+	691.52	8.7	1383.69	19.1
022	3	F	+	4480	AB+	368.50	5.2	230.02	3.4
023	7	F	+	240	O+	713.74	13.2	217.56	4.3
024	5	M	+	32960	A+	194.06	7.4	166.28	4.1
025	4	M	-	-	O+	314.1	11.6	192.12	4.1
026	4	F	-	-	A+	456.75	11.8	366.73	6.2
027	2	F	+	120	O+	925.69	22.3	468.85	9.6
028	1	M	+	160	O+	966.85	19.0	134.14	4.7
029	6	M	-	-	O+	293.49	12.0	128.87	5.5
030	5	M	-	-	O+	507.64	6.3	1153.59	14.8
031	5	F	+	200	B+	1176.76	11.2	476.64	4.1
032	4	F	-	-	O+	632.96	9.5	492.80	6.9
033	2	M	+	7200	A+	1058.58	20.0	590.54	9.7
034	4	M	-	-	B+	233.84	9.9	150.65	3.3
035	3	F	+	280	O+	1902.63	14.9	847.21	5.1
036	2	F	-	-	A+	1076.35	15.2	657.87	9.4
037	8	M	+	5600	O+	696.07	19.6	604.01	15.7

SClient No.	Age Years	Sex	MPS	Parasitaemia Level per µl	Blood Group	CD_4 Count/µL	% CD4	CD_8 Count/µl	% CD_8
038	7	M	-	-	O+	381.39	3.5	356.07	3.1
039	10	F	-	-	O+	900.64	18.7	823.63	9.7
040	2	F	-	-	O+	364.04	10.2	371.23	8.2
041	4	M	+	360	AB+	70.79	1.9	102.57	0.7
042	8	M	-	-	O+	Not haemolysed	***	Not haemolysed	***
043	1	M	-	-	A+	Not haemolysed	***	Not haemolysed	***
044	4	F	-	-	A+	316.72	16.1	162.54	3.7
045	4	M	-	-	O+	483.71	8.7	284.07	4.6
046	4	M	-	-	B+	481.83	8.7	287.0	4.9
047	5	F	+	32960	B+	192.06	7.4	165.1	4.2
048	2	F	+	2560	O+	712.72	13.0	217.56	4.3
049	4	F	-	-	O+	373.91	2.6	264.71	3.2
050	1	M	+	3760	A+	611.62	11.1	405.0	8.0
051	1	M	-	-	O+	692.62	11.3	1410.31	13.3
052	5	F	+	480	O+	114.7	9.9	234.15	4.6
053	5	M	-	-	A+	150.76	9.6	195.03	3.8
054	8	M	+	7920	A+	74.62	5.4	152.86	1.4
055	4	M	-	-	A+	463.86	20.6	359.12	6.0
056	7	M	+	120	O+	211.06	11.1	220.15	0.8
057	6	M	-	-	O+	206.41	2.0	2212.82	6.9
058	6	F	-	-	O+	755.13	13.9	451.77	7.4
059	7	M	-	-	B+	451.64	4.9	131.36	1.2
060	5	F	+	7520	A+	101.86	0.7	270.91	3.6
061	8	M	-	-	O+	820.03	11.5	1009.57	3.4
062	2	F	-	-	B+	749.36	13.8	1213.98	18.4
063	4	M	-	-	O+	434.78	4.1	494.27	9.9
064	7	F	+	480	A+	167.61	3.1	154.11	2.9
065	2	M	-	-	A+	1831.18	18.9	592.12	8.0
066	4	M	-	-	O+	777.90	6.5	344.0	7.6
067	2	M	-	-	B+	1730.05	19.5	587.11	13.4
068	2	F	+	360	B+	1354.47	6.8	644.7	9.4
069	10	F	+	120	B+	1299.41	6.0	567.13	12.1
070	3	M	-	-	O^-	1428.49	3.2	456.1	12.9
071	5	M	+	1600	A+	312.4	6.1	276.0	7.0
072	4	M	+	680	A+	508.2	6.4	466.1	4.1
073	2	F	+	240	B+	318.7	16.2	162.4	3.8
074	6	M	-	-	O+	340.6	4.2	158.1	1.8
075		M	-	-	O+	371.2	2.5	261.9	3.1
076		F	-	-	A+	423.8	5.1	278.0	8.7
077		F	+	720	O+	302.1	2.70	169.1	7.1
078		M	+	1200	A+	162.8	2.2	244.6	3.2