EFFECT OF INTERLEUKIN 4 AND RECEPTOR GENE POLYMORPHISMS IN RELATION TO OXIDATIVE STRESS DURING UNCOMPLICATED MALARIA INFECTION

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THESIS SUBMITTED TO THE DEPARTMENT OF CHEMICAL PATHOLOGY, UNIVERSITY OF GHANA MEDICAL SCHOOL (UGMS), IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A MASTER OF PHILOSOPHY (M. PHIL) DEGREE IN CHEMICAL PATHOLOGY

JUNE 2011

DECLARATION

I hereby declare that with the exception of references to other people's work, which have been duly acknowledged, this thesis is the outcome of my own research conducted at the Department of Chemical Pathology (UGMS, Korle-Bu) and the Life Sciences Unit, Department of Medical Laboratory Sciences, School of Allied Health Sciences, Korle-Bu).

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DEDICATION

Not unto us, O LORD, not unto us, but unto thy name give glory, for thy loving-kindness. I dedicate this work to my family, most particularly to my mum, who has never ceased to spur me to attain greater heights in academia and integrity in the LORD. My profound appreciation goes to my sweet wife and lovely children for their priceless support and encouragement.

ACKNOWLEDGEMENT

I could never have made it without the Almighty God, who has honoured His promise to help me with His victorious right hand. He walked me through, from the beginning to the completion of this project, and I owe it all to Him.

I am extremely grateful to my supervisors, Dr. Henry Asare-Anane, Dr. Ben Gyan and Mr. Richard Harry Asmah for committing themselves through collaboration, to ensuring the final outcome of this evidence of academic achievement. May God reward your invaluable efforts.

I thank the staff of the Life Sciences Unit (School of Allied Health Sciences), Clinical Virology Unit and Molecular laboratory (Department of Microbiology, UGMS) for permitting me to use resources belonging to their outfit. I thank Mr. Isaac Boamah of the Virology Unit (Microbiology Department, UGMS) for his words of encouragement and technical assistance particularly during the bench phase of this project. My appreciation is extended to Mr. Selorm Adukpo and Mr. Kofi Badu for their timely help in analysing my samples.

My sincere gratitude goes to my Head of department, Senior Staff, Administrative Assistants and all other members of the Chemical Pathology Department (UGMS) for their diverse contribution towards this work. I really appreciate the help offered me by the Staff of the Polyclinic (Korle Bu Teaching Hospital) in getting my samples for my study in this project. To my classmates: Richmond, Ofori, Bani and Tijani, I say that I value all your constructive comments that helped fine-tune the relevant portions of my

proposal, presentations and finally the defence of this thesis. Many thanks for contributing to this academic feat!

ABSTRACT

Parasitic infections such as malaria in host organisms often lead to oxidative stress condition resulting in the constant generation of free radicals and other reactive species *in vivo* that lead to extensive oxidative damage in bio-molecules such as DNA and proteins. Susceptibility of Plasmodium parasite to oxidative stress is a well-established feature and advantage has been taken of this property to design some pro-oxidant anti-malarial drugs. This study was carried out with the aim of determining single nucleotide polymorphisms in interleukin (IL) 4 gene and its receptor gene, and their relationship to the generation of free radicals by the human host during uncomplicated malaria infection.

The study population were one hundred subjects, reporting for medical care at the Polyclinic of the Korle Bu Teaching Hospital, Accra with uncomplicated malaria. Apparently healthy children (n = 41) without detectable malaria parasites were used as controls. Haematological analysis was done for all the study population. The gene regions containing the +33 C/T polymorphism of IL-4, and Pro-478-Ser of the IL-4Rα were amplified by Polymerase Chain Reaction (PCR) and the various genotypes determined by Restriction Fragment Length Polymorphism (RFLP) using the restriction enzymes (B*smF* I for IL-4 and *Kpn* I for IL-4Rα gene regions respectively). Oxidative stress situations in the human host and its effect on malaria parasites were determined using the DNA comet assay determined by a commercial kit, and levels of reactive oxygen species in the infected RBCs of cases and uninfected controls was measured using the superoxide dismutase assay.

A significant mean difference in neutrophil levels was observed when the uncomplicated malaria cases were compared with the controls (p = 0.001). It was observed that the mean Hb value of the control group did not differ significantly when compared with the cases (p = 0.07). Moderate to extensive DNA damage of the malaria parasite was demonstrated in increasing levels of estimated parasitaemia among the uncomplicated malaria cases, using the DNA comet assay. Significant correlation was observed between SOD levels and IL4R (Pro-478-Ser) (p = 0.017) polymorphism as well as between neutrophils and IL4 (+33) SNP (p = 0.002), indicating a likely interaction between the gene and neutrophil in parastite clearance in malaria infection, via the genotoxic effects of the super oxide anion.

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AMA	Apical membrane antigen
CD	Cluster of differentiation
CM	Cerebral malaria
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
DALYs	Disability-adjusted life years
DMSO	Dimethylsulphoxide
dNTPs	deoxynucleotide triphosphates
EBA	Erythrocyte binding antigen
EDTA	ethylenediaminetetraacetic acid
EMP	Erythrocyte membrane protein
GSH	Glutathione
G6PD	Glucose-6-phosphate dehydrogenase
Hb	Haemoglobin
HRP	Histidine-rich protein
ICAM	Intracellular adhesion molecule
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
iNOS	inducible nitric oxide synthase
IRF	Interferon regulatory factor
MoH	Ministry of Health
MCV	Mean corpuscular volume

MSP	Merozoite surface protein
NADP	Nicotinamide adenine dinucleotide phosphat
NKCs	Natural killer cells
NO	Nitric oxide
P. falciparum	*Plasmodium falciparum*
PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline
PECAM	Platelet endothelial cellular adhesion molecule
RBCs	Red blood cells
RFLP	Restriction fragment length polymorphism
RNI	Reactive nitrogen intermediates
RON	Rhoptry neck
ROS	Reactive oxygen species
ROI	Reactive oxygen intermediates
SCGE	Sickle cell gel electrophoresis
SOD	Super oxide dismutase
STEVOR	Subtelomeric variable open reading frame
TBE	Tris borate EDTA
Th	T-helper
TNF	Tumour necrosis factor
VSA	Variant surface antigen
VCAM	Vascular cellular adhesion molecule
WBCs	White blood cells
WHO	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria remains one of the leading causes of morbidity and mortality worldwide and in sub-Saharan Africa (WHO, 2008). Mortality from the disease is due to complications arising as a result of severe infections usually caused by *P. falciparum* (WHO, 2000), which is an obligate intracellular Apicomplexa parasitic protozoa (Pierce and Miller, 2009). Studies on mortality have shown that deaths occur predominantly among young children (WHO, 2008). In Ghana, malaria is hyperendemic and presents a serious health problem in the country (Asante and Asenso-Okyere, 2003). It is the nation's leading cause of deaths in children less than five years old and accounts for over 40% of outpatient attendance with annual reported cases of 2.2 million between 1995 and 2001 (Asante and Asenso-Okyere, 2003). The impact of malaria goes beyond mortality and morbidity as the disease results in reduction in school attendance amongst children and productivity at work by their parents (Asante *et al.*, 2004).

Parasitic infections such as malaria in host organisms often lead to oxidative stress condition which is a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defences (Becker *et al.*, 2004). The constant generation of free radicals and other reactive species *in vivo* lead to extensive oxidative damage in parasite bio-molecules such as DNA, lipids and proteins,

resulting from oxidative stress. Susceptibility of Plasmodium parasite to oxidative stress is a well-established feature and advantage has been taken of this property to design some pro-oxidant anti-malarial drugs (Long *et al.*, 2006).

Previous work also suggests that oxidative damage in red blood cells (RBCs) is accelerated upon infection with *P. falciparum* and that this leads to their enhanced removal from circulation by phagocytosis (Famin and Ginsburg, 2003; Becker *et al.*, 2004). It has also been shown that malaria parasites are particularly vulnerable to oxidative stress during their erythrocytic life cycle (Winter *et al.*, 1997). This is seen in polymorphisms such as sickle cell anaemia, β-thalassaaemia and Glucose-6-phosphate dehydrogenase deficiency (G6PD) that confer enhanced oxidative stress in RBCs and allow a certain amount of resistance to infection with Plasmodium and limit the severity of malaria disease.

In humans, malaria provides a clear example of host genetic factors influencing the onset, progression, type of disease developed, and ultimate outcome of infection (Hill, 1998).

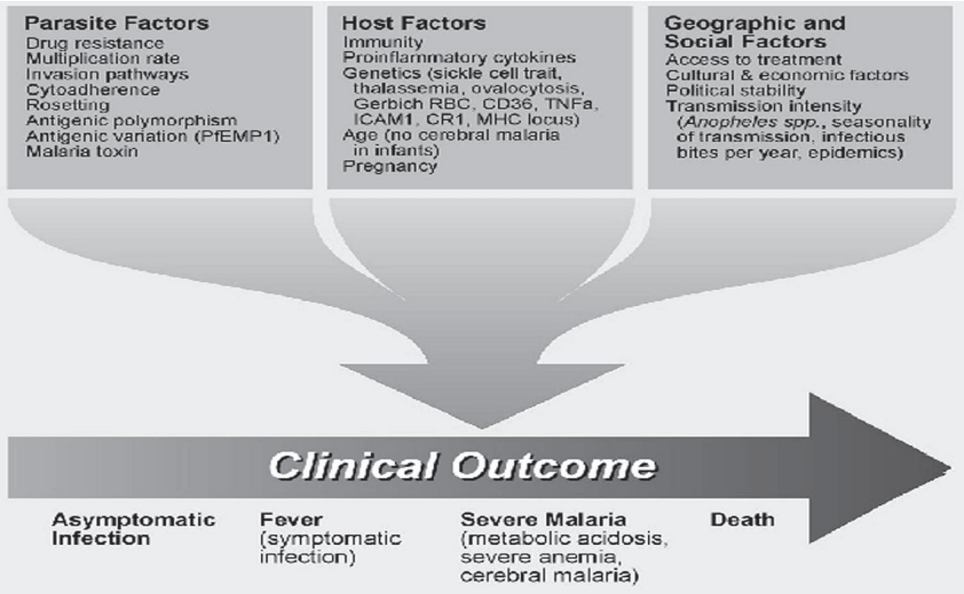


Figure 1.0: The clinical outcome of a malarial infection in an Africa child depends on many parasite, host, geographic and social factors. These converge in the child to result in a range of outcomes, from an asymptomatic infection to severe disease and death (adapted from Weatherall et al., 1992).

Epidemiological data together with linkage and association studies have shown that selection pressure from the parasite has caused retention of disease-associated but malaria-protective alleles in the human population, suggesting co-evolution of the host and parasite. Such otherwise deleterious alleles include those causing sickle cell anemia (Willcox *et al.*, 1983), thalassemias (Weatherall, 2001), and glucose-6-phosphate dehydrogenase deficiency (Ruwende, 1995).

Polymorphisms in other erythroid proteins, including common variants of the Duffy antigen, the erythrocyte band 3 (Allen, 1999), and glycophorin C (Patel, 2001), as well as variants in the TNFα cytokine (McGuire *et al.*, 1994) and the CD36 scavenger receptor (Aitman *et al.*, 2000) are also associated with protection against malaria. Over the course of the last decade, a number of studies have provided evidence for a linkage between the blood infection level of *Plasmodium falciparum* and the human

chromosome 5q31 region in African populations (Garcia *et al.*, 1998; Flori *et al.*, 2003; Sakuntabhai *et al.*, 2008). Studies conducted in Burkina Faso suggested the genetic component of susceptibility showing linkage between parasitemia levels and 5q31-q33 region (Rihet, 1998).

The 5q31-33 region contains genes encoding the T helper 2-type cytokines (the interleukin genes *IL3*, *IL4*, *IL5*, *IL9*, and *IL13*) and other immunologically active genes such as interferon regulatory factor-1 (*IRF1*). These genes are strong candidates for controlling the outcome of malaria infection. Overall, the genetic component of malaria susceptibility is acknowledged to be very complex and heterogeneous in humans and is further modified by environmental factors (Kwiatkowski, 2000).

1.2 Problem Statement

The malaria parasite is a prevalent human pathogen with at least 300 million acute cases of malaria each year globally and more than a million deaths (Kristoff, 2007). About 90% of all malaria deaths in the world today occur in Africa south of the Sahara. This is because the majority of infections in Africa are caused by *Plasmodium falciparum*, the most dangerous of the four human malaria parasites. Studies on mortality have shown that deaths occur predominantly among young children and mortality rates among patients with an illness severe enough to warrant hospitalization are consistently high with case fatality rates varying from 10% to 30% (Murphy and Breman, 2001).

In Ghana, malaria is hyperendemic and presents a serious health problem in the country (Asante and Asenso-Okyere, 2003). It is also the nation's leading cause of deaths and accounts for over 40% of outpatient attendance with annual reported cases of 2.2 million between 1995 and 2001. Children less than five years are the most affected (Roll Back Malaria, 2008). A study conducted by Ministry of Health in 2006 showed that more than 17 million of Ghana's 20 million people are infected with malaria every year, with cost of $85 million for treatment (ASI-Ghana, 2009).

Despite the importance *P. falciparum* as a human pathogen, the pathophysiologic basis of the disease is not well understood. In erythrocytes, *P. falciparum* encounters enhanced oxidative stress, resulting largely from its digestion of haemoglobin and thus its redox balance is fragile (Sylke, 2004). Intra-erythrocytic malaria parasites ingest and digest the cytosol of their host cell which consists mostly of haemoglobin. Superoxide (O_2^-) is normally produced when oxidized haemoglobin is exposed to the acid environment of the food vacuole, and can therefore be considered as the major generator of ROS. Inside the parasite, regardless of its origin, O_2^- is dismutated by superoxide dismutase (SOD) to H_2O_2. Two genes coding for iron-containing SODs have been identified in the *P. falciparum* genome (Becker *et al.*, 2004).

Previous work suggests that upon infection with *P. falciparum*, the human host induces oxidative stress to the parasite in the erythrocyte and this leads to their enhanced removal from circulation by phagocytosis (Kodjo *et al.*, 2004). It has also been shown that the parasites are vulnerable to oxidative stress during their erythrocytic life stages (Postma *et al.*, 1996; Becker *et al.*, 2003).

Genetic factors are a major determinant of child survival in malaria endemic countries. Identifying which genes are involved and how they affect the malaria disease risk will potentially offer a powerful mechanism to better appreciate the host-parasite relationship. Understanding the molecular basis of the parasite-host interaction during infection in relation to oxidative stress will help in the search for new potential drugs targets and vaccine candidates.

1.3 Justification

Malaria, caused by parasites transmitted to humans by mosquitoes, is one of the world's most common and serious tropical diseases. Half the world's population, which is found in more than 100 countries, is at risk of malaria. Children are at particular risk, accounting for most malaria deaths globally (WHO, 2008). Children are at risk because they lack specific active anti-malaria immunity to protect against the disease (Roll Back Malaria, 2008). Although preventable and treatable, malaria causes significant morbidity and mortality, particularly in resource-poor regions.

Sub-Saharan Africa is the hardest hit region in the world, and parts of Asia and Latin America also face significant malaria epidemics (Guerra *et al.*, 2008). While anyone living in or visiting an endemic country may be at risk, certain groups, particularly children and pregnant women, are more vulnerable. The World Health Organization (WHO) estimates that in 2006, there were 109 malaria-endemic countries and approximately 3.3 billion people at risk for infection, worldwide. There were 247 million cases of malaria and 881,000 deaths, mostly among children, under the age of five (Roll Back Malaria, 2008; WHO, 2008).

As a disease, malaria is not only a serious public health problem but is also a major development problem in all endemic countries of the African Region (Asante *et al.*, 2004). From a macroeconomic perspective, malaria mortality and morbidity slow economic growth by reducing capacity and efficiency of the labour force. Malaria also presents significant costs to affected households since it is possible to experience multiple and repeated episodes in a year (Asante *et al.*, 2004).

The total cost of malaria to Africa was an estimated US$12 billion in 2000, which is about 3% of the total GDP of the Region (WHO, 2004). This therefore makes malaria an important development problem in Africa. In Ghana, the cost of illness due to malaria represented a substantial burden on poorer households. The total cost of illness due to malaria in 2002 was estimated at per capita average cost of US$2.63 or US$13.51 per household (Asante *et al.*, 2004).

The generation of the genotoxic superoxide anion, together with other reactive oxygen intermediates, serve to combat a diverse array of pathogens. These reactive oxygen species (ROS), effectively generated by neutrophils, have been shown to be highly toxic for intra-erythrocytic malaria parasites (Bouharoun-Tayoun *et al.*, 1995), and correlated with fast parasite clearance in children with *P. falciparum* malaria (Greve *et al.*, 1999). Opsonized *P. falciparum* merozoites are known to participate in triggering neutrophil respiratory bursts, and are enhanced by cytokines (Kumaratilake *et al.*, 1992) including IL-4 and its receptor. This study would provide insight into the likely relationship between IL-4 and IL-4Rα genes in parasite clearance via the genotoxic effects of the super oxide anion.

1.4 Hypothesis

In this study, it is hypothesized that IL 4 and IL 4R genes play a role in the generation of free radical by the host resulting in oxidative stress on *P. falciparum* parasite during uncomplicated malaria infection.

1.5 Aim

To determine single nucleotide polymorphisms in IL 4 gene and IL 4Rα gene and their relationship to the generation of reactive oxygen species by the host during uncomplicated malaria infection.

1.6 Specific Objectives

1. To analyse haematological parameters of study subjects in relation to controls.
2. To detect oxidative DNA damage using comet assay in parasite-infected RBCs during uncomplicated malaria infections.
3. To measure reactive oxygen species during malaria infection using the superoxide dismutase assay.
4. To determine single nucleotide polymorphisms in IL 4/IL 4R genes involved in oxidative stress effects using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of Malaria

Malaria has infected humans for over 50,000 years, and *Plasmodium* may have been a human pathogen for the entire history of the species (Joy *et al.,* 2003). References to the unique periodic fevers of malaria are found throughout recorded history, beginning in 2700 BC in China (Cox, 2002). The term malaria originates from Medieval Italian: *mala aria*—"bad air"; and the disease was formerly called *ague* or *marsh fever* due to its association with swamps and marshland (Bruce-Chwatt, 1981; Biggs and Brown, 2001). Malaria was once common in most of Europe and North America (Mary, 1999) where it is no longer endemic (Norman, 2006) though imported cases do occur.

Scientific studies on malaria made their first significant advance in 1880, when a French doctor, Charles Louis Alphonse Laveran, observed parasites for the first time, inside the red blood cells of people suffering from malaria. He, therefore, proposed that malaria is caused by this organism, the first time a protist was identified as causing disease (Bruce-Chwatt, 1981; Gilles, 1993). However, it was Britain's Sir Ronald Ross who finally proved in 1898 that malaria is transmitted by mosquitoes. He did this by showing that certain mosquito species transmit malaria to birds and isolating malaria parasites from the salivary glands of mosquitoes that had fed on infected birds (Ross, 1897; Gilles, 1993).

Although the blood stage and mosquito stages of the malaria life cycle were identified in the 19th and early 20th centuries, it was not until the 1980s that the latent liver form of the parasite was observed (Krotoski *et al.,* 1982; Meis *et al.,* 1983). The discovery of this latent form of the parasite finally explained why people could appear to be cured of malaria but still relapse years after the parasite had disappeared from their bloodstreams.

2.2 The Global Malaria Epidemic

Malaria, caused by parasites transmitted to humans by mosquitoes, is one of the world's most common and serious tropical diseases. Half the world's population is at risk for malaria, which is endemic in more than 100 countries. Children are at particular risk, accounting for most malaria deaths globally (WHO, 2008). Malaria causes considerable morbidity and mortality, although a preventable and treatable disease, particularly in poorly-resourced regions. Parts of Asia and Latin America have encountered major malaria epidemics, with Sub- Saharan Africa being the hardest hit region in the world (WHO, 2010).

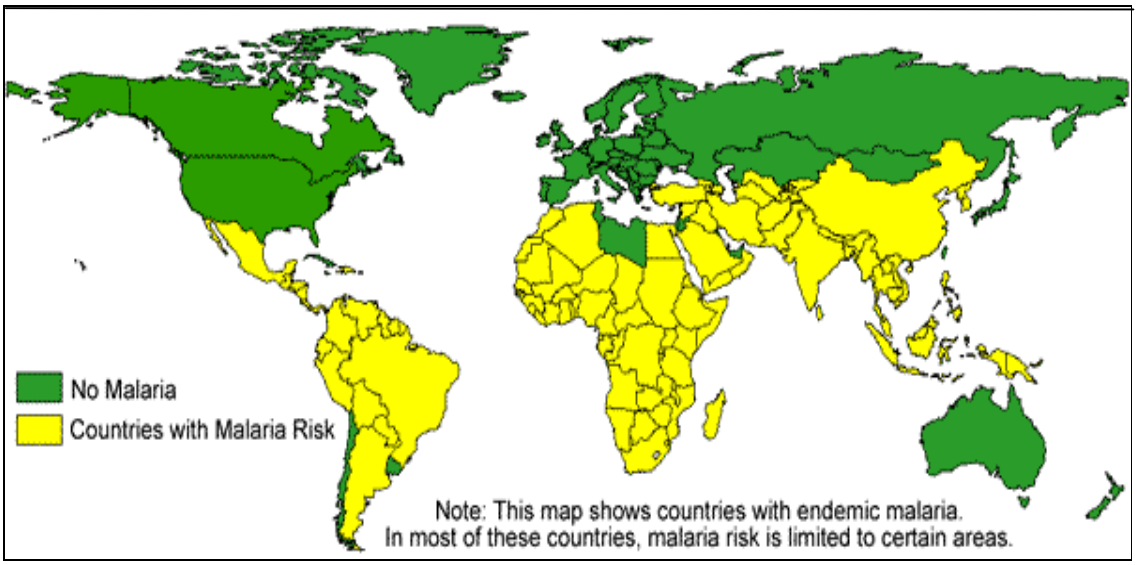


Figure 2.0 Map of malaria endemic countries (Adapted from CDC, 2003).

Regional and international efforts to address malaria began in the 1940s and 1950s, and strategies have evolved over time (Tanner and Savigny, 2008). From the early 1950s until 1978, malaria was eliminated in parts of the Americas, Europe, and Asia. However, such efforts did not reach or were unsuccessful in many of the endemic areas, particularly sub-Saharan Africa (Tanner and Savigny, 2008). More recent attention to these regions by the United States, other donor governments, multilateral institutions, and affected countries, has helped to increase access to prevention and treatment and reduce cases and deaths (Roll Back Malaria, 2008; United Nations Report, 2008; WHO, 2008).

Still, while access to interventions has increased, gaps remain and many challenges continue to complicate malaria-control efforts in hard hit areas, including poverty, poor sanitation, weak health systems, limited disease surveillance capabilities, drug

and insecticide resistance, natural disasters, armed conflict, migration, and climate change (WHO, 2008; Tanner and Savigny, 2008; Roll Back Malaria, 2008; Senior, 2008). Other high-risk groups include travellers, refugees, displaced persons, and migrant workers entering endemic areas (Roll Back Malaria, 2008).

The expanded malaria control programmes have helped decrease malaria cases and deaths significantly (UN, 2008). Since 2000, seven African countries have experienced at least a 50% reduction in malaria cases and deaths; 22 countries outside of Africa have experienced at least a 50% reduction in malaria cases (WHO, 2008). Ninety two formerly endemic countries or territories are now considered malaria-free by WHO (Roll Back Malaria, 2008).

2.2.1 Burden of Malaria in the African Region

As a disease, malaria is not only a serious public health problem but is also a major development problem in all endemic countries of the African Region. From a macroeconomic perspective, malaria mortality and morbidity slow economic growth by reducing capacity and efficiency of the labour force. The economic burden of malaria is the total loss or reduction in output (gross domestic product) associated with malaria morbidity and mortality. Malaria also presents significant costs to affected households since it is possible to experience multiple and repeated episodes in a year. It traps the most vulnerable households in a vicious cycle of poverty.

Sub-Saharan Africa accounts for 90% of the world's 300 million to 500 million cases of malaria and 1.5 million to 2.7 million deaths annually (Roll Back Malaria, 2002).

In sub-Saharan Africa, 12.5% of all disability adjusted live years (DALYs) were lost to malaria in 2003 (WHO, 2004). Only with the upsurge of the HIV/AIDS pandemic has malaria been pushed to the second rank as the highest contributor to the disease burden in the African Region. While malaria contributed 2.05% to the total global deaths in 2000, it was responsible for 9.0% of all deaths in Africa (WHO, 2002).

It has been estimated that the countries with intensive malaria have roughly one-third the income levels of the others, globally (Gallup and Sachs, 2001). Furthermore, it was shown that from 1965 to 1990, countries with intensive malaria transmission grew by 1.3 percent less per person annually, controlling for factors such as initial poverty, economic policy, tropical location and life expectancy. The total cost of malaria to Africa was an estimated US$12 billion in 2000, which is about 3% of the total GDP of the Region (WHO-AFRO, 2004). This therefore makes malaria an important development problem in Africa.

2.2.2 Evidence from Ghana

Malaria is hyperendemic in Ghana, with a crude parasite rate ranging from 10–70% and *Plasmodium falciparum* dominating. It is the number one cause of morbidity, accounting for 40-60% of outpatient attendance in public health facilities, with average annual reported cases of about 2.2 million between 1995 and 2001, with over 10% admitted (MoH- Ghana, 2002).

In Ghana, the disease is a major killer and the leading cause of mortality among children who are less than five years. It accounts for an average of 13.2% of all mortality cases in Ghana and 22% of all mortalities in children under five. In the case of pregnant women, of the total number reporting at health institutions, 13.8% suffer from malaria and 9.4% of all deaths in pregnant women are malaria-related (Antwi and Marfo, 1998). The disease is the leading cause of workdays lost due to illness in Ghana and thereby contributing more to potential income lost than any other disease. It has been shown that economically active persons who suffer from malaria lose approximately nine working days per episode, with males losing more time off than females. In addition, caretakers sacrificed more than five workdays on average to care for the sick, who were mostly children (Antwi and Marfo, 1998).

The total cost of illness due to malaria in Ghana in 2002 was estimated at per capita average cost of US$2.63 or US$13.51 per household. This figure is equivalent to 9.74% of the per capita government expenditure on health. The average cost per case to the MoH/GHS was estimated at US$2.94 (Asante *et al.*, 2004).

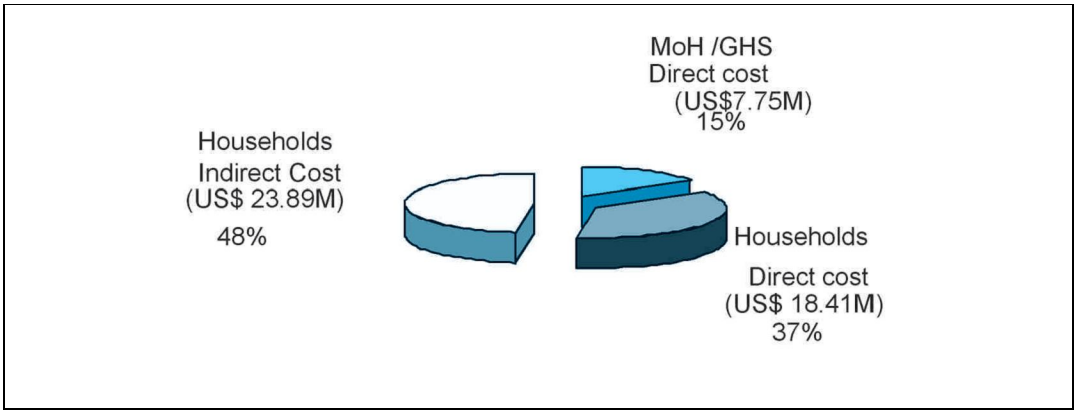


Figure 2.1 Total cost of malaria in Ghana: 2002 (Adapted from Survey data, 2003)

2.3 THE LIFE CYCLE OF MALARIA PARASITES

Plasmodia present a sexual multiplication phase in the insect vector, a first asexual multiplication phase in the tissue of the vertebrate host, followed by the main asexual multiplication phase in the blood. The comprehensive life-cycle of the species of Plasmodium comprises the exoerythrocytic and erythrocytic stages in the vertebrate host and the sporogonic cycle in the mosquito (Fig. 2.2). Humans and other vertebrates act as the intermediate host for the parasite, while the mosquito, in which the sexual reproduction takes place, is considered to be the definitive one.

2.3.1 The Exoerythrocytic stage

Malaria infection in the human host starts when the sporozoites are injected into the blood stream during a blood meal by an infectious mosquito. The sporozoites remain in circulation for a few minutes, before they actively enter the liver of the host (Lopez-Antunano and Shmunis, 1980). The Kuppfer cells in the liver may be invaded (or the parasite may be phagocytosed) but the sporozoites are not able to develop in those cells and die shortly after invasion. Most parasites however invade the hepatocytes and start the asexual exo-erythrocytic schizogonic cycle. The sporozoite initially appears as a mononucleated round body into the cytoplasm of the host cell; subsequently it begins to develop and multiply asexually, a mature schizont (the multinucleated stage of the parasite) is formed, and finally a large number of merozoites are released. The liver cycle ends when the mature schizont ruptures and releases the merozoites into the sinusoids of the liver.

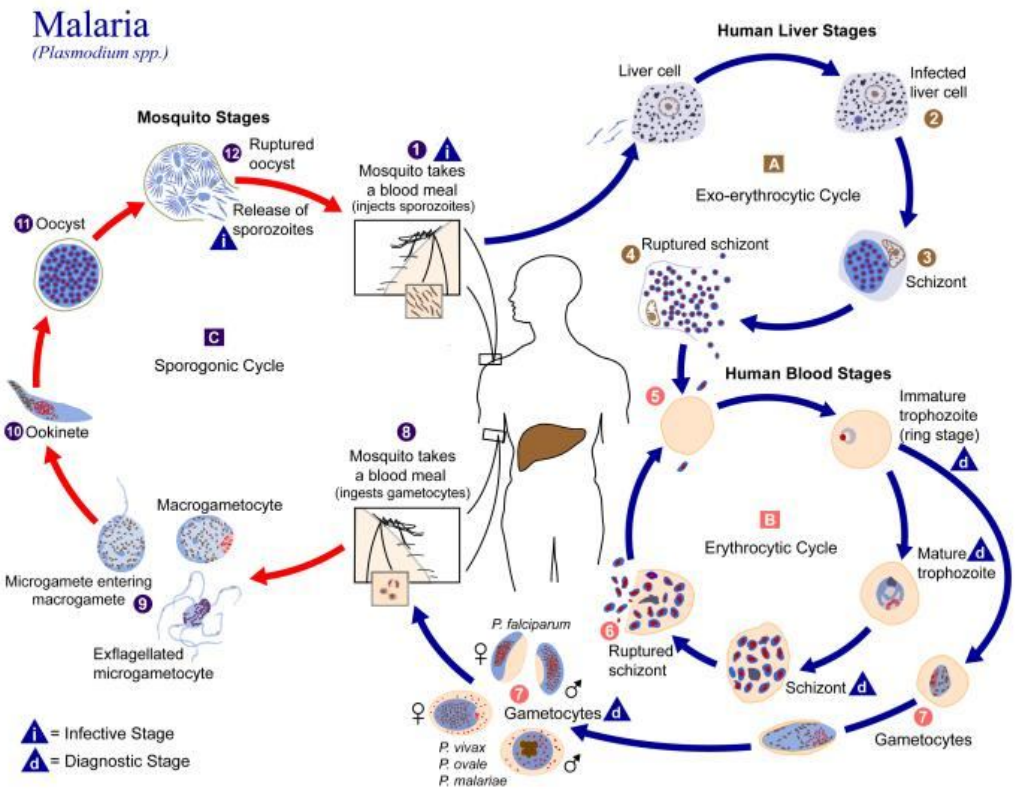


Figure 2.2 Life cycle of *Plasmodium sp.* (Adapted from CDC, 2004).

2.3.2 The Erythrocytic stage

The blood phase of the life-cycle is initiated when the merozoites from liver schizonts are discharged into the circulation (Garnham, 1988). After initial contact and reorientation of the merozoite, the apical tip of the merozoite comes in contact with the erythrocyte membrane, resulting in the formation of a tight junction and translocation of the rhoptry neck protein (RON) complex across the host cell membrane (Fig. 2.3).

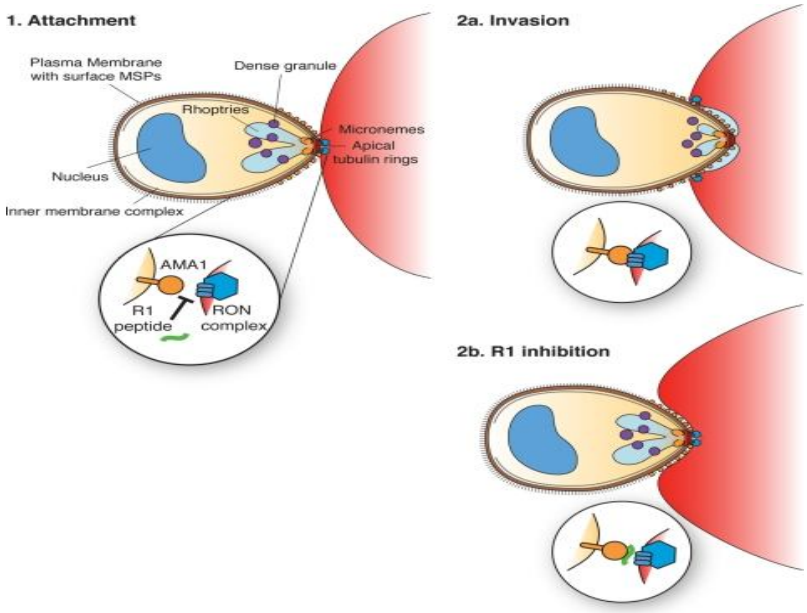


Figure 2.3 Early step of merozoite invasion of red cells (Adapted from : Dave *et al.*, 2010)

Interaction between the RON complex and apical membrane antigen (AMA) 1 triggers secretion of the rhoptry bulb contents, which are then used in the generation of the nascent parasitophorous vacuole (Cowman and Crabb, 2006). The parasite can then pull itself through the tight junction powered by its actomyosin motor. Binding of the R1 peptide to the AMA1 hydrophobic trough prevents its interaction with the RON complex and subsequent rhoptry secretion (Dave *et al.*, 2010). However, because the tight junction and the invasion motor are established, the merozoite is able to pull on the red blood cell (Bannister *et al.*, 1986) which ends up wrapping around the parasite because of the absence of a nascent parasitophorous vacuole.

The merozoite almost immediately invades an erythrocyte to enter its trophozoite stage. A vacuole is produced by the parasite which assumes the characteristic ring form (the young trophozoite). The parasite enters the stage of a schizont following

nuclear division of the trophozoite (Pavithra *et al.*, 2004). At the end of this phase, the schizogonic cycle is completed, the erythrocyte ruptures releasing the merozoites into the blood stream, determining the typical malaria paroxism. The merozoites discharged into the circulation invade new erythrocytes to repeat the schizogonic cycle (Cowman and Crabb, 2006) until the process is inhibited by the specific immune response or by chemotherapy. During the schizogonic cycle (within a red blood cell) some of the merozoites become differentiated into sexual forms (the gametocytes).

The erythrocytic stage of malaria parasites has several important implications in clinical practice: first, this is the only stage causing the complex and varying spectrum of symptoms characterizing the disease in humans. Again, the recognition of parasites in the blood of a patient allows the diagnosis of the infection and the differentiation of the various species of the causing agent.

2.3.3 The Sporogonic cycle

The sporogonic cycle starts with the ingestion of mature female and male gametocytes by the female *Anopheles* during a blood meal. As soon as gametocytes reach the midgut of the insect, the female gametocyte shed the red blood cell and remains free in the extracellular space as a macrogamete. The male gametocyte nucleus divides into eight sperm-like flagellated microgametes each of which also leaves the erythrocyte, reaches the midgut and actively moves to fertilize a macrogamete. Exflagellation of the microgametocyte is triggered by factors present in the mosquito midgut (Nijhout and Carter, 1978; Sinden, 1983; Billker *et al.*, 1998; Garcia *et al.*,

1998). The result of the fertilization process is the zygote, which develops into the elongated, slowly motile ookinete.

The ookinete actively penetrates the peritrophic membrane and the epithelium of the midgut and settles beneath the basal lamina of the outer gut wall (Sieber *et al.*, 1991; Adini and Warburg, 1999; Vlachou *et al.*, 2001) where it develops into a non-motile oocyst after the blood meal. The product of the mature oocyst are the sporozoites which actively leave the cyst passing through small perforations without destroying the wall, at least till most of the parasites have been released, and move into the haemocelomic space of the insect. The sporozoites employ a chemotactic response to locate the salivary glands (Akaki and Dvorak, 2005) where they penetrate the basal membrane, pass intracellularly through a secretory cell and settle into the salivary duct (Wernsdorfer, 1980). When the mosquito feeds, the salivary fluid (which has anti-clotting properties) and its content of sporozoites are actively injected into the vertebrate host to start another asexual replicative cycle (Ribeiro and Francischetti, 2003).

2.4 MALARIA AND THE IMMUNE SYSTEM IN HUMANS

The complexity of immune responses to malaria has been increasingly recognized over recent years, together with an appreciation that any single antigen-specific response is unlikely to afford much immunity on its own. This is reflected in the multiple life-stages in the life cycle of *Plasmodium* and the large genome. With around 5000 genes, there are myriad potentially important immune targets, making the identification of protective responses highly challenging. Furthermore, immune

responses are not only involved in preventing infection and clearing parasites but also might instead contribute to the pathogenesis of severe malaria if they are inappropriate in their nature and extent (Mackintosh *et al.*, 2004).

After repeated exposure to malaria, individuals eventually develop effective immunity that controls parasitemia and prevents severe and life-threatening complications (Marsh and Kinyanjui, 2006). Similarly, effective immunity can be induced by repeated experimental infections in animals, and have also been induced by experimental infections in humans (Pombo *et al.*, 2002). These observations continue to provide a strong rationale that an effective vaccine against malaria is achievable. Effective immunity seems to require both humoral and cellular immune responses, probably in co-operation, although the relative importance of each remains unclear. The acquired response is thought to target predominantly blood-stage parasites, but antigens expressed by sporozoites and malaria-infected hepatocytes also seem to be important.

2.4.1 Innate Immunity

The innate response to malaria has, until recently, received relatively little attention. However, studies in both mice and humans have repeatedly shown that proinflammatory cytokines, specifically IL-12, IFN-γ, and TNF-α, are essential mediators of protective immunity to erythrocytic malaria (Stevenson *et al.*, 1995; Favre *et al.*, 1997); these cytokines are derived from either the innate or adaptive arm of the immune response. In humans, IFN-γ production is correlated with resistance to

re-infection with *Plasmodium falciparum* (Luty *et al.,* 1994) and protection from clinical attacks of malaria (Dodoo *et al.,* 2002), plasma TNF-α and nitrogen oxide levels are associated with resolution of fever and parasite clearance (Kremsner *et al.,* 1995; Kremsner *et al.,* 1996), and plasma TNF-α and IFN-γ mediate loss of infectivity of circulating gametocytes (Naotunne *et al.,* 1991). Many vaccine developers now regard IFN-γ production to be the hallmark of effector T-cell function for malaria (Doolan and Good, 1999; Plebanski and Hill, 2000).

A critical review of the literature (Fell and Smith, 1998) concluded that control of the early peak of parasitemia in murine malaria infections was dependent on innate rather than adaptive cellular immune mechanisms, raising important questions about the role of innate immunity in control of human malaria. There is less information regarding the role of innate immune mechanisms in controlling blood stage malaria infections; however, depletion of natural killer (NK) cells from *Plasmodium chabaudi*-infected mice results in a more severe course of infection with higher parasitemia and increased mortality (Mohan *et al.,* 1997).

Regarding the human immune response to malaria, it has been shown that peripheral blood mononuclear cells (PBMCs) from malaria-unexposed donors can produce IFN-γ in response to stimulation by either live or dead schizont antigens (Currier *et al.,* 1992; Zevering *et al.,* 1992; Dick *et al.,* 1996; Waterfall *et al.,* 1998). Cytokine induction is dependent on the presence of both monocytes and lymphocytes indicating that this is not a classical endotoxin-like response as had previously been thought (Schofield and Hackett, 1993). Increased NK-like cytotoxicity has been reported

during mild malaria infection (Ojo-Amaize *et al.*, 1981), but appears to be depressed in children with severe disease (Stach *et al.*, 1986).

2.4.2 Humoral Immunity

Passive transfer studies in which antibodies from malaria-immune adults were successfully used to treat patients with severe malaria have provided the most direct evidence that antibodies are important mediators of immunity to malaria (Sabchareon *et al.*, 1991). Protective antibodies are thought to target primarily merozoite surface antigens, erythrocyte invasion ligands and variant surface antigens expressed by *P. falciparum*-infected erythrocytes (IEs) (Bull and Marsh, 2002; Good *et al.*, 2004). In an approach that considered both the magnitude and breadth of the antibody response to a large panel of merozoite antigens, Osier *et al.* (2008) found that a broad antibody response was strongly associated with protection from clinical malaria and the strongest associations with protection were found with combined responses to merozoite surface protein (MSP) 2 and MSP3. Antibodies to merozoite antigens are also believed to act by directly inhibiting merozoite invasion of erythrocytes or opsonising merozoites for phagocytosis (Bouharoun-Tayoun *et al.*, 1990).

The IgG subclass response could also be important for antibody function. Studies in Papua New Guinea children found that IgG3 to apical membrane antigen 1 (AMA1) was strongly associated with protection from malaria, whereas there was only a weak association with IgG1 (Stanisic *et al.*, 2010). Other studies have also pointed to the importance of IgG3 responses in protection from malaria (Roussilhon *et al.*, 2007).

P. falciparum can use different pathways for invasion of erythrocytes during blood-stage infection by varying the expression and/or use of the erythrocyte binding antigens (EBAs) and *P. falciparum* reticulocyte binding homologs (PfRhs) (Duraisingh *et al.*, 2003; Stubbs *et al.*, 2005). Recent findings indicate that variation in invasion phenotype alters parasite susceptibility to human inhibitory antibodies and that this parasite property might exist as a mechanism that facilitates immune evasion (Persson *et al.*, 2008).

During intra-erythrocytic development, *P. falciparum* expresses highly variant antigens on the erythrocyte surface, known as variant surface antigens (VSAs). These antigens include *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), rifins, subtelomeric variable open reading frame (STEVOR) and others (Beeson and Brown, 2002). The importance of each of these antigens is unclear, but PfEMP1 is thought to be the most important target of antibodies (Leech *et al.*, 1984; Biggs *et al.*, 1991). PfEMP1 is encoded by the *var* multigene family, and different *var* genes encode PfEMP1 variants with different antigenic and adhesive properties (Smith *et al.*, 1995). Antigenic diversity and variation by *P. falciparum*-infected erythrocytes, through expression of different VSAs, enables *P. falciparum* to cause repeated infections over time and new infections seem to exploit gaps in the repertoire of variant-specific antibodies (Marsh and Howard, 1986). With increasing exposure, a broad repertoire of antibodies is obtained that eventually provides protection against most variants.

2.4.3 Cell-Mediated Immunity

T-cells are crucial for malaria immunity, especially during the erythrocytic stage of infection. T-cells help produce immunoglobulins (IgG1, IgG2a, IgM, IgE) (Phillips *et al.*, 1997; Taylor-Robinson 1995) to activate macrophages and Th1 immune response, which are essential in the early stage of malaria. Later, there is an immunity switch to a Th2 response with antibody-mediated mechanisms to eliminate parasites (Collier *et al.*, 1998). Macrophages, neutrophils and other phagocytic cells are key components of the antimicrobial and tumoricidal immune responses, because these cells are capable of generating large amounts of highly toxic molecules, reactive oxygen and reactive nitrogen intermediates (ROI, RNI) (Bogdan *et al.*, 2000).

Both Th1 and Th2 cells can protect the host from malaria infection. Th1 cells protect, in part at least, by the NO pathway, whereas Th2 cells protect by enhancing a specific IgG1 antibody response (Taylor *et al.*, 1997). Both CD4+ and CD8+ T-cells appear to serve a protective role with IFN-γ (Hommel and Barnish, 1998). In addition, early NO production may promote the proliferation of specific CD8+ T-cells, or perhaps a subset required to eliminate parasites (Scheller *et al.*, 1997). Both CD4+ T-cells and IFN-γ are necessary to induce NO synthesis in infected hepatocytes or hepatic iNOS (Klotz *et al.*, 1995).

The effector functions of macrophages include release of H_2O_2, ROI, RNI, NO, TNF and production at least eighty other cytokines and enzymes (Clark *et al.*, 1996). Macrophages can be stimulated by IFN-γ and subsequently TNF-α to produce high levels of NO. They can kill erythrocytic-stage of malaria parasites by different mechanisms, including phagocytosis of smaller parasites and secretion of many

cytotoxic factors. Macrophages also act as killer cells by antibody-dependent cell-mediated cytotoxicity (ADCC) (Roitt *et al.*, 1998). In addition, TNF-α and IFN-γ can induce production of RNI by neutrophils, Kupffer cells and hepatocytes (Gyan *et al.*, 1994). There are some possible effects of Ab action during parasite development, including blockage of merozoite dispersion, inhibition of cell invasion, intracellular killing of erythrocytic stages, inhibition of reverse cytoadherence and cooperation with various cells to increase cell-mediated killing (Hommel, 1996).

2.4.4 Cytokines and soluble mediators

Cytokines play an important role in the defence against malaria and some have long been recognized to have anti-parasitic effects on different stages of malaria. This protective effect was further demonstrated by administration *in vivo* of some key cytokines (Grau *et al.*, 1992). A large number of cytokines appear to be involved in malaria, that is, TNF-α, IFN-γ, GM-CSF, IL-1, IL-4, IL-6, IL-8, and IL-10 (Hommel, 1996). Nitric oxide production during murine malaria is regulated *in vivo* by the Th1-cytokines (TNF-α and IFN-γ), but not by IL-4, which is a Th2 cytokine. TNF-α and IFN-γ induce high amounts of NO involved in controlling the peak level of parasitemia (Jacobs *et al.*, 1996). To date, NO is known to affect the production of more than 20 cytokines, including IL-1, IL-6, IL-10, IL-12, IFN-γ, TNF-α, and TGF-β by various immune cells, namely, macrophages, T-lymphocytes, natural killer cells (NKC) and endothelial cells (Bogdan *et al.*, 2000).

Conversely, more than 30 cytokines or cytokine-like factors have been described that increase or inhibit the expression of inducible nitric oxide synthase (iNOS) activity in cells participating in the immune response: macrophages, microglia, Kupffer cells, neutrophils, eosinophils, mast cells and NKC (Bogdan *et al.*, 2000). The importance of a balance in the cytokine network to achieve protective immunity has been emphasized; eventual effects will depend on the amount of these cytokines released and the rate, time and site of production (Grau *et al.*, 1992).

Plasmodia have the ability to promote the secretion of TNF-α, IL-1, lymphotoxin (LT) with some overlapping functions (Rockett *et al.*, 1992). TNF-α and LT are able to increase RNI production, which may serve as anti-microbial cooperation between them (Rockett *et al.*, 1991). IFN-γ and TNF-α transmit a series of immune signals leading to expression of NOS (Jones *et al.*, 1996) or activation of iNOS (Good and Doolan, 1999). IFN-γ, IL-1, IL-6, TNF-α, C- reactive protein (CRP) and NO have all been implicated in killing exoerythrocytic stage *Plasmodium* (Collier *et al.*, 1998).
A close association was found between expression of spleen IFN-γ and iNOS mRNA and levels of IFN-γ and NO in serum (Tsutsui and Kamiyama, 1999). Nitric oxide produced in high concentrations by iNOS can inhibit Th1 cell proliferation, which may act by blocking the synthesis of IL-2, a major Th1 cell factor (Taylor *et al.*, 1997). Interleukin 6 and TNF-α increase acute phase proteins and these molecules may trap RNI and ROI (Motard *et al.*, 1993). Levels of IL-10 also rise in cerebral malaria (CM) and other severe forms of malaria (Jakobsen *et al.*, 1995). It is likely that the effect of NO is highly specific, since it has little or no effect on the secretion of IL-4 and IL-10 (Taylor *et al.*, 1997), whereas, IL-4 does not appear to be involved in regulating NO production *in vivo* (Jacobs *et al.*, 1996). Nitric oxide synthesis by

macrophages is induced by IFN-γ and TNF-α; therefore, when they act together it is greatly increased (Roitt *et al.*, 1998). TGF-β can also inhibit NO synthesis, whereas migration inhibitory factor (MIF) activates macrophages to produce NO (Liew, 1992).

2.4.4.1 IL-4 and Malaria Severity

The IL-4 gene is pleiotropic, located in the 5q31–q33 region, with multiple immune-modulating functions on a variety of cell types (Marsh *et al.*, 1994). IL-4 serves as an important regulator in isotype switching from IgM/IgG to IgE (Vitetta *et al.*, 1984; Del Prete *et al.*, 1988). It also regulates the differentiation of precursor T helper-cells into the Th2 subset that regulates humoral immunity and specific-antibody production (Romagnani, 1995). In the human *P. falciparum* system IL-4 has been shown to be involved in the regulation of antimalarial antibody responses, including antimalarial IgE (Troye-Blomberg *et al.*, 1990; Elghazali *et al.*, 1997).

Several polymorphisms in the IL-4 gene have been described, four of which are located in the promoter region of the gene (Nakayama *et al.*, 2000). Some of these polymorphisms have been implicated in the regulation of total IgE production (Marsh *et al.*, 1995; Hizawa *et al.*, 2000). A study was conducted to analyse three known IL-4 polymorphisms, namely, a single nucleotide polymorphism (SNP) in the IL-4 promoter region (C→T) at position - 590 base pairs from the open reading frame, one SNP at position + 33 relative to the transcription initiation site and the variable number of tandem repeat (VNTR) region in intron 3 of the IL-4 gene (Gyan *et al.*, 2004). This was carried out in children with cerebral malaria, severe anaemia, uncomplicated malaria or controls to see if any of the polymorphisms were correlated

with severity of disease and total IgE and antibody levels. Data from the study suggested that IL-4 and/or IgE play a regulatory role in the pathogenesis of severe or complicated malaria.

2.5 Cerebral malaria (CM)

Cerebral malaria is a neurological syndrome and a severe complication of *P. falciparum* malaria occurring 6-14 days after infection, which generally leads to death even after treatment (Hommel, 1996). One million victims of CM are reported annually in African children (Grau *et al.*, 1992). The majority of animals in experimental rodent CM die early in week two after infection, with progressive hypothermia, histological observations of brain hemorrhages, mental disturbances and adherence of WBC to the endothelial lining (Curfs *et al.*, 1992; Hermsen *et al.*, 1997). In *P. falciparum* CM, peripheral red blood cells (PRBCs) are sequestered in the brain capillaries, leading to macrophage activation and NO release (Favre *et al.*, 1999). Electron microscopy shows multiple electron-dense knobs protruding from the membrane of the PRBC in capillaries (Aikawa, 1988), which is attached to the cerebral capillary endothelial cells by the knobs. In addition, knobs contain proteins produced by the parasite, on the surface of PRBC, which have a key role in cytoadherence (Wyler, 1990).

A variety of cytoadherent receptor molecules have been recognized, including cluster differentiation 36 (CD36), intracellular adhesion molecule-1 (ICAM-1), thrombospondin (TSP), E-selectin, P-selectin, vascular cellular adhesion molecule-1

(VCAM-1), platelet endothelial cellular adhesion molecule-1 (PECAM-1)/CD31, αv β3 and chondritin sulfate (Collier *et al.*, 1998; Mazier *et al.*, 2000). Moreover, at least 4 malarial proteins have been identified on the surfaces of *P. falciparum* PRBC, including histidine-rich protein 1 (HRP1), HRP2, erythrocyte membrane protein 1 (EMP1), and EMP2 (Aikawa, 1988).

Hypotheses that describe the etiology of CM include micro-vascular obstruction by coagulation-induced thrombus formation, deposition of immune complexes and local inflammation leading to alteration of cerebral permeability and oedema (Wakelin, 1988; Hommel, 1996). The current hypothesis defines a central role for intracapillary sequestration of PRBC cytoadherence to endothelial receptors (Hommel, 1996). The explanation for the mechanism of coma in CM may be the association between cytokines, such as TNF-α and free diffuseable NO (Rockett *et al.*, 1992).

2.6 Severe malarial anaemia

The degree of anaemia and the rate at which it develops during conditions of severe malaria varies enormously. The haemoglobin concentration of patients may fall as low up to 2 g/dl every 24 hours. In children this can turn out a serious problem as sudden death can occur particularly at Hb value of less than 4 g/dl.

The pathogenesis of severe malaria anaemia is multi-factorial and it includes obligatory destruction of RBCs containing parasites at the merogony stage; accelerated destruction of non-parasitised RBC also contributes to the disease severity (Davis *et al.*, 1990). Again, severe malaria anaemia arises due to decreased

concentrations of IL-10 which has inhibitory effect on TNF alpha (Meisel *et al.*, 1996). The latter contributes to anaemia by its influence in bone marrow suppression and destruction of RBCs.

2.7 Uncomplicated Malaria

The accompanying signs of an uncomplicated malaria infection are few, with the notable absence of lymphadenopathy or rash, but include splenomegaly and mild jaundice. Follow-up of treated cases is essential as parasites may recrudesce and repeat a latent infection if the course of treatment is incomplete or in the case of administering parasite-resistant medication (Weatherall *et al.*, 2002).

2.8 Oxidative Stress in Malaria Infection

Alterations in redox metabolism in malaria may be important in two ways. First, oxidative changes form a central aspect of the host response to the disease. In children with malaria, plasma lipid peroxides are increased, especially in those with concomitant riboflavin deficiency (Das *et al.*, 1990). Erythrocyte lipid peroxidation is also increased, and erythrocyte GSH, catalase and tocopherol are all significantly lower in malaria patients than in control subjects (Das and Nanda, 1999). Oxygen radicals have been demonstrated to be important in mice and humans for clearance of disease (Clark and Hunt, 1983; Greve *et al.*, 1999). A study carried out showed that paracetamol decreased oxygen radical production and hence, delayed parasite clearance (Brandts *et al.*, 1997). Knockout mice lacking NADPH oxidase (due to

disruption of the gp91phox gene) cannot produce superoxide, and suffer more rapid increases in malaria parasite densities than wild type mice (Sanni *et al.*, 1999).

These observations suggest that impaired production of ROS by monocytes might exacerbate infection. However, altered redox metabolism at the level of the host cell (especially endothelial cells) may also contribute to disease manifestations, and enhanced oxidative stress on erythrocytes may contribute to haemolysis and development of anaemia.

2.8.1 Oxidative Stress and RBC sequestration

Plasmodium falciparum IRBCs sequester in the deep circulation, adhering to endothelial cell receptors including non-class A scavenger receptor, or CD36, intracellular adhesion molecule 1 (ICAM-1 or CD54), platelet-endothelial cell adhesion molecule (PECAM-1 or CD31) and P-selectin in the microvasculature. In the placenta, *P. falciparum* IRBC adherence is via glycosaminoglycans, chondroitin sulphate A (CSA) and hyaluronic acid (Barnwell *et al.*, 1985; Berendt *et al.*, 1989; Fried and Duffy, 1996; Treutiger *et al.*, 1997; Udomsangpetch *et al.*, 1997; Beeson *et al.*, 2000). Other endothelial receptors supporting adhesion of IRBCs have also been described, but their correlation with clinical disease is lacking. The principal parasite-derived adhesin is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a family of variable proteins, members of which have been shown to mediate adhesion to CD36, CSA, ICAM-1 and P-selectin (Baruch *et al.*, 1996; Reeder *et al.*, 1999; Senczuk *et al.*, 2001). The effect of oxidative stress on PfEMP1 expression or conformation is unknown.

Expression of some of these endothelial adhesion molecule receptors for IRBCs is upregulated by oxidant stress. In falciparum malaria, levels of tumour necrosis factor (TNF) correlate with disease severity and prognosis (Grau *et al.,* 1989; Kwiatkowski *et al.,* 1990). Tumour necrosis factor synergises with intracellular oxidants to increase or induce expression of ICAM-1 and VCAM-1 (Terada, 2002), whereas reducing agents significantly decrease cytoadhesion of IRBCs to CD36-expressing cells (Gruarin *et al.,* 2001).

Oxidative stress may also increase adhesion of IRBCs through increased expression of phosphatidylserine on the surface of the infected erythrocytes, which appears at least partially to mediate adhesion of IRBCs to CD36 (Eda and Sherman, 2002). CD36 adhesion is common in patient isolates (Ockenhouse *et al.,* 1991) and the relative contributions of PfEMP1 and phosphatidylserine on IRBCs to such adhesion may vary between parasite isolates, although most papers suggest PfEMP1 to be the dominant ligand (Cooke *et al.,* 1998; Baruch *et al.,* 2002).

2.8.2 Oxidative stress and severe anaemia

The increase in lipid peroxidation reported in human malaria (Das and Nanda, 1999) may affect the membrane of both IRBCs and uninfected erythrocytes (Omodeo-Sale *et al.,* 2003). Uninfected erythrocytes co-cultured with IRBCs show accelerated senescence. Decreased red cell deformability-a feature of senescent red cells, is seen in malaria infection, with the greatest decrease in severe anaemia (Dondorp *et al.,* 1999). Decreased deformability was associated with increased mortality from malaria

in adults and children (Dondorp *et al.*, 1997; Dondorp *et al.*, 2002). The deleterious consequences of increased erythrocyte rigidity may include microcirculatory obstruction (exacerbating tissue hypoperfusion), and rigid red cells may be more likely to be removed by the spleen, exacerbating anaemia.

Also thrombocytopenia is common in malaria. Haemorrhage is rare, but is associated with poor prognosis (Clemens *et al.*, 1994). Intravascular coagulation occurs in placental malaria (where fibrin deposition is common) (Walter *et al.*, 1982), and fibrin thrombi and platelet deposition are commonly seen in cerebral vessels of children with fatal cerebral malaria (Grau *et al.*, 2003). Activity of tissue factor, the dominant intravascular initiator of coagulation, is increased in inflammatory conditions including sepsis and acute lung injury (reviewed by Abraham, 2000) and is upregulated by reactive oxygen species (Cadroy *et al.*, 2000). Recently, tissue factor expression by placental monocytes was shown to be increased (Imamura *et al.*, 2002). Although it has not been studied in other tissues in malaria, increased tissue factor activity may explain the common finding of fibrin thrombus deposition in cerebral microvessels at autopsy.

2.8.3 Oxidative Stress and Th cell differentiation

Upon activation, naive Th cells differentiate into at least two types of polarized responses (Street and Mosmann, 1991, Murphy *et al.*, 2000). Th1 cells secrete IFN-γ, TNF, and lymphotoxin (Street and Mosmann, 1991; Szabo *et al.*, 2003). They are associated with cell-mediated immunity and pathological autoimmune states characterized by organ-specific inflammation (King and Sarvetnick, 1997; Singh *et*

al., 1999). Th2 cells secrete IL-4, IL-5, and IL-13 (Street and Mosmann, 1991; Murphy *et al.,* 2000; Paul, 1991). They are important for Ab-mediated immunity and resistance to parasitic infection, and are associated with pathologic states such as allergy and asthma (Pritchard *et al.,* 1997; Romagnani, 2000; O'Shea *et al.,* 2002).

There are a number of factors that influence the decision of naive $CD4^+$ T cells to differentiate into Th1 or Th2 effectors. The primary factor is the presence of key cytokines at the time of T cell activation (O'Garra, 1998). T cells exposed to IL-4, a product of other $CD4^+$ T cells as well as mast cells, tend to become Th2 cells (Street and Mosmann, 1991; Murphy *et al.,* 2000; O'Garra, 1998; Trinchieri, 2003).

Modulation of intracellular signaling pathways also occurs when cells are exposed to reactive oxygen species (ROS) (Szabo *et al.,* 2003) such as peroxide or superoxide. It is well-documented that oxidative stress activates NF-κB, although the exact mechanism is unclear. Exposure of freshly isolated peripheral blood T cells to the antioxidant vitamin α-tocopherol (vitamin E) results in a reduction in IL-4 production (Li-Weber *et al.,* 2002). Possible mechanisms for this effect include the inhibition of NF-κB binding to chromatin, or the activation of NF-κB in a protein kinase C-dependent manner (Li-Weber and Kramer, 2003). Reactive oxygen species are produced at sites of inflammation by myelophagocytic cells as well as in response to exogenous factors such as aryl hydrocarbons contained in environmental tobacco smoke and diesel exhaust particles. Miranda *et al.,* (2006) investigated the role of chronic oxidative stress in the polarization of Th2 responses. They demonstrated that exposure of $CD4^+$ T cells to low levels of superoxide anion leads to up-regulation of

the entire family of Th2-specific cytokines, as well as modulation of chemokine receptors associated with T cell polarization.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study design, site and samples

This case-control study was carried out at the Polyclinic of the Korle Bu Teaching Hospital, Accra. One hundred blood samples were collected from children reporting for medical care with symptomatic uncomplicated malaria; this was after consent had been obtained from their parents/guardians. Information relating to the demography of study participants was documented using a standard questionnaire. Patients selected for the study were from different ethnic groups in Ghana who enrolled at the hospital.

3.2 Inclusion criteria

The study covered malaria patients aged 14 years or below. Written consent was obtained from parent or guardian concerning the requirements of the protocol. Apparently healthy children without detectable malaria parasites were used as controls.

3.3 Malaria Case Definition

The criteria for diagnosis of malaria were based on these criteria as follows: fever (>37.5^{o}C) measured within 24 hours of admission, malaria parasitaemia and at least one other sign of malaria (vomiting, diarrhoea, malaise). Uncomplicated malaria (UM) was defined as fully conscious patients with hemoglobin (Hb) value of at least 8g/dL, microscopically confirmed malaria parasitemia and with no clinical features of

severe malaria. Thick blood films stained with Giemsa stain for the detection of plasmodium parasites was taken from all subjects, i.e. cases and controls.

3.4 Exclusion Criteria

A parent/ guardian's refusal to give informed consent on behalf of a child or comply with requirements of the protocol ethically excluded such child from participation in the study. Subjects who had any other disease or were found to be positive for sickling test (metabisulphite method) were also excluded from the study.

3.5 Sample size determination

The minimum sample size of children selected was determined by the formula;

$$N = \frac{Z^2 (P)(1-P)}{(ERROR)^2}$$

Where Z, 1.96 is the standard score for the confidence interval of 95%

P, 0.5 is the sample proportion

A 6% allowable error, ERROR was used.

Minimum sample size, $N = \frac{1.96^2(0.5)(1-0.5)}{(7/100))^2}$

= 196 samples.

A total of one hundred and forty one (141) samples were however obtained for the study.

3.6 Specimen collection and transportation

Four (4) ml of venous blood samples was collected into EDTA tubes and stored at $4^{O}C$ until required for use.

3.7 Parasitological and Haematological measurements

The asexual form of *P. falciparum* was detected by light microscopy using thick and thin blood smear stained with Giemsa. Haematological parameters such as haemoglobin level, total RBC count and MCV were measured with an auto haematological analyser (Sysmex K21, Japan).

3.8 Genomic DNA Extraction

Genomic DNA was extracted from the buffy coat of EDTA-preserved whole blood samples, using QIAGEN DNeasy tissue kit (QIAGEN Co., Germany). The extracted genomic DNA was stored at $-20^{0}C$ until required for use.

3.8.1 Genotyping of IL 4/IL 4R Polymorphisms

IL 4/IL 4R polymorphisms were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis.

3.8.2 Genotyping IL-4 Gene Polymorphism (Locus +33 C/T)

Using the method described by Gyan *et al.*, (2004), amplification of the IL 4 gene regions that contain the +33 polymorphism was carried out using the oligonucleotide

primer set 5' -GTG CTG ATT GGC CCC AAG TGA CTG- 3' and 5' –GGA CTG CCA CCA ACC ACC AGT- 3'(forward and reverse primers, respectively). For the DNA amplification, 50µl PCR reaction mix containing 10µl of 5X PCR (with $MgCl_2$) buffer, 0.5µl of each of the four deoxyribonucleotide triphospates (dNTPs) at 10mM, 1.0µl of each of the oligonucleotide primers and 0.25µl of the *Taq* polymerase enzyme (5U/µl) (Sigma, Missouri, USA) were used. Three microlitre (3µl) of the extracted genomic DNA was used as template for the amplification using a PTC 100 thermal cycler (MJ Research Inc., USA). The thermocycling process included an initial denaturation at 95 ^{O}C for 10min, followed by 30 cycles at 95 ^{O}C for 50 sec, 62 ^{O}C for 50 sec and 72 ^{O}C for 50 sec. A final extension step of one cycle at 72 ^{O}C for 5 min concluded the reaction. For each reaction, a 47.0µl reaction mix that contained no DNA template (but ddH_2O) was included as negative control. The PCR products were analysed directly by electrophoresis on a 2% agarose gel stained with ethidium bromide.

3.8.2.1 RFLP Locus +33C/T

The PCR products were further analysed by Restriction Fragment Length Polymorphism Analysis (RFLP) with the restriction enzyme B*smA* I for +33 C/T. The B*smF* I enzyme digestions were carried out using the protocol as described by the manufacturers (New England Biolabs Inc., USA). The 20µl reaction volume contained 12 µl of the amplified product, 0.5µl of 1U B*smA* I, 2µl of NEBuffer 4 (New England Biolabs Inc., USA) and 5.5µl sterile double-distilled water. The PCR products were digested at 65 ^{O}C for five hours. Electrophoresis was carried out on the digested fragments using a 2% agarose gel stained with ethidium bromide.

3.8.3 Genotyping IL-4R (Pro-478-Ser) Single Nucleotide Polymorphism

The polymorphism Pro-478-Ser of the IL 4R gene region, was amplified using the primer set 5' -CTT ACC GCA GCT TCA GGT AC- 3' and 5' -TTT CTG GCT CAG GTT GGG GC- 3' (Eurogentec, Seriaing, Belgium). The 50µl PCR reaction contained 10µl of 5X PCR (with $MgCl_2$) buffer, 0.5µl of each of the four deoxyribonucleotide triphospates (dNTPs) at 10mM, 1.0µl of each of the oligonucleotide primers and 0.25µl of the *Taq* polymerase enzyme (5U/µl) (Sigma, Missouri, USA). The extracted genomic DNA was used as template for the amplification using a PTC 100 thermal cycler (MJ Research Inc., USA). The thermocycling process involved an initial denaturation at 95 ^{O}C for 12min, followed by 35 cycles at 95 ^{O}C for 30 sec, 56 ^{O}C for 2min and 72 ^{O}C for 40 sec. A final extension step of one cycle at 72 ^{O}C for 5 min concluded the reaction. For each reaction, a negative control that contained no DNA template was included. The PCR products were analysed directly by electrophoresis on 2% agarose gel stained with ethidium bromide.

3.8.3.1 RFLP Analysis - Pro-478-Ser

The PCR products were analysed by RFLP with the restriction enzyme *Kpn I* to genotype the Pro-478-Ser polymorphism using the recommended protocol of the manufacturers (New England Biolabs Inc., USA) The 20µl reaction volume contained 12 µl of the amplified product, 0.5 µl of 1U *Kpn I*, 2 µl of NEBuffer 4 (New England Biolabs Inc., USA) and sterile double-distilled water, and digested at 37^{O}C overnight. Electrophoresis was carried out on the digested fragments using a 4% agarose gel stained with ethidium bromide. The sizes of the PCR product were

determined by comparison with the mobility of a 100 base-pair molecular marker (Promega, Madison, USA).

3.9.1 Oxidative DNA damage analysis in parasite infected RBCs

DNA Comet Assay ™ (Trevigen Inc, Gaithersburg, MD, USA) was carried out as described by manufacturer. In this assay, red blood cells were immobilized in a bed of low melting agarose on a Trevigen Comet Slide. After a gentle cell lyses, samples were treated with alkali to unwind and denature the DNA and hydrolyze sites of damage. Electrophoresis was conducted on the samples using TBE as buffer. For visualization of the cells on the slides for DNA damage, observations of the Silver-stained DNA was done by using the X10 objective of the Olympus BX51 standard light microscope fitted with DP2-BSW microscope digital camera (Olympus Co., Japan), for comet assay results analysis (Collins, 2004).

3.9.2 Measurement of reactive oxygen species during infections using the superoxide dismutase assay

The levels of the superoxide anion were determined in the serum of the subjects involved in the study following the centrifugation of the whole blood samples. The supernatant (serum) was diluted 100X using ddH_2O and 20µl added to micro-plate well including the blank. Two hundred microlitres (200µl) of WST working solution was added to each well, and 20µl of dilution buffer added to the blanks. Twenty microlitres (20µl) of enzyme working solution was added to the samples and blanks and mixed thoroughly. Absorbance was read at 450nm after incubating for 20min at 37ºC. The rate of the reduction with O_2^- is linearly related to the Xanthine oxidase

(XO) activity and is inhibited by SOD (Droge, 2002). The inhibitory concentration, IC_{50} (50% inhibition activity of SOD), was therefore determined by a colorimetric method (Peng *et al.*, 2000). The SOD activity as an inhibition activity was quantified by measuring the decrease in the colour development.

3.10 Ethics

This project was carried out with prior approval and ethical clearance obtained from the Research and Protocol Review Committee of the University of Ghana Medical School (UGMS).

3.11 Statistical analysis

Results obtained from this study were analysed using the version 16 of SPSS software. Basic descriptive statistics were determined for the study population with regards to their haematological parameters; a *p*-value of ≤ 0.05 was considered statistically significant. A comparison, with $\chi 2$ tests for the frequencies of interleukin 4 (IL 4) and interleukin 4 receptor (IL 4R) genotypes, was carried out between the uncomplicated malaria phenotype and controls. Odds ratio with corresponding (95%) confidence interval and *p*-values were tested for protective effects of genotypes in the case-control study; the analysis was conducted at each locus, +33 and Pro-478-Ser, for IL 4 and IL 4R respectively. The protective effect, upon carriage of genotype combinations, was determined by Odds ratios with corresponding (95%) confidence intervals; a *p*-value of ≤ 0.05 was considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Characteristics of Study participants

A total of 141 children aged 1 to 14 years were involved in this study, based on the study selection criteria. The number of uncomplicated malaria cases recruited were 100 (mean age = 9.9) (Table 4.1); the number of controls were 41. Uncomplicated malaria (UM) definition was based on the microscopic examination of parasitemia with haemoglobin (Hb) value of at least 8g/dL and without clinical features of severe malaria in the cases. Study participants were fully conscious and had no other identifiable symptoms of severe malaria. The overall mean age of cases of uncomplicated malaria was 9.9 and standard deviation, 3.1. The total number of participants involved in the study was 141.

Table 4.1: Characteristics of study participants

Sex	Total number	% number	Mean age(years)	Standard deviation
Male	56	56.0	10.0	3.0
Female	44	44.4	9.8	3.3

A comparison of haematological parameters between the cases and controls in Table 4.2 showed significant differences for the total white blood cell count (p = 0.001), level of neutrophils (p = 0.001) and lymphocytes (p = 0.001). The concentration of

haemoglobin among the uncomplicated malaria cases did not differ significantly from the control group.

Table 4.2: Comparison of cases and controls using haematological parameters

Variables	Group	N	Mean	S.D.	F-value	P-value
Hb (g/dL)	**Controls**	41	10.96	0.54	3.307	0.071
	Cases	100	9.94	0.89		
RBCs (X 10^{12}/L)	**Controls**	41	5.17	0.70	1.563	0.214
	Cases	100	4.70	0.64		
WBC (X 10^{9}/L)	**Controls**	41	5.57	0.85	31.484	0.001
	Cases	100	7.41	3.41		
NEUT (%)	**Controls**	41	71.98	5.66	29.744	0.001
	Cases	100	72.01	14.48		
LYMPHO (%)	**Controls**	41	39.17	7.48	14.371	0.001
	Cases	100	27.20	14.26		
EOSINO (%)	**Controls**	41	0.20	0.51	1.611	0.207
	Cases	100	0.28	0.64		
MCHC (g/dL)	**Controls**	41	33.27	1.57	0.075	0.785
	Cases	100	33.35	1.59		
MCV (fL)	**Controls**	41	84.71	5.80	1.203	0.275
	Cases	100	80.29	7.16		
PLT (X 10^{9}/L)	**Controls**	41	220.98	53.85	0.591	0.443
	Cases	100	132.99	66.70		

4.2 IL-4 +33 PCR-RFLP Analysis

The product of DNA amplification of IL-4 +33 showed a band size of 190bp, which upon digestion with the restriction enzyme *BsmAI*, resulted in 150 bp and 38 bp for the +33C allele; the intact 190 bp corresponded to the +33T allele (Fig. 4.1).

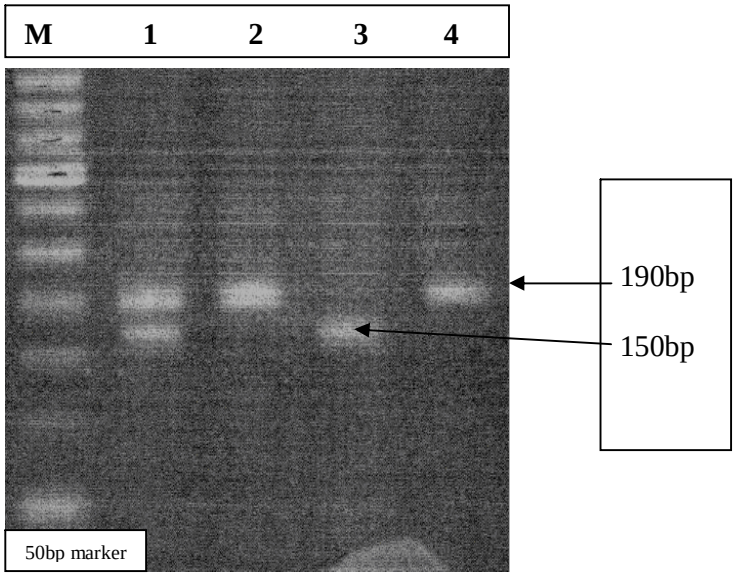


Fig 4.1:Electrophoregram showing IL-4 (+33) amplicon digestion with *BsmAI*. Lane M represents molecular weight ladder. Lanes 1 to 4 show products of digestion with the restriction enzyme.

4.3 IL-4Rα PCR-RFLP Analysis

The restriction enzyme, *Kpn I* digestion of the 159bp amplicon resulted in 138 bp and 21 bp for the P and S alleles respectively. The product of DNA amplification (Fig 4.2) and digestion are shown in Fig 4.3.

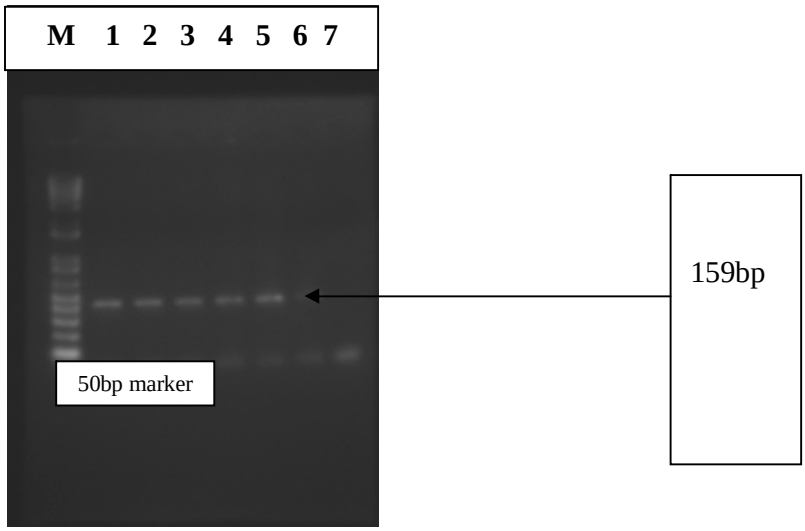


Fig 4.2: Electrophoregram showing IL-4Rα (Pro-478-Ser) amplicon of 159bp. M represents a molecular ladder. Lane 7 shows the negative control which did not contain DNA. Each of the remaining bands represents the amplified DNA fragment of samples.

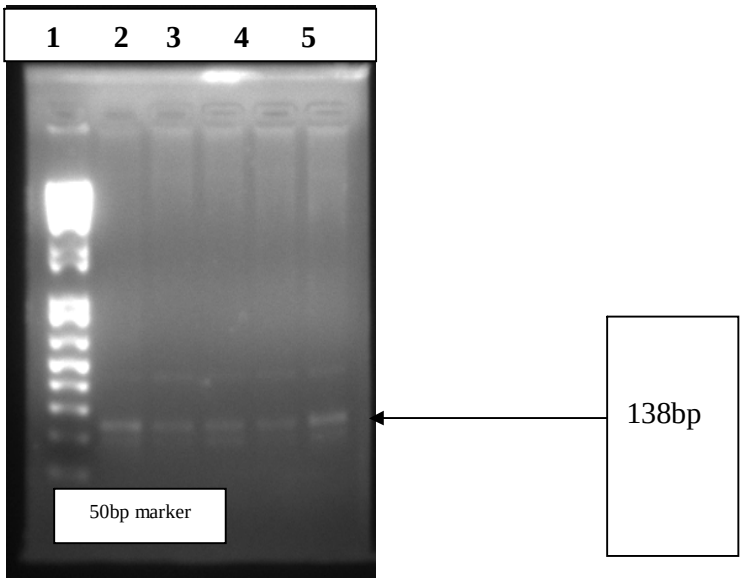


Figure 4.3: Electrophoregram showing *Kpn I* digestion of IL-4Rα (Pro-478-Ser) amplicon. Lane 1 represents the molecular weight ladder; lanes 1 to 5 represent the products of restriction enzyme digestion.

4.4 Comparison of IL 4 (+33) and IL 4Rα genotypes in patients and controls

The distribution of the various genotypes of IL-4 (+33) and IL 4Rα were determined among the uncomplicated malaria cases and controls (Table 43). The genotype distributions of the polymorphisms were in agreement with the Hardy-Weinberg equilibrium. The comparison between cases and controls for the IL-4 (+33) genotypes after a chi square analysis showed $\chi^2 = 1.26$, and $p = 0.53$. A chi square value of 0.55, and $p = 0.76$, were obtained for the IL-4Rα gene following the analysis of the various genotypes of Pro-478-Ser polymorphism (Table 4.4).

Table 4.3 Distribution of IL 4 (+33) genotypes among study participants

	C/C	C/T	T/T	AF (C)	χ^2	*P*
UM	31(31)	43(43)	26(26)	0.37	1.26	0.53
Controls	9(22)	19(46.3)	13(31.7)	0.45		
Total	40	62	39			

Table 4.3 shows distribution of IL 4 (+33) genotypes among study participants. Values are the number of subjects in each group; % is shown in parenthesis. AF represents allele frequency of allele shown in bracket.

Table 4.4 Distribution of IL 4Rα (Pro-478-Ser) genotypes among study participants

	P/P	P/S	S/S	AF (P)	χ^2	*P*
UM	23(23)	42(42)	35(35)	0.45	0.55	0.76
Controls	8(19.5)	16(39)	17(41.5)	0.39		
Total	31	58	52			

The summary of distribution of IL 4Rα (Pro-478-Ser) genotypes among study participants. Values are the number of subjects in each group; % is shown in parenthesis. AF represents allele frequency of allele shown in bracket.

4.5 Comet assay analysis of selected study subjects

Single cell gel electrophoresis of infected RBCs showed smears of *P. falciparum* DNA. The DNA comet assay analysis below showed malaria infected RBCs with comets tails in comparison to control RBCs (Figures 4.4 - 4.6).

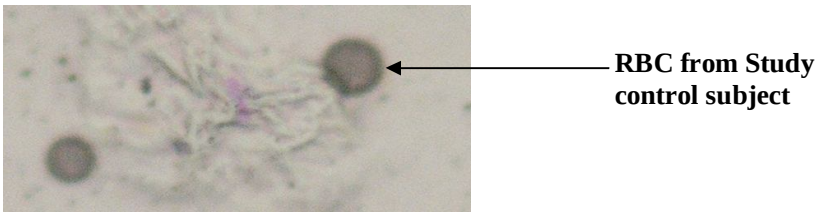


Fig 4.4: Negative control slide with uninfected RBCs after DNA comet assay (Mag. X100).

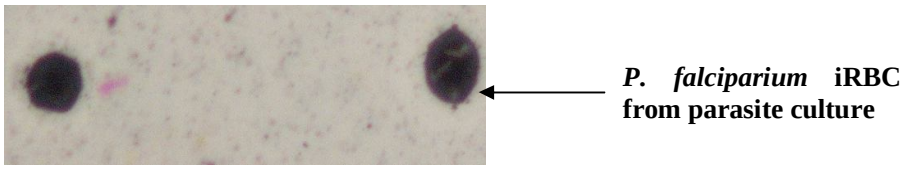


Fig 4.5: Positive control slide with *in vitro* parasite-infected RBCs (with *P. falciprium*) taken through the Comet assay. Notable is the absence of a comet tail (Mag. X100).

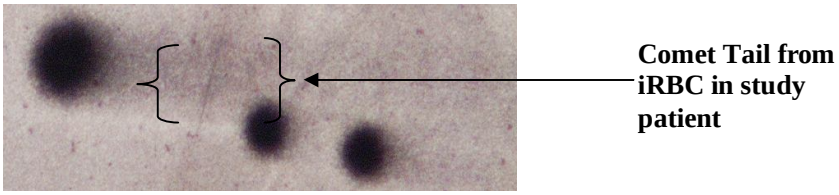


Fig 4.6: Slide with damaged infected RBCs from study subject showing comet tails from Comet assay (Mag. X100).

4.6 Independent assessment of haematological parameters in controls and uncomplicated malaria cases in relation to IL-4 (loci +33) and IL4Rα (Pro-478-Ser) genotypes

The haematological parameters measured in controls and the uncomplicated malaria cases were compared with the genotypes of IL-4 (+33) and IL4Rα (Pro-478-Ser) and summarised in Tables 4.5 to 4.8. The mean lymphocyte concentration was observed to be significant among the control group ($p = 0.047$) for the CT genotype (Table 4.5). Comparison of mean eosinophil and platelet levels for the IL 4Rα (Pro-478-Ser) gene was found to be significant ($p = 0.048$; $p = 0.015$ respectively) for the PS genotype (Table 4.8).

Table 4.5: Comparison of haematological parameters among genotypes (IL 4 gene loci +33) for controls

Variables	Genotypes	N	Mean	S.D.	F-value	P-value
Hb	CC	9	11.17	0.39		
(g/dL)	CT	19	10.86	0.56	1.014	0.372
	TT	13	10.97	0.59		
	Total	41	10.96	0.54		
RBCs	CC	9	5.16	0.55		
(X 10^{12}/L)	CT	19	5.19	0.80	0.032	0.968
	TT	13	5.13	0.68		
	Total	41	5.17	0.70		
WBC	CC	9	5.48	0.81		
(X 10^{9}/L)	CT	19	5.59	0.93	0.059	0.942
	TT	13	5.58	0.81		
	Total	41	5.57	0.85		
NEUT	CC	9	71.11	5.60		
(%)	CT	19	72.74	5.73	0.319	0.729
	TT	13	71.46	5.91		
	Total	41	71.98	5.66		
LYMPHO	CC	9	41.44	6.62		
(%)	CT	19	40.95	7.39	3.317	0.047
	TT	13	35.00	6.90		
	Total	41	39.17	7.48		
EOSINOPHILS	CC	9	0.22	0.44		
(%)	CT	19	0.26	0.65	0.516	0.601
	TT	13	0.08	0.28		
	Total	41	0.20	0.51		
MCHC	CC	9	32.78	1.64		
(g/dL)	CT	19	33.47	1.43	0.597	0.556
	TT	13	33.31	1.75		
	Total	41	33.27	1.57		
MCV	CC	9	79.78	4.18		
(fL)	CT	19	86.21	5.64	5.007	0.012
	TT	13	85.92	5.42		
	Total	41	84.71	5.80		
PLT	CC	9	224.00	57.62		
(X 10^{9}/L)	CT	19	226.58	57.06	0.342	0.712
	TT	13	210.69	48.96		
	Total	41	220.98	53.85		

Table shows summary of comparison of haematological parameters for the IL 4 (+33) gene. Significant associations were shown in MCV and lymphocyte concentrations.

Table 4.6: Comparison of haematological parameters among genotypes (IL 4 gene loci +33) for cases

Variables	Genotypes	N	Mean	S.D.	F-value	P-value
Hb	CC	31	9.96	0.72		
(g/dL)	CT	43	9.84	1.10	0.608	0.546
	TT	26	10.08	0.65		
	Total	100	9.94	0.89		
RBCs	CC	31	4.70	0.66		
(X 10^{12}/L)	CT	43	4.73	0.64	0.17	0.844
	TT	26	4.63	0.63		
	Total	100	4.70	0.64		
WBC	CC	31	6.75	2.55		
(X 10^{9}/L)	CT	43	7.96	3.78	1.155	0.319
	TT	26	7.29	3.65		
	Total	100	7.41	3.41		
NEUT	CC	31	70.61	15.65		
(%)	CT	43	72.95	13.69	0.233	0.793
	TT	26	72.12	14.76		
	Total	100	72.01	14.48		
LYMPHO	CC	31	28.61	15.95		
(%)	CT	43	26.35	13.09	0.23	0.795
	TT	26	26.92	14.45		
	Total	100	27.20	14.26		
EOSINOPHILS	CC	31	0.16	0.37		
(%)	CT	43	0.23	0.48	2.265	0.109
	TT	26	0.50	0.99		
	Total	100	0.28	0.64		
MCHC	CC	31	33.26	1.69		
(g/dL)	CT	43	33.53	1.49	0.534	0.588
	TT	26	33.15	1.67		
	Total	100	33.35	1.59		
MCV	CC	31	79.03	7.19		
(fL)	CT	43	80.16	7.14	1.231	0.297
	TT	26	82.00	7.10		
	Total	100	80.29	7.16		
MCH	CC	31	26.97	2.83		
(X 10^{-12} g)	CT	43	26.95	2.90	1.332	0.269
	TT	26	28.04	2.97		
	Total	100	27.24	2.91		
PLT	CC	31	135.65	68.47		
(X 10^{9}/L)	CT	43	136.65	75.79	0.333	0.717
	TT	26	123.77	47.12		
	Total	100	132.99	66.70		

Table 4.7 : Comparison of haematological parameters among genotypes (IL 4Rα for controls

Variables	Genotypes	N	Mean	S.D	F-value	P-value
Hb	**PP**	8	10.90	0.55	0.131	0.878
(g/dL)	**PS**	16	11.01	0.51		
	SS	17	10.94	0.58		
	Total	41	10.96	0.54		
RBCs	**PP**	8	5.00	0.77		
(X 10^{12}/L)	**PS**	16	5.10	0.70	0.628	0.539
	SS	17	5.31	0.68		
	Total	41	5.17	0.70		
WBC	**PP**	8	5.70	0.93		
(X 10^{9}/L)	**PS**	16	5.55	0.84	0.124	0.884
	SS	17	5.52	0.87		
	Total	41	5.57	0.85		
NEUT	**PP**	8	71.13	4.73		
(%)	**PS**	16	70.81	5.80	1.021	0.370
	SS	17	73.47	5.90		
	Total	41	71.98	5.66		
LYMPHO	**PP**	8	41.88	8.36		
(%)	**PS**	16	38.81	6.57	0.663	0.521
	SS	17	38.24	8.00		
	Total	41	39.17	7.48		
EOSINOPHILS	**PP**	8	0.25	0.46		
(%)	**PS**	16	0.25	0.58	0.323	0.726
	SS	17	0.12	0.49		
	Total	41	0.20	0.51		
MCHC	**PP**	8	32.63	1.85		
(g/dL)	**PS**	16	33.44	1.41	0.833	0.442
	SS	17	33.41	1.58		
	Total	41	33.27	1.57		
MCH	**PP**	8	85.25	5.87		
(X 10^{-12} g)	**PS**	16	82.56	5.21	2.01	0.148
	SS	17	86.47	5.96		
	Total	41	84.71	5.80		
PLT	**PP**	8	225.38	46.61		
(X 10^{9}/L)	**PS**	16	233.25	57.20	0.986	0.383
	SS	17	207.35	53.60		
	Total	41	220.98	53.85		

Table 4.8: Comparison of haematological parameters among genotypes (IL 4Rα gene) for cases

Variables	Genotypes	N	Mean	S.D.	F-value	P-value
Hb	**PP**	23	9.93	0.76		
(g/dL)	**PS**	42	10.02	0.75	0.308	0.735
	SS	35	9.86	1.10		
	Total	100	9.94	0.89		
RBCs	**PP**	23	4.70	0.71		
(X 10^{12}/L)	**PS**	42	4.68	0.61	0.038	0.963
	SS	35	4.72	0.64		
	Total	100	4.70	0.64		
WBC	**PP**	23	8.27	4.29		
(X 10^{12}/L)	**PS**	42	7.04	2.74	1.006	0.369
	SS	35	7.29	3.51		
	Total	100	7.41	3.41		
NEUT	**PP**	23	69.96	13.43		
(%)	**PS**	42	72.83	16.27	0.306	0.737
	SS	35	72.37	13.07		
	Total	100	72.01	14.48		
LYMPHO	**PP**	23	29.22	13.49		
(%)	**PS**	42	25.76	14.88	0.452	0.637
	SS	35	27.60	14.20		
	Total	100	27.20	14.26		
EOSINOPHILS	**PP**	23	0.57	1.04		
(%)	**PS**	42	0.19	0.40	3.126	0.048
	SS	35	0.20	0.47		
	Total	100	0.28	0.64		
MCHC	**PP**	23	33.22	1.65		
(g/dL)	**PS**	42	33.31	1.60	0.217	0.805
	SS	35	33.49	1.58		
	Total	100	33.35	1.59		
MCV	**PP**	23	79.13	8.53		
(fL)	**PS**	42	80.21	6.98	0.547	0.581
	SS	35	81.14	6.48		
	Total	100	80.29	7.16		
MCH	**PP**	23	26.30	3.44		
(X 10^{-12} g)	**PS**	42	27.17	2.72	2.281	0.108
	SS	35	27.94	2.63		
	Total	100	27.24	2.91		
PLT	**PP**	23	166.17	79.64		
(X 10^{9}/L)	**PS**	42	129.50	51.95	4.409	0.015
	SS	35	115.37	67.19		
	Total	100	132.99	66.70		

4.7 Measurement of reactive oxygen species (super oxide anion) levels in cases and controls using superoxide dismutase assay (SOD)

The level of the superoxide anion was measured as an index of the reactive oxygen species in the sera of both cases and controls using the mean inhibition activity of the superoxide dismutase (SOD) (Table 4.9). The mean level of SOD inhibition activity (mean ROS level) was found to be significant in the uncomplicated malaria group as compared to the controls ($p = 0.005$).

Table 4.9 SOD analysis of cases and controls for ROS levels

	Mean	SD	*p*- value
Cases	50.71893	30.74505	0.005
Controls	34.61266	17.74475	

Summary of inhibition activity of SOD (expressed in %) measured at 440nm for all cases and controls.

4.8 Correlation analysis between IL4+33 and IL 4Rα (Pro-478-Ser) genes and SOD values

The amount of superoxide anion generated and its subsequent inhibition by the dismutase enzyme was analysed for strength of relationship with the IL4+33 and IL 4Rα (Pro-478-Ser) genes. The controls and the uncomplicated malaria group did not

show significant level of association with the amount of superoxide anion measured for the IL4 gene and its receptor (Table 4.10).

Table 4.10 Correlation between IL4 (+33) and IL 4Rα (Pro-478-Ser) genotypes and SOD values

	Pearsons correlation	*P*-value
IL 4 gene & SOD Controls	0.008	0.934
IL 4 gene & SOD Cases	0.123	0.373
IL 4Rα gene SOD Controls	0.019	0.913
IL 4Rα gene SOD Controls	0.235	0.439

The relationship between the IL4+33 and IL 4Rα (Pro-478-Ser) genes and SOD activity as expressed by the Pearson's correlation value.

4.9 Univariate analysis of haematological parameters among controls and uncomplicated malaria group

A generalised linear model using SOD values as dependent variable and haematological parameters as covariates showed that neutrophils and platelet levels have significant effect on the SOD values in the control group (Table 4.11). Partial Eta values showed that neutrophils exerted the greatest effect (0.197) followed by MCHC (0.129). The remaining parameters did not show any significant effect on the SOD values for controls. Summary of the generalized linear model using SOD values as dependent variable and haematological parameters as covariates is shown in Table 4.12. HB, neutrophils and MCHC levels were found to have significant effect on the

SOD values. Partial Eta values showed that neutrophils exerted the greatest effect (0.144) followed by HB (0.123) and then MCHC (0.115).

Table 4.11: Generalised linear models (univariate analysis) of haematological parameters among controls

Source	**Sum of Squares**	**Mean Square**	**F-value**	***p*-value**	**Partial Eta Squared**
Corrected Model	15871.364	1587.14	2.789	0.014	0.482
Intercept	339.79	339.79	0.597	0.446	0.020
Hb (g/dL)	587.94	587.94	1.033	0.318	0.033
RBCs (X 10^{12}/L)	179.48	179.48	0.315	0.579	0.010
WBC (X 10^{9}/L)	267.71	267.71	0.47	0.498	0.015
NEUT (%)	4186.46	4186.46	7.357	0.011	0.197
LYMPHO (%)	1573.51	1573.51	2.765	0.107	0.084
EOSINOPHILS(%)	17.00	17.00	0.03	0.864	0.001
MCHC (g/dL)	243.13	243.13	0.427	0.518	0.014
MCV (fL)	86.33	86.33	0.152	0.700	0.005
MCH (X 10^{-12}/L)	12.16	12.16	0.021	0.885	0.001
PLT (X 10^{9}/L)	2532.80	2532.80	4.451	0.043	0.129

Table 4.12: Generalised linear models (univariate analysis) of haematological parameters among the uncomplicated malaria group

Source	Type III Sum of Squares	Mean Square	F	*p*-value	Partial Eta Squared
Corrected Model	13305.272	1330.53	1.734	0.119	0.366
Intercept	2055.19	2055.19	2.678	0.112	0.082
Hb (g/dL)	5156.55	5156.55	6.72	0.015	0.123
RBCs (X 10^{12}/L)	580.69	580.69	0.757	0.391	0.025
WBC (X 10^{9}/L)	49.49	49.49	0.064	0.801	0.002
NEUT (%)	3870.02	3870.02	5.044	0.032	0.144
LYMPHO (%)	2796.90	2796.90	3.645	0.066	0.108
EOSINOPHILS(%)	11.91	11.91	0.016	0.902	0.001
MCHC (g/dL)	3278.64	3278.64	4.273	0.047	0.115
MCV (fL)	0.42	0.42	0.001	0.981	0.001
MCH (X 10^{-12}/L)	20.73	20.73	0.027	0.871	0.001
PLT (X 10^{9}/L)	97.31	97.31	0.127	0.724	0.004

4.10 Correlation analysis of neutrophils, SOD levels and genotypes of IL4 (+33) and IL4Rα (Pro-478-Ser)

An independent correlation analysis of SOD levels carried out in the uncomplicated malaria group and controls did not show significant association (Table 4.13). The only haematological parameter used in the analysis was neutrophil concentration, together

with SOD levels and genotypes of IL4 (+33) and IL4Rα (Pro-478-Ser). Table 4.14 showed significant association (p = 0.002) following a similar analysis carried out for the uncomplicated malaria group.

Table 4.13: Pearsons correlation analysis of neutrophils, SOD levels and genotypes of IL4 (+33) and IL4Rα (Pro-478-Ser) for controls

	Pearson's correlation	*p*-value
Neutrophils & IL4-(+33)	-0.106	0.51
SOD & IL4 Rα (Pro-478-Ser)	-0.054	0.77

Pearson's correlation analysis between IL4-(+33) gene and neutrophils, and between mean SOD level and IL4 Rα (Pro-478-Ser).

Table 4.14: Pearsons Correlation analysis of neutrophils, SOD levels and genotypes of IL4-33 and IL4Rα PRO-478 for Cases

	Pearson's correlation	*p*-value
Neutrophils & IL4 (+33)	0.345	0.002
SOD & IL4 Rα Pro-478-Ser	0.457	0.017

Correlation study between IL4-(+33) gene and neutrophils, and between mean SOD level and IL4 Rα (Pro-478-Ser) among uncomplicated malaria group.

CHAPTER FIVE

5.0 DISCUSSION

The clinical outcome of malaria infection in African children depends on multiple factors and is particularly influenced by the age, immune status and genotype of the host, and to a lesser extent, the geographical origin of the parasite (Weatherall *et al.*, 2002). In those with acquired or innate immunity to malaria, an infection may turn out asymptomatic whereas others with partial or no immunity may suffer from a severe acute illness. A constant feature of the epidemiology of clinical malaria, though yet unexplained, is the age distribution of syndromes of severe disease. In the first 6 months of life, children born in malaria-endemic areas are protected from severity of the disease, and this is due to the passive transfer of maternal immunoglobulins and by expression of foetal haemoglobin (Weatherall *et al.*, 2002).

From birth till infancy, the presentation of malaria is predominantly severe anaemia, in children aged between 1 and 3 in areas of high transmission (Preiser *et al.*,1999). A likely explanation for this trend is that in these areas where mosquito-human transmission of *P. falciparum* is intense, almost all of the children become infected with the parasite and subsequently acquire a form of partial immunity, allowing them to resist malaria without experiencing any of its associated illnesses. However, the fact that some children do become ill with malaria while others manage to resist suggests that some parasites are tolerated better than others. In this study, the IL-4 (+33) single nucleotide polymorphism was investigated together with IL-4 receptor

(Pro-478-Ser), to determine their relationship with ROS production in uncomplicated malaria infection in children aged fourteen years, or younger.

Anaemia has been shown to be a predictable outcome of malaria infection and its degree reflects the duration and severity of infection with a multi-factorial pathogenesis (White, 1998) which may be related to the degree of parasitaemia and erythrocyte destruction (Hommel, 1996). In this study, haemoglobin concentration was measured as an index of the anaemic status of the subjects. It was observed that the mean Hb value of the control group did not differ significantly when compared with the cases (p = 0.07). Kulkarni *et al.*, in 2003, however reported significantly lower Hb concentration in a similar study. A number of factors including the lysis of parasitised red blood cells and dyserythropoietic changes have been linked with the cause of malarial anaemia (Cusick *et al.*, 2005). In *P. falciparum* malaria, both infected and uninfected red cells have structural and functional defects as a result of an interaction between the membrane cytoskeleton proteins (Omodeo-Sale *et al.*, 2003). Infected erythrocytes are identified via the exposure of phosphatidylserine at their surfaces by macrophages which engulf and degrade these eryptotic cells, resulting finally in the reduction of Hb concentration (Föller *et al.*, 2009).

Being the most numerous blood leucocytes, neutrophils are considered paramount phagocytes and are the probable immune effectors for the control of *Plasmodium* blood stage infection. In this study, a significant mean difference (p = 0.001) in neutrophil levels was observed when the uncomplicated malaria cases were compared with the controls. Neutrophils undergo a critical mechanism of oxidative burst involving the catalytic conversion of dimolecular oxygen into superoxide anion (Graham *et al.*, 2007) which together with other reactive oxygen intermediates serve

to combat a diverse array of pathogens (Lambeth, 2004). These reactive oxygen species (ROS), effectively generated by neutrophils, have been shown to be highly toxic for intra-erythrocytic malaria parasites (Bouharoun-Tayoun *et al.*, 1995; Allison and Eugui, 1983), and correlated with fast parasite clearance in Gabonese children with *P. falciparum* malaria (Greve *et al.*, 1999). Opsonized *P. falciparum* merozoites are known to participate in triggering neutrophil respiratory bursts, and is enhanced by cytokines (Kumaratilake *et al.*, 1992). These observations suggest that cytokine-mediated release of ROS might be more involved in immune protection from malaria than is generally appreciated. Significantly differing levels (p = 0.005) of the super oxide anion was observed when the mean values of the uncomplicated malaria cases were compared with the controls of this study.

The damaging effect of ROS on *Plasmodium* DNA was demonstrated in this study by the comet assay in each of the varying degrees of parasitaemia. The various lengths of comet tail observed following the single cell gel electrophoresis of the uncomplicated malaria samples provided evidence of the parasite DNA strand breaks that occur within iRBCs upon release of the genotoxic superoxide anion. The difficulty encountered in this study in determining the end of the comet tails in assessing DNA damage has been similarly reported in previous work (Collins, 2004). Slides from the control group showed only uninfected RBCs after carrying out the comet assay, indicating absence of genetic material which could have undergone electrophoretic migration.

Out of the several polymorphisms in the IL-4 gene discussed in other studies (Nakayama *et al.*, 2000) this work focused on the +33 SNP, and the Pro-478-Ser

polymorphism located in the α subunit of the IL-4 receptor. The relevance of the +33 SNP, relative to the transcription initiation site, is indicated in its promoter-enhancing activity of the IL-4 gene and has also been shown to be associated with severity of malaria infection. A study conducted among Ghanaian children to investigate the interleukin 4 gene and malaria severity did not show difference between the study groups for the +33 genotype and its alleles (Gyan *et al.*, 2004). In this work, a similar no-association trend was observed as the +33 and Pro-478-Ser genotypes and alleles did not show any significant relationship with the outcome of malaria infection. Correlation analysis carried out in this study between the SOD levels and IL4 Rα (Pro-478-Ser) and also between neutrophils and IL4 (+33) showed significant relationship ($p = 0.017$; $p = 0.002$).

5.1 CONCLUSION

This study demonstrated ROS-mediated damage of *P. falciparum* DNA in infected red blood cells with haematological analysis revealing significantly higher mean concentration of neutrophil, and the super oxide anion in the uncomplicated malaria population. The assessment of correlation between SOD levels and IL4 Rα (Pro-478-Ser) polymorphism as well between neutrophils and IL4 (+33) SNP showed significant relationships. Considering that human neutrophils express complete functional receptors for IL-4 including IL-4Rα, and also undergoes respiratory burst, it is likely that the interaction between the gene and neutrophils could be involved with parasite clearance in malaria infection via the genotoxic effects of the super oxide anion.

5.2 LIMITATION/RECOMMENDATION

This study did not cover other notable functional polymorphisms of IL-4 such as (590C/T) and (Arg551Gln) of IL-4Rα which have been investigated in other malaria studies and showed significant associations with clinical outcome of the infection. For further studies, it is recommended that a larger sample size should be used to cover the remaining clinical improve the understanding of the basis of the observations documented in this work on malaria infection, and the other IL-4 and IL-4Rα polymorphisms determined. Haematological and ROS analysis in such a study could provide in-depth understanding of the cell signalling events occurring in IL-4/IL-Rα induced human neutrophil cells in malaria infection.

REFERENCES

Abraham E. (2000). Coagulation abnormalities in acute lung injury and sepsis. *Am. J. Respir. Cell Mol. Biol.* **22**: 401–404.

Adini A. and Warburg A. (1999). Interaction of *Plasmodium gallinaceum* ookinetes and oocysts with extracellular matrix proteins. *Parasitology* **119**: 331–336.

African Scientific Institute (ASI). (2009). Funding Needs for 2009. ASI Malaria Travel Clinic Project In Ghana, West Africa.

Aikawa M. (1988). Human cerebral malaria. *Am J Trop Med Hyg*. **39**: 3-10.

Aitman T. J., Cooper L.D., Norsworthy P.J., Wahid F.N., Gray J. K., Curtis B.R., McKeigue P.M., Kwiatkowski D., Greenwood B.M., Snow R.W. (2000). Malaria susceptibility and CD36 mutation. *Nature.* **405**: 1015–1016.

Akaki M. and Dvorak A. J. (2005). A chemotactic response facilitates mosquito salivary gland infection by malaria sporozoites. *J Expt Biol,* **208**: 3211-3218.

Allen S. J., O'Donnell A., Alexander N. D., Mgone C. S., Peto T. E., Clegg J. B., Alpers M. P., Weatherall D. J. (1999). Prevention of cerebral malaria in children in Papua New Guinea by Southeast Asian ovalocytosis band 3. *Am. J. Trop. Med. Hyg.* **60**: 1056–1060.

Allison A., and Eugui E. (1983). The role of cell-mediated immune responses in resistance to malaria, with special reference to oxidant stress. *Annu Rev Immunol* **1**: 361–392.

Antwi K. Y. and Marfo C. (1998).Ghana moves toward intermittent presumptive treatment in pregnancy. Ghana Health Services, Accra.

Asante F. A. and Asenso-Okyere K. (2003). Economic Burden of Malaria in Ghana. A Technical Report Submitted to the World Health Organisation (WHO), African Regional Office (AFRO).

Asante F. A., Asenso-Okyere K., d'Almeida S., Mwabu G. Okorosobo T. (2004). Economic Burden of Malaria in the African Region: Evidence from Ghana. Communicable Diseases Bulletin for the African Region. World Health Organisation (WHO).

Bannister L. H., Mitchell G. H., Butcher G. A., Dennis E. D. (1986). Lamellar membranes associated with rhoptries in erythrocytic merozoites of *Plasmodium knowlesi*: a clue to the mechanism of invasion *Parasitology* **92:** 291–303.

Barnwell J. W., Ockenhouse C. F., Knowles D. M. (1985). Monoclonal antibody OKM5 inhibits the in vitro binding of Plasmodium falciparum-infected erythrocytes to monocytes, endothelial, and C32 melanoma cels. *J. Immunol.* **135**: 3494–3497.

Baruch D. I., Gormley J. A., Ma C., Howard R. J., Pasloske B. L. (1996). *Plasmodium falciparum* erythrocyte membrane protein 1 is a parasitized erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. USA.* **93**: 3497–3502.

Baruch D. I., Gamain B., Barnwell J. W., Sullivan J. S., Stowers A., Galland G. G., Miller L. H., Collins W. E. (2002). Immunization of Aotus monkeys with a functional domain of the *Plasmodium falciparum* variant antigen induces protection against a lethal parasite line. *Proc. Natl. Acad. Sci. USA.* **99:** 3860–3865.

Becker K., Tilley L., Vennerstrom J. L., Roberts D., Rogerson S., Ginsburg H. (2004). Oxidative stress in malaria parasite-infected erythrocytes: host-parasite interactions. *Int J Parasitol* **34**: 163-89.

Becker, K., Rahlfs, S., Nickel, C., Schirmer, R. H., (2003). Glutathione— function and metabolism in the malarial parasite *Plasmodium falciparum. Biol. Chem*. 348, 551–566.

Beeson J. G., Rogerson S. J., Cooke B. M., Reeder J. C., Chai W., Lawson A. M., Molyneux M. E., Brown G. V. (2000). Adhesion of *Plasmodium falciparum*-infected erythrocytes to hyaluronic acid in placental malaria. *Nat. Med*. **6**: 86–90.

Beeson J. G. and Brown G. (2002). Pathogenesis of *Plasmodium falciparum* malaria: the roles of parasite adhesion and antigenic variation. *Cell. Mol. Life Sci*. **59**: 258–271.

Berendt A. R., Simmons D. L., Tansey J., Newbold C. I., Marsh K. (1989). Intercellular adhesion molecule-1 is an endothelial cell adhesion molecule for *Plasmodium falciparum. Nature,* **341**: 57–59.

Biggs B. A., Gooze L., Wycherley K., Wollish W., Southwell B., Leech J. H., Brown G. V. (1991). Antigenic variation in *Plasmodium falciparum. Proc. Natl. Acad. Sci. USA*. **88**: 9171–9174.

Biggs B. A. and Brown G.V. (2001). Malaria In: Principles and Practice of Clinical . Edited by Gillespie S. and Pearson, R.D. John Wiley & Sons Ltd, USA. *Pg*. 53-98.

Billker O., Lindo V., Panico M., Etienne A. E., Paxton T., Dell A., Rogers M., Sinden R. E., Morris H. R. (1998). Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito. *Nature,* **392**: 289–292.

Bogdan C., Rollinghoff M., Diefenbach A. (2000). Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Curr Opin Immunol,* **12**:64-76.

Bouharoun-Tayoun H., Attanath P., Sabchareon A., Chongsuphajaisiddhi T., Druilhe P. (1990). Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. *J. Exp. Med.* **172**, 1633–1641.

Bouharoun-Tayoun H., Oeuvray C., Lunel F., Druilhe P. (1995). Mechanisms underlying the monocyte-mediated antibody-dependent killing of *P. falciparum* asexual blood stages. *J Exp Med* **182**: 409–418.

Brandts C. H., Ndjave M., Graninger W., Kremsner P. G. (1997). Effect of paracetamol on parasite clearance time in *Plasmodium falciparum* malaria. *Lancet,* **350**: 704–709.

Bruce-Chwatt L. J. (1981). Alphonse Laveran's discovery 100 years ago and today's global fight against malaria. *J R Soc Med.,* **74**: 531-536.

Bull P. C. and Marsh K. (2002). The role of antibodies to *Plasmodium falciparum*-infected-erythrocyte surface antigens in naturally acquired immunity to malaria. *Trends Microbiol*. **10**: 55–58.

Cadroy Y., Dupouy D., Boneu B., Plaisancie H. (2000). Polymorphonuclear leukocytes modulate tissue factor production by mononuclear cells: role of reactive oxygen species. *J. Immunol.* **164**: 3822–3828.

CDC.(2003).Malaria,*http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Malaria_il.htm.*(2003).17/01/11, 7:31pm.

CDC.(2004).Malaria*http://www.dpd.cdc.gov/dpdx/html/PDF.../Parasitemia_and_Life Cycle.pdf* (2004): 12/02/11, 11:35pm.

Clark I. A., al-Yaman F. M., Cowden W. B., Rockett K. A. (1996). Does malarial tolerance, through nitric oxide, explain the low incidence of autoimmune disease in tropical Africa? *Lancet,* **348**: 1492-4.

Clark I. A. and Hunt N. H. (1983). Evidence for reactive oxygen intermediates causing hemolysis and parasite death in malaria. *Infect. Immun.* **39**: 1–6.

Clemens R., Pramoolsinsap C., Lorenz R., Pukrittayakamee S., Bock H. L., White N. J. (1994). Activation of the coagulation cascade in severe malaria through the intrinsic pathway. *Br. J. Haematol.* **87**: 100–105.

Collier L., Balows A., Sussman M. (1998). In: Toply and Wilson's microbiology and microbial infection, Vol. 5. 9th ed. London: Toply and Wilson; p. 378-85.

Collins A. R. (2004). Comet Assay for DNA damage and repair: principles, applications and limitations. *Mol. Biotechnol*. **26**: 249-61.

Cooke B. M., Nicoll C. L., Baruch D. I., Coppel R. L. (1998). A recombinant peptide based on PfEMP1 blocks and reverses adhesion of malaria-infected red blood cells to CD36 under flow. *Mol. Microbiol*. **30**: 83–90.

Cowman A. F. and Crabb B. S. (2006). "Invasion of Red Blood Cells by Malaria Parasites". *Cell* **124** (4): 755-766.

Cox F. (2002). History of human parasitology. *Clin Microbiol Rev* **15** (4): 595–612.

Curfs J. H., Hermsen C. C., Meuwissen J. H., Eling W. M. (1992). Immunization against cerebral pathology in *Plasmodium berghei*-infected mice. *Parasitology*, **105**:7-14.

Currier J., Sattabongkot J., Rosenberg R., Good M. F. (1992). Natural T cells responsive to malaria: evidence implicating immunological cross reactivity in the maintenance of $TCR_{\alpha}\beta^{+}$ malaria specific responses from non-exposed donors. *Int. Immunol.* **4**: *985*.

Cusick S. E., Tielsch J. M., Ramsan M., Jape J. K., Sazawal S., Black R. E., Stolzfus R. J. (2005). Short-term effects of vitamin A and antimalarial treatment on erythropoiesis in severely anemic Zanzibari preschool children. *Am. J. Clin. Nutr*. **82**(2): 406-412.

Das B. S., **and Nanda N. K**. (1999). Evidence for erythrocyte lipid peroxidation in acute falciparum malaria. Trans. R. Soc. Trop. Med. *Hyg*. **93**: 58–62.

Das B. S., Thurnham D. I., Patnaik J. K., Das D. B., Satpathy R., Bose T. K. (1990). Increased plasma lipid peroxidation in riboflavin-deficient, malaria-infected children. *Am. J. Clin. Nutr*. **51:** 859–863.

Dave R., Christopher A. M., David T. R., Jo-Anne C., Michael F., Jake B., Stuart A. R., Raymond S. N., and Alan F. C. (2010). Interaction between *Plasmodium falciparum* Apical Membrane Antigen 1 and the Rhoptry Neck Protein Complex Defines a Key Step in the Erythrocyte Invasion Process of Malaria Parasites. *J Biol Chem*. **285**(19): 14815–14822.

Davis T. M. E., Krishna S., Looareesuwan S., Supanaranond W., Pukrittayakamee S., Attatamsoonthorn K., White N. J. (1990). Erythrocyte sequestration and anemia in severe falciparum malaria. Analysis of acute changes in venous hematocrit using a simple mathematical model. *J Clin Invest* **86**:793.

Del Prete G., Maggi E., Parronchi P. *et al.* (1988). IL-4 is an essential factor for the IgE synthesis induced *in vitro* by human T cell clones and theirsupernatants. *J Immunol;* **140**:4193–8.

Dick S., Waterfall M., Currie J., Maddy A., Riley E. (1996). Naive human $\alpha\beta$T cells respond to membrane-associated components of malaria-infected erythrocytes by proliferation and production of IFNγ. *Immunology* **88**: 412.

Dodoo D., Omer F., Todd J., Akanmori B., Koram K., Riley E. (2002). Absolute levels and ratios of pro-inflammatory and anti-inflammatory cytokine production in vitro predict clinical immunity to *P. falciparum* malaria. *J. Infect. Dis.* **185**:971.

Dondorp A. M., Angus B. J., Hardeman M. R., Chotivanich K. T., Silamut K., Ruangveerayuth R., Kager P. A., White N. J., Vreeken J. (1997). Prognostic significance of reduced red blood cell deformability in severe falciparum malaria. *Am. J. Trop. Med. Hyg*. **57**: 507–511.

Dondorp A. M., Nyanoti M., Kager P. A., Mithwani S., Vreeken J., Marsh K. (2002). The role of reduced red cell deformability in the pathogenesis of severe falciparum malaria and its restoration by blood transfusion. *Trans. R. Soc. Trop. Med. Hyg*. **96**: 282–286.

Doolan D. and Good M. (1999). Immune effector mechanisms in malaria. *Curr. Opin. Immunol.* **11**: 412.

Droge W. (2002). Free radicals in the physiological control of cell function. *Physiol Rev,* **82**: 47–95.

Duraisingh M. T. Triglia T., Ralph S. A., Rayner J. C., Barnwell J. W., McFadden G. I. Cowman A. F. (2003). Phenotypic variation of Plasmodium falciparum merozoite proteins directs receptor targeting for invasion of human erythrocytes. *EMBO J*. **22**: 1047–1057.

Eda S. and Sherman I. W. (2002). Cytoadherence of malaria-infected red blood cells involves exposure of phosphatidylserine. *Cell. Physiol. Biochem.* **12**: 373–384.

Elghazali G., Perlmann H., Rutta A. S., Perlmann P., Troye-Blomberg M. (1997). Elevated plasma levels of IgE in *Plasmodium falciparum*-primed individuals reflect an increased ratio of IL-4 to interferon-gamma (IFN-γ) - producing cells. *Clin Exp Immunol*, **109**: 84–9.

Famin, O., and Ginsburg, H. (2003). The treatment of *Plasmodium falciparum* infected erythrocytes with chloroquine leads to accumulation of ferriprotoporphyrin IX bound to particular parasite proteins and to the inhibition of the parasite's 6-phosphogluconate dehydrogenase. *Parasite* **10**: 39–50.

Favre N., Ryffel B., Rudin W. (1999). The developmenof murine cerebral malaria does not require nitric oxide production. *Parasitology*;**118**:135-8.

Favre N., Ryffel B., Bordmann G., Rudin W. (1997). The course of *Plasmodium chabaudi chabaudi* infections in interferon-γ receptor deficient mice. *Parasite Immunol.* **19**: *375.*

Fell A. H. and Smith N. C. (1998). Immunity to asexual blood stages of *Plasmodium*: is resistance to acute malaria adaptive or innate? *Parasitol. Today*, **14**: *364.*

Flori L., Kumulungui B., Aucan C., Esnault C., Traore A. S., Fumoux F., Rihet P. (2003). Linkage and association between *Plasmodium falciparum* blood infection levels and chromosome 5q31-q33. *Genes Immun*, **4:**265-268.

Föller M., Bobbala D., Koka S., Huber S. M., Gulbins E., Lang F. (2009). Suicide for Survival - Death of Infected Erythrocytes as a Host Mechanism to Survive Malaria. *Cell Physiol Biochem* **24**:133-140.

Fried M. and Duffy P. E. (1996). Adherence of *Plasmodium falciparum* to chondroitin sulphate A in the human placenta. *Science*, **272**: 1502–1504.

Gallup J. L and Sachs J. D. (2001). The economic burden of malaria. *American Journal of Tropical Medicine and Hygiene.* **64**(1, 2) S., pp85 – 96.

Garcia G. E., Wirtz R. A., Barr J. R., Woolfitt A., Rosenberg R. (1998). Xanthurenic acid induces gametogenesis in *Plasmodium*, the malaria parasite. *J Biol Chem* **273**: 12003–12005.

Garham P. C. C. Malaria parasites of man: life-cycles and morphology (excluding ultrastructure). (1988). In Malaria - Principles and practice of malariology, Wernsdorfer WH and McGregor I eds. Vol I pp. 65-96 Churchill Livingstone.

Gilles H. M. (1993). Historical Outline In: Bruce-Chwatt's Essential Malariology. 3rd Edition, The Bath Press, UK.

Good M. F. and Doolan D. L. (1999). Immune effector mechanisms in malaria. *Curr Opin Immunol*; **11**:412-9.

Good M. F. Stanisic D., Xu H., Elliott S., Wykes M. (2004). The immunological challenge to developing a vaccine to the blood stages of malaria parasites. *Immunol. Rev.* **201**: 254–267.

Graham D. B., Robertson C. M., Bautista J., Mascarenhas F., M. Diacovo J., Montgrain V., Lam S. K., Cremasco V., W. Dunne M., Faccio R, Coopersmith C. M., Swat W. (2007). Neutrophil-mediated oxidative burst and host defense are controlled by a Vav-PLCγ2 signaling axis in mice. *J Clin Invest*. **117**(11):3445–3452.

Grau G. E., Mackenzie C. D., Carr R. A., Redard M., Pizzolato G., Allasia C., Cataldo C., Taylor T. E., Molyneux M. E. (2003). Platelet accumulation in brain microvessels in fatal pediatric cerebral malaria. *J. Infect. Dis.* **187:** 461–466.

Grau G., Piguet P. F., Pointaire P. (1992). Cytokines and malaria; duality of effects in pathology and protection. In: Kunkel SL, Remick DG, editors. Cytokines in health and disease. New York: Marcel Dekker, Inc; p. 197-213.

Grau G. E., Taylor T. E., Molyneux M. E., Wirima J. J., Vassalli P. (1989). Tumor necrosis factor and disease severity in children with falciparum malaria. *N. Engl. J. Med*. **320**: 1586–1591.

Greve B., Lehman L. G., Lell B., Luckner D., Schmidt-Ott R., Kremsner P. G. (1999). High oxygen radical production is associated with fast parasite

clearance in children with Plasmodium falciparum malaria. *J. Infect. Dis.* **179:** 1584–1586.

Gruarin P., Primo L., Ferrandi C., Bussolino F., Tandon N. N., Arese P., Ulliers D., Alessio M. (2001). Cytoadherence of *Plasmodium falciparum*-infected erythrocytes is mediated by a redox-dependent conformational fraction of CD36. *J. Immunol.* **167**: 6510–6517.

Gyan B. A., Goka B., Cvetkovic J. T., Kurtzhals J. L., Adabayeri V., Perlmann H., Lefvert A.-K., Akanmori B. D., Troye-blomberg M. (2004). Allelic polymorphisms in the repeat and promoter regions of the interleukin-4 gene and malaria severity in Ghanaian children. *Clin Exp Immunol* 2004;**138**:145–150.

Gyan B., Troye-Blomberg M., Perlmann P., Bjorkman A. (1994). Human monocytes cultured with and without interferon-gamma inhibit *Plasmodium falciparum* parasite growth *in vitro* via secretion of reactive nitrogen intermediates. *Parasite Immunol,* **16**: 371-5.

Hermsen C. C., Crommert J. V., Fredix H., Sauerwein R. W., Eling W. M. (1997). Circulating tumour necrosis factor alpha is not involved in the development of cerebral malaria in *Plasmodium berghei*-infected C57Bl mice. *Parasite Immunol,* **19**: 571-7.

Hill A.V. (1998). The immunogenetics of human infectious diseases. *Annu. Rev. Immunol.* **16**: 593–617.

Hizawa N., Yamaguchi E., Kawayami Y. (2000). Association between a C+33T polymorphism in the IL-4 promoter region and total serum IgE levels. *Clin Exp Allergy*; **30**:1746–9.

Hommel M. (1996). Immunology of malaria. In: WHO, health co-operation papers, quaderni di cooperazion sanitaria. World Health Organisation. p. 53-70.

Hommel M. and Barnish G. (1998). A broader view of malaria: from cell biology to health economies. *Parasitol Today*, **14**: 337- 40.

Imamura T., Sugiyama T., Cuevas L.E., Makunde R., Nakamura S. (2002). Expression of tissue factor, the clotting initiator, on macrophages in *Plasmodium falciparum*-infected placentas. *J. Infect. Dis.* **186**: 436–440.

Jacobs P., Radzioch D., Stevenson M. M. (1996). *In vivo* regulation of nitric oxide production by tumor *necrosis* factor alpha and gamma interferon, but not by interleukin-4, during blood stage malaria in mice. *Infect Immun*, **64**: 44-9.

Jakobsen P. H., Bate C. A., Taverne J., Playfair J. H. (1995). Malaria: toxins, cytokines and disease. *Parasite Immunol*, **17**: 223-31.

Jones I. W., Thomsen L. L., Knowles R., Gutteridge W. E., Butcher G. A, Sinden R. E. (1996). Nitric oxide synthase activity in malaria-infected mice. *Parasite Immunol*, **18**: 535-8.

Joy D., Feng X., Mu J. (2003). "Early origin and recent expansion of *Plasmodium falciparum*". *Science* **300** (5617): 318–21.

King C. and Sarvetnick N. (1997). Organ-specific autoimmunity. *Curr. Opin. Immunol.* **9:** 863-871.

Klotz F.W., Scheller L. F., Seguin M. C., Kumar N., Marletta M. A., Green S. J., Azad A. F. (1995). Co-localization of inducible-nitric oxide synthase and *Plasmodium berghei* in hepatocytes from rats immunized with irradiated sporozoites. *J Immunol*, **154**:3391-5.

Kodjo A., Franco T., Antonio P., Paolo A. (2004). Enhanced phagocytosis of ring-parasitized mutant erythrocytes: a common mechanism that may explain protection against falciparum malaria in sickle trait and beta-thalassemia trait. *Blood* **104** (10): 3364-71.

Kremsner P. G., Winkler X., Brandts C., Wilding E., Jenne L., Graninger W., Prada J., Bienzle U., Juillard P., Grau G. E. (1995). Prediction of accelerated cure in *Plasmodium falciparum* malaria by the elevated capacity of tumor necrosis factor production. *Am. J. Trop. Med. Hyg.* **53**:532.

Kremsner P. G., Winkler X., Wilding E., Prada J., Bienzle U., Graninger W., Nussler A. (1996). High plasma levels of nitrogen oxides are associated with severe disease and correlate with rapid parasitological and clinical cure in *Plasmodium falciparum* malaria. *Trans. R. Soc. Trop. Med. Hyg*. **90**:44.

Kristoff J. (2007). Malaria stage-specific vaccine candidates. Curr Pharm *Des* **13**:1989-99.

Krotoski W., Collins W., Bray R., Garnham P. C. C., Cogswell F. B., Gwadz R. W., Killick-Kendrick T., Wolf R., Sinden R., Koontz L. C., Stanfill P. S. (1982). Demonstration of hypnozoites in sporozoite-transmitted *Plasmodium vivax* infection. *Am J Trop Med Hyg* **31** (6): 1291–3.

Kulkarni A. G., Suryakar A. N., Sardeshmukh A. S., Rathi D. B .(2003). Studies on biochemical changes with special reference to oxidants and antioxidants in malaria patients. *Ind. J. Clin. Biochem*. **18**(2): 136- 149.

Kumaratilake L. M., Ferrante A., Jaeger T., Rzepczyk C. M. (1992). Effects of cytokines, complement, and antibody on the neutrophil respiratory burst and phagocytic response to *Plasmodium falciparum* merozoites. *Infect Immun* **60**: 3731–3738.

Kwiatkowski D. (2000). Genetic susceptibility to malaria getting complex. *Curr. Opin. Genet. Dev.* **10**:320–324.

Kwiatkowski D., Hill A.V.S., Sambou I., Twumasi P., Castracane J., Manogue K. R., Cerami A., Brewster D.R., Greenwood B.M. (1990). TNF concentration in fatal cerebral, non-fatal cerebral and uncomplicated *Plasmodium falciparum* malaria. *Lancet* **336**: 1201–1204.

Lambeth J. D. (2004). NOX enzymes and the biology of reactive oxygen. *Nat. Rev. Immunol.* **4:**181-189.

Leech J. H., Barnwell J. W., Miller L. H. (1984). Identification of a strain-specific malarial antigen exposed on the surface of *Plasmodium falciparum*-infected erythrocytes. *J. Exp. Med.* **159**: 1567–1575.

Liew F. Y. (1992). Regulation of nitric oxide synthase in macrophages. In: Moncada S, Stamler J, Gross S, Higgs EA. The biology of nitric oxide: 2. Enzymology, biochemistry and immunology. London: Portland Press; p. 223-9.

Li-Weber M., Krammer P. H. (2003). Regulation of IL4 gene expression by T cells and therapeutic perspectives. *Nat. Rev. Immunol*. **3**: 534-543.

Li-Weber M., Giaisi M., Treiber M. K., Krammer P. H. (2002). Vitamin E inhibits IL-4 gene expression in peripheral blood T cells. *Eur. J. Immunol.* **32**: 2401-2408.

Long C., Jun M., Liwang C. (2006). Cytotoxic Effect of Curcumin on Malaria Parasite *Plasmodium falciparum*: Inhibition of Histone Acetylation and Generation of Reactive Oxygen Species. *Antimicrob. Agents Chemother.* **10**:01238-06.

Luty A. J. F., Mayombo J., Lekoulou F., Mshana R. (1994). Immunologic Responses to Soluble Exoantigens of *Plasmodium falciparum* in Gabonese Children Exposed to Continuous Intense Infection. *Am J Trop Med Hyg*, **51:**720-729.

Mackintosh C. L. Beeson J. G, Marsh K. (2004). Clinical features and pathogenesis of severe malaria. *Trends Parasitol.* **20**: 597–603.

Marsh K. and Kinyanjui S. (2006). Immune effector mechanisms in malaria. *Parasite Immunol.* **28**: 51–60.

Marsh D. G., Neely J. D., Breazeale D. R. *et al.* (1995). Total serum IgE levels and chromosome 5q. *Clin Exp Allergy*; **25**:79–83.

Marsh D. G., Neely J. D., Breazeale D. R *et al.* (1994). Linkage analysis of IL-4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. *Science*; **264**:1152–6.

Marsh K. and Howard R. J. (1986). Antigens induced on erythrocytes by *P. falciparum*: expression of diverse and conserved determinants. *Science,* **231**: 150–153.

Mary L. (1999). Medicine and society in early modern Europe. Cambridge University Press. p.62.

Mazier D., Nitcheu J., Idrissa-Boubou M. (2000). Cerebral malaria and immunogenetics. *Parasite Immunol*, **22**:613-23.

McGuire W., Hill A. V., Allsopp C. E., Greenwood B. M., Kwiatkowski D. (1994). Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. *Nature,* **371**:508–510.

Meis J., Verhave J., Jap P., Sinden R., Meuwissen J. (1983). Malaria parasites-discovery of the early liver form. *Nature,* **302**: 424–6.

Meisel C., Vogt K., Platzer C., Randow F**,** Liebenthal C., Volk H. D. (1996). Differential regulation of monocytic tumor necrosis factor-alpha and interleukin-10 expression. *Eur. J. Immunol*.; **26**(7):1580-6.

Ministry of Health, Ghana (2002). Information for action; A Bulletin of Health Information. Accra.

Miranda King R., Anisa I. S., Laurie D. S., David K. R. (2006). Oxidative Stress Promotes Polarization of Human T Cell Differentiation Toward a T Helper 2 Phenotype. *J. Immunol*., 176: 2765-2772.

Mohan K., Moulin P., Stevenson M. M. (1997). Natural killer cell cytokine production, not cytotoxicity, contributes to resistance against blood-stage *Plasmodium chabaudi AS* infection. *J. Immunol.* **159**:4990.

Motard A., Landau I., Nussler A., Grau G., Baccam D., Mazier D. (1993). The role of reactive nitrogen intermediates in modulation of gametocyte infectivity of rodent malaria parasites. *Parasite Immunol*, **15**:21-6.

Murphy K. M., Ouyang W., Farrar J. D., Yang J., Ranganath S., Asnagli H., Afkarian M., Murphy T.L. (2000). Signaling and transcription in T helper development. *Annu. Rev. Immunol.* **18**: 451-494.

Murphy S. C. and Breman J. G. (2001). Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia and complications of pregnancy. *Am J Trop Med Hyg*, **64:**57-67.

Nakayama E. E., Hoshino Y., Xin X., Liu H., Goto M., Watanabe N., Taguchi H., Hitani A., Kawana-Tachikawa A., Fukushima M., Yamada K., Sugiura W., Oka S. I, Ajisawa A., Sato H., Takebe Y., Nakamura T., Nagai Y., Iwamoto A., Shioda T. (2000). Polymorphism in the interleukin-4 promoter affects acquisition of human immunodeficiency virus type 1 syncytium-inducing phenotype. *J. Virol*, **74**: 5452-9.

Naotunne T. S., Karunaweera N. D., del Giudice G., Kularatne M. U., Grau G. E., Carter R., Mendis K. N. (1991). Cytokines kill malaria parasites during infection crisis: extracellular complementary factors are essential. *J. Exp. Med.* **173**:*523*.

Nijhout M. M. and Carter R. (1978). Gamete development in malaria parasites: bicarbonate-dependent stimulation by pH *in vitro*. *Parasitology* **76**: 39–53.

Norman G. G. (2006). Vector- and Rodent-Borne Diseases in Europe and North America. World Health Organization, Geneva.

Ockenhouse C. F., Ho M., Tandon N. N., Van-Seventer G. A., Shaw S., White N.J., Jamieson G. A., Chulay J. D., Webster H. K. (1991). Molecular basis of sequestration in severe and uncomplicated *Plasmodium falciparum* malaria: differential adhesion of infected erythrocytes to CD36 and ICAM-1. *J. Infect. Dis.* **164**: 163–169.

O'Garra A. (1998). Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* **8**: 275-283.

Ojo-Amaize E. A., Salimonu L. S., Williams A. I. O., Akinwolere O. A. O., Shabo R., Alm G., Wigzell H. (1981). Positive correlation between degree of parasitaemia, interferon titers and natural killer cell activity in *Plasmodium falciparum*-infected children. *J. Immunol.* **127**: 2296.

Omodeo-Sale M. F., Motti A., Basilico N., Parapini S., Olliaro P. (2003). Accelerated senescence of human erythrocytes cultured with *Plasmodium falciparum*. *Blood* **102**: 705–711.

O'Shea J. J., Ma A., Lipsky P. (2002). Cytokines and autoimmunity. *Nat. Rev. Immunol.* **2**: 37-45.

Osier F. H. Fegan G., Polley S. D., Murungi L., Verra F., Tetteh K. K. A.., Lowe B., Mwangi T.,. Bull P. C, Thomas A. W., Cavanagh D. R., McBride J. S., Lanar D. E., Mackinnon M. J., Conway D. J. Marsh K. (2008). Breadth and magnitude of antibody responses to multiple *Plasmodium falciparum* merozoite antigens are associated with protection from clinical malaria. *Infect. Immun.* **76**: 2240–2248.

Patel S. S., Mehlotra R. K., Kastens W., Mgone C. S., Kazura J. W., Zimmerman P. A. (2001). The association of the glycophorin C exon 3 deletion with ovalocytosis and malaria susceptibility in the Wosera, Papua New Guinea. *Blood,* **98**:3489–3491.

Paul W. E. (1991). Interleukin-4: a prototypic immunoregulatory lymphokine. *Blood,* **77**: 1859-1870.

Pavithra S. R., Banumathy G., Joy O., Singh V., Tatu U. (2004). Recurrent Fever Promotes *Plasmodium falciparum* Development in Human Erythrocytes**.** The *J Biol Chem* **279**: 46692-46699.

Peng H., Li F., Elizabeth A.O., Michael J.K., William P., (2000). Superoxide dismutase as a target for the selective killing of cancer cells.

Persson K.E.M., McCallum F. J., Reiling L., Lister N. A., Stubbs J., Cowman A. F., Marsh K., Beeson J. G. (2008). Variation in use of erythrocyte invasion pathways by *Plasmodium falciparum* mediates evasion of human inhibitory antibodies. *J. Clin. Invest.* **118**: 342–351.

Phillips R. S., Brannan L. R., Balmer P., Neuville P. (1997). Antigenic variation during malaria infection: the contribution from the murine parasite *Plasmodium chabaudi*. *Parasite Immunol*, **19**:427-34.

Pierce S. K. and Miller L. H. (2009). World Malaria Day 2009: What Malaria Knows about the Immune System That Immunologists Still Do Not. *J Immunol*, **182***: 5171 – 5177.*

Plebanski M. and Hill A. (2000). The immunology of malaria infection. *Curr. Opin. Immunol.* **12**:437.

Pombo D. J. Lawrence G., Hirunpetcharat C., Rzepczyk C., Bryden M., Cloonan N., Anderson K., Mahakunkijcharoen Y., Martin L. B., Wilson D., Elliott S, Eisen D. P., Weinberg J. B., Saul A., Good M. F. (2002). Immunity to malaria after administration of ultra-low doses of red cells infected with *Plasmodium falciparum*. *Lancet*, **360**: 610–617.

Postma N. S., Zuidema J., Mommérs E. C., Eling W. M. C. (1996). Oxidative stress in malaria; implications for prevention and therapy**.** *Pharmacy World and Science* **18**: 121-129.

Preiser P. R., Jarra W., Capiod T., Snounou G. (1999). A rhoptryprotein- associated mechanism of clonal phenotypic variation in rodent malaria. *Nature*; **398**:618-622.

Pritchard D. I., Hewitt C., Moqbel R. (1997). The relationship between immunological responsiveness controlled by T-helper 2 lymphocytes and infections with parasitic helminths. *Parasitology*, **115**: *(Suppl.)*:S33-S44.

Reeder J. C., Cowman A. F., Davern K. M., Beeson J. G., Thompson J. K., Rogerson S. J., Brown G. V. (1999). The adhesion of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulphate A is mediated by PfEMP1. *Proc. Natl. Acad. Sci. USA*, **96**: 5198–5202.

Ribeiro J. M. and Francischetti I. M. (2003). Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Ann. Rev. Entomol.* **48**: 73–88.

Rihet P., Traore Y., Abel L., Aucan C., Traore-Leroux T., Fumoux F. (1998). Malaria in humans: *Plasmodium falciparum* blood infection levels are linked to chromosome 5q31-q33. *Am J Hum Genet*, **63:**498-505.

Rockett K. A., Awburn M. M., Cowden W. B., Clark I. A. (1991). Killing of *Plasmodium falciparum in vitro* by nitric oxide derivatives. *Infect Immun*, **59**:3280-3.

Rockett K. A., Cowden W. B., Awburn M. M., Clark I. A. (1992). Arginine utilization and induction of nitric oxide synthase: cytokine-induced release of nitric oxide *in vivo* and its implications for the pathogenesis of cerebral malaria. In: Moncada S, Martella MA, Hibbs Jr JB, Higgs EA, editors. The biology of nitric oxide: 2. Enzymology, biochemistry and immunology. London: Portland Press; p. 135-7.

Roitt I.M., Brostoff J., Male D. (1998). *Immunology*. 5th ed. London: Mosby Publication.

Roll Back Malaria. (2002). Malaria Consortium. Final report of the external evaluation of Roll Back Malaria. Achieving impact.

Roll Back Malaria. (2008). The Global Malaria Action Plan.

Romagnani, P., Annunziato M. F., Piccinni P., Maggi E., Romagnani S. (2000). Th1/Th2 cells, their associated molecules and role in pathophysiology. *Eur. Cytokine Netw.* **11**: 510-511.

Romagnani S. (1995). Biology of human TH1 and TH2 cells. *J Clin Immunol*, **15**:1219.

Ross R. (1897). On some peculiar pigmented cells found in two mosquitoes fed on malarial blood. *Br Med J*, **2**: 1786–8.

Roussilhon C., Oeuvray C., Müller-Graf C., Tall A., Rogier C., Jean-François T., Theisen M., Balde A., Jean-Louis P., Druilhe P. (2007). Long-term clinical protection from falciparum malaria is strongly associated with IgG3 antibodies to merozoite surface protein 3. *PLoS Med*. **4**, e320.

Ruwende C., Khoo S. C., Snow R. W., Yates S. N., Kwiatkowski D., Gupta S., Warn P., Allsopp C. E., Gilbert S. C., Peschu N. (1995). Natural selection of hemi- and heterozygotes for G6PD deficiency in Africa by resistance to severe malaria. *Nature*. **376**:246–249.

Sabchareon A. Burnouf T., Owattara D., Attanath P., Bouharoun- Tayoun H., Chantavanich P., Foucault C., Chongsuphajaisiddhi T., Druilhe P. (1991). Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. *Am. J. Trop. Med. Hyg*, **45**: 297–308.

Sakuntabhai A., Ndiaye R., Casademont I., Peerapittayamongkol C., Rogier C., Tortevoye P., Tall A., Paul R., Turbpaiboon C., Phimpraphi W., Trape J. F., Spiegel A., Heath S., Mercereau-Puijalon O., Dieye A., Julier C. (2008). Genetic determination and linkage mapping of *Plasmodium falciparum* malaria related traits in Senegal. *PLoS ONE*, **3:**e2000.

Sanni L. A., Fu S., Dean R. T., Bloomfield G., Stocker R., Chaudhri G., Dinauer M. C., Hunt N.H. (1999). Are reactive oxygen species involved in the pathogenesis of murine cerebral malaria? *J. Infect. Dis*, **179**: 217–222.

Scheller L. F., Green S. J., Azad A. F. (1997). Inhibition of nitric oxide interrupts the accumulation of CD8+ T cells surrounding *Plasmodium berghei*-infected hepatocytes. *Infect Immun*, **65**: 3882-8.

Schofield L., Hackett F. (1993). Signal transduction in host cells by a glycophosphatidylinositol toxin of malaria parasites. *J. Exp. Med.* **177**:145.

Senczuk A. M., Reeder J. C., Kosmala M. M., Ho M. (2001). *Plasmodium falciparum* erythrocyte membrane protein 1 functions as a ligand for P selectin. *Blood*, **98**: 3132–3135.

Senior K., (2008). Climate Change and Infectious Disease: A Dangerous Liaison? *The Lancet*, **8** (2).

Sieber K. P., Huber M., Kaslow D., Banks S. M., Torii M., Aikawa M., Miller L. H. (1991). The peritrophic membrane as a barrier: its penetration by *Plasmodium gallinaceum* and the effect of a monoclonal antibody to ookinetes. *Exp Parasitol*, **72**: 145–156.

Sinden R. E. (1983). Sexual development of malarial parasites. *Adv Parasitol*, **22**: 153–216.

Singh V. K., Mehrotra S., Agarwal S. S. (1999). The paradigm of Th1 and Th2 cytokines: its relevance to autoimmunity and allergy. *Immunol. Res.* **20**: 147-161.

Smith J. D. Chitnis, C. E., Craig, A. G., Roberts, D. J., Hudson-Taylor, D. E., Peterson, D. S. (1995). Switches in expression of *Plasmodium falciparum* var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell,* **82**: 101–110.

Stach J., Dufrenoy E., Roffi J., Bach M. (1986). T-cell subsets and natural killer activity in *Plasmodium falciparum*-infected children. *Clin. Immunol. Immunopathol.* **38**:129.

Stanisic D. I., Mueller I., Betuela I., Siba P., Schofield L. (2010). Robert Koch redux: malaria immunology in Papua New Guinea. *Parasite Immunol.* **32**: 623–632.

Stubbs J. Simpson K. M., Triglia T., Plouffe D., Tonkin C. J., Duraisingh M. T., Maier A. G., Winzeler Elizabeth A., Cowman A. F. (2005). Molecular mechanism for switching of *P. falciparum* invasion pathways into human erythrocytes. *Science,* **309**: 1384–1387.

Street N. E., Mosmann T. R. (1991). Functional diversity of T lymphocytes due to secretion of different cytokine patterns. *FASEB J.* **5**: 171-177.

Stevenson M. M., Tam M. F., Wolf S.F., Sher A. (1995). IL-12-induced protection against blood-stage *Plasmodium chabaudi AS* requires IFNγ and TNFα and occurs via a nitric oxide-dependent mechanism. *J. Immunol.* **155**: 2545.

Sylke M. (2004). Redox and antioxidant systems of the malaria parasite *Plasmodium falciparum*. *Molecular Biology*, 53 (5): 1291-1305.

Szabo S. J., Sullivan B. M., Peng S. L., Glimcher L. H. (2003). Molecular mechanisms regulating Th1 immune responses. *Annu. Rev. Immunol.* **21**: 713-758.

Taylor-Robinson A.W. (1995). Regulation of immunity to malaria: valuable lessons learned from murine models. *Parasitol Today*, **11**:334-42.

Taylor B. S., Kim Y. M., Wang Q., Shapiro R. A., Billiar T.R., Geller D. A. (1997). Nitric oxide down-regulates hepatocyte-inducible nitric oxide synthase gene expression. *Arch Surg*, **132**:1177-83.

Tanner M. and de Savigny D. (2008). Malaria Eradication Back on the Table? Bulletin of WHO. **86**: 81-160.

Terada L. S. (2002). Oxidative stress and endothelial activation. *Crit. Care Med*. **30**: 186–191.

Treutiger C. J., Heddini A., Fernandez V., Muller W. A., Wahlgren M. (1997). PECAM-1/CD31, an endothelial receptor for binding *Plasmodium falciparum*-infected erythrocytes. *Nat. Med.* **2**: 1405–1408.

Trinchieri G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* **3**: 133-146.

Troye-Blomberg M., Riley E. M., Kabilan L., Holmberg M., Perlmann H., Andersson U., Heusser C. H., Perlmann P. (1990). Production by activated human T cells of interleukin 4 but not interferon-gamma is associated with elevated levels of serum antibodies to activating malaria antigens. *Proc Natl Acad Sci USA*, **87**:5484–8.

Tsutsui N. and Kamiyama T. (1999). Transforming growth factor beta-induced failure of resistance to infection with blood-stage *Plasmodium chabaudi* in mice. *Infect Immun*, **67**: 2306-11.

Udomsangpetch R., Reinhardt P.H., Schollaardt T., Elliott J.F., Kubes P., Ho M. (1997). Promiscuity of clinical *Plasmodium falciparum* isolates for multiple adhesion molecules under flow conditions. *J. Immunol.* **158**: 4358–4364.

United Nations. (2008). The Millennium Development Goals Report, September 2008.

Vitetta E. S., Brooks K., Chen Y. W. *et al.* (1984). T-cell-derived lymphokines that induce IgM and IgG secretion in activated murine B cells. *Immunol Rev;* **78**:137–57.

Vlachou D., Lycett G., Siden-Kiamos I., Blass C., Sinden R. E., Louis C. (2001). *Anopheles gambiae* laminin interacts with the P25 surface protein of *Plasmodium berghei* ookinetes. *Mol Biochem Parasitol*, **112**: 229–237.

Wakelin D. (1988). Intracellular Protozoa; survival within cells. In: Wakelin D, editor. Immunity to parasites, how animals control parasite infections; p. 32-47.

Walter P. R., Garin Y., Blot P. (1982). Placental pathologic changes in malaria. A histologic and ultra structural study. *Am. J. Pathol*. **109:** 330–342.

Waterfall M., Black A., Riley E. (1998). $\gamma\delta^+$ T cells preferentially respond to live rather than killed malaria parasites. *Infect. Immun.* **66**:2393.

Weatherall D. J. (2001). Phenotype-genotype relationships in monogenic disease: lessons from the thalassaemias. *Nat. Rev. Genet.* **2**:245–255.

Weatherall D. J., Miller L. H., Baruch D. I., Marsh K., Doumbo O. K., Casals-Pascual C and Roberts D J. (2002). Malaria and the Red Cell. *Haematology*, The American Society of Hematology.

Wernsdorfer W. (1980). The Importance of Malaria in the World. In *Malaria*. Vol. 1 (ed. J. Kreier), pp. 1-93. New York: Academic Press.

White N. J. (1998). Malaria pathophysiology. In: Sherman IW, editor. Malaria, parasite biology, pathogenesis and protection. 1st ed. Washington DC: ASM Press; 1998. p. 371-85.

WHO. (2002). Macroeconomics and health initiative in Ghana. Press Release No. 013/02.(http:/www.who.int/macrohealth/infocentre/press/bulletin/en/html;12/12/10: 11:05am).

WHO. (2004). The World Health Report: 2004 – changing history. (http:// www.who.int/whr/2004/en/overview_en.pdf; 20/01/11; 10:15pm)

Willcox M. A., Bjorkman J., Brohult P. O., Pehrson L., Rombo., Bengtsson E. (1983). A case-control study in northern Liberia of *Plasmodium falciparum* malaria in haemoglobin S and beta-thalassaemia traits. *Ann. Trop. Med. Parasitol.* **77**:239–246.

Winter R. W., Ignatushchenko M., Ogundahunsi O. A. T., Cornell K. A., Oduola A. M. J., Hinrichs D. J., Riscoe M. K. (1997). Potentiation of an Antimalarial Oxidant Drug. *Antimicrobial agents and chemotherapy* **41**(7):1449-54.

World Health Organization (WHO). (2000). Severe falciparum malaria. *Trans R Soc Trop Med Hyg*, **94**(suppl 1):S1-S90.

World Health Organization (WHO). (2008). World Malaria Report. WHO Global Malaria Programme.(http://whqlibdoc.who.int/publications/2008/9789241563697_eng.pdf; 10/11/10; 6:30pm)

World Health Organization (WHO). (2010). World Malaria Report. (http://www.who.int/malaria/world_malaria_report_2010/worldmalariareport2010.pdf; 15/01/11; 3:30pm).

Wyler D. J. (1990). Molecular biology of parasites. In: Wyler DJ WH, editor. Modern parasite biology: cellular, immunological and molecular aspects. USA: Freeman and Company; 1990. p. 313-32.

Zevering Y., Amante F., Smillie A., Currier J., Smith G., Houghten R. A., Good M. F. (1992). High frequency of malaria-specific T cells in non-exposed humans. *Eur. J. Immunol.* **22**:689.

APPENDICES

APPENDIX A

GENOMIC DNA EXTRACTION FROM BUFFY COAT (Qiagen Co. Ltd., UK)

Principle

DNeasy Tissue Kits are advanced silica-gel membrane technology for rapid and efficient purification of total cellular DNA without organic extraction or ethanol precipitation. The buffer system is optimised to allow direct cell lysis followed by selective binding of DNA to the DNeasy membrane. After lysis, DNeasy procedure can be completed in as little as 20 minutes. Simple purification processing completely removes contaminants and enzyme inhibitors such as proteins and divalent cations, and allows simultaneous processing of multiple samples in parallel. In addition, the DNeasy procedure is suitable for a wide range of samples.

Procedure:

1. 20µl of proteinase K was pipetted into 1.5ml microcentrifuge tube.
2. 100µl of buffy coat was added onto the proteinase K.
3. 100µl of PBS was added to adjust the volume to 220µl.
4. 200µl Buffer AL was added and mixed thoroughly by vortexing.
5. The mixture was incubated for 10min at 56ºC.
6. 200µl of absolute ethanol was added to the sample and mixed thoroughly by vortexing.
7. The mixture from step 6 (including precipitate) was pipetted into Dneasy column and centrifuged at 8000rpm for 1 min. Flow-through and collection tube were discarded.

8. The Dneasy column was placed in a new 2ml collection tube. 500µl Buffer AW1 was added and centrifuged at 8000rpm for 1 min. Flow-through and collection tube were discarded.

9. The Dneasy mini spin column was placed in a new 2ml collection tube and 500µl

   Buffer AW2 added and centrifuged for 3min at 14000rpm to dry the Dneasy membrane. Flow- through and collection tube were discarded.

10. Dneasy mini spin column was placed in a 1.5ml microcentrifuge tube. 50µl buffer AE was added and incubated at room temperature for 1min and centrifuged at 8000rpm for 1min. The resulting DNA sample was divided into 2 aliquots of 25µl each and stored at -20ºC.

Table showing Master Mix for PCR for IL-4 (+33) gene

REAGENT	**X 1 µl**	**X n µl**
Nuclease-free water	32.75	
5X buffer + $MgCl_2$	10.0	
dATP (10mM)	0.5	
dTTP (10mM)	0.5	
dGTP (10mM)	0.5	
dCTP (10mM)	0.5	
Primer 1 (F) [20pmol]	1.0	
Primer 2 (R) [20pmol]	1.0	
Genomic DNA	3.0	
***Taq* polymerase (5U/ µl)**	0.25	
TOTAL	**50.0**	

n represents the total number of samples run at any given time plus one negative control

Restriction enzyme digestion reaction for IL-4 (+33) gene

Reagent	X 1 µl	X n µl
1 X NE Buffer 3	2.0	
BSA	0.2	
PCR product	12.0	
Restriction enzyme (*BsmAI*)	1.0	
DdH_2O	4.8	
TOTAL	20 µl	

n represents the total number of samples run at any given time plus one negative control

Table showing Master Mix for PCR for IL-4Rα (Pro-478-Ser) gene

REAGENT	X 1 µl	X n µl
Nuclease-free water	32.75	
5X buffer + $MgCl_2$	10.0	
dATP (10mM)	0.5	
dTTP (10mM)	0.5	
dGTP (10mM)	0.5	
dCTP (10mM)	0.5	
Primer 1 (F) [20pmol]	1.0	
Primer 2 (R) [20pmol]	1.0	
Genomic DNA	3.0	
***Taq* polymerase (5U/ µl)**	0.25	
TOTAL	**50.0**	

n represents the total number of samples run plus one negative control

Restriction enzyme digestion reaction for IL-4Rα (Pro-478-Ser) gene

Reagent	X 1 µl	X n µl
1 X NE Buffer 3	2.0	
BSA	0.2	
PCR product	12.0	
Restriction enzyme (*Kpn* I)	1.0	
DdH_2O	4.8	
TOTAL	20 µl	

n represents the total number of samples run at any given time plus one negative control

APPENDIX B

COMET ASSAY PROTOCOL

Materials and Equipment

Lysis Low Melting Agarose (LMA)

Trevigen CometSlide™

200mM EDTA, pH 10

10X PBS, Ca^{2+} and Mg^{2+}

NaOH pellets

Dimethylsulfoxide (DMSO)

10X TBE Buffer

Silver Staining kit

Methanol

Deionised water

Temperature-regulated water bath

Eppendorf tubes

Refrigerator

Horizontal electrophoresis apparatus

Improved Neubauer counting chamber

Light microscope

Pipettes and pipette tips

REAGENT PREPARATION

Principle of Comet assay

The principle of the assay is based upon the ability of denatured, deaved DNA fragments to migrate out of the cell under the influence of electric field; undamaged DNA migrates slower and remains within the confines of the nucleoid when a current is applied. Evaluation of the resulting DNA "comet" tail shape and migration patterns allows for assessment of DNA damage.

In this assay cells are immobilized in a bed of low melting point agarose on a Trevigen Comet Slide. Following a gentle cell lysis, samples are treated with alkali to denature the DNA and hydrolyse sites of damage. The samples are then subjected to horizontal gel electrophoresis. The samples are then visualised after silver staining which allows standard light microscopy analysis. The Comet or Single Cell Gel Electrophoresis (SCGE) assay provides a simple and effective procedure for assessing DNA damage in cells.

PREPARATION OF SOLUTIONS

1. **TBE (1X)**

100mls of TBE (10X) was added to 900mls of distilled water to obtain TBE (1X).

To prepare 10X TBE:

Tris Base = 108g

Boric acid = 55g

EDTA = 9.3g

Tris base was dissolved in 900mls of distilled water; the volume was adjusted to 1 litre and stored at room temperature.

2. **5% Acetic acid v/v**

25mls of acetic acid was added to 475mls of distilled water to obtain the needed total volume of 500mls.

3. **70% ethanol**

280mls of absolute ethanol was added to 120mls of distilled water to obtain the required total volume of 400mls.

4. **PBS (1X) (Ca^{2+} and Mg^{2+}-free)**

Used concentration w/v: weighed 9.55g of the PBS is dissolved in litre of distilled water; it is homogenised, and then autoclaved.

LYSIS SOLUTION

40mls of lysis solution (from manufacturer) / 4mls of DMSO

40mls of the lysis solution is added to 4mls of DMSO and chilled at 4ºC (to prevent damage) or on ice for at least at least 20mins before use. (Addition of DMSO is optional and is required only for samples containing haeme, such as blood cells or tissue samples).

SAMPLE PREPARATION

1. Melt LMAgarose in a beaker or boiling water (1004ºC) for 5min (loosened cap).
2. Transfer to water bath (37ºC) for at least 20min to cool.
3. Add LMAgarose (37ºC) 500µl + 50µl PBS + cells
4. 500µl + 50µl cells 1:10
5. 1:10 cells (PBS) + 50µl agarose

6. Pipette 75µl immediately onto comet slide and spread evenly.

(When working with many samples, place aliquots of molten agarose in a pre-warmed micro centrifuge tubes placed at 37 ºC to prevent hardening. If cells (sample) are not spreading evenly on the slide, warm the slide at 37 ºC before application).

7. Place slide flat at 4ºC in the dark (refrigerator) for 10mins. A 0.5mm clear ring appears at the edge of CometSlide area. Increasing gelling to 30mins improves adherence of samples in high humidity environments.

8. Prepare lysis solution 20mins after chilled on ice before use. For ten slides, prepare 40mls lysis + 4ml DMSO chilled on ice for 20mins before use.

9. Immerse slide in pre-chilled lysis solution and leave on ice or at 4ºC for 30mins to 60mins.

10. Tap excess buffer from slide and immerse in freshly prepared alkaline solution, pH >13. (Alkaline solution is prepared by dissolving 0.6g NaOH in a mixture of EDTA (200mM, 250 µl) and distilled water (49.75ml). the solution warms during preparation and so should be stirred and allowed to cool to room temperature. Gloves should be worn when preparing or handling the solution.

11. Leave CometSlide in alkaline solution in the dark for 20mins to 60mins, at room temperature.

TBE ELECTROPHORESIS

12. Remove slide from alkaline solution and gently tap excess buffer from slide; wash by immersing in 1X TBE buffer for 5mins, twice.

13. Transfer slide from 1X TBE buffer to a horizontal electrophoresis apparatus. Place slides flat onto gel tray and align equidistant from the electrodes. Pour 1X TBE buffer until level just covers samples. Set power supply to 1 volt per cm (measured electrode to electrode). Apply voltage for 10mins.

14. Gently tap off excess TBE, and dip slide in 70% ethanol for 5mins.

15. Air-dry samples. Drying brings all the cells in a single plane to facilitate observation. At this stage samples may be stored at room temperature, with desiccant. Samples must be well-dried before staining.

COMET ASSAY SILVER STAINING

Trevigen's CometAssay™ Silver staining kit (Gaithersburg, MD, USA) is designed for the convenient silver staining of Comet Assay or Single cell gel electrophoresis results. Using the silver staining kit, permanent records that can be visualized using standard light microscopy are prepared, thereby avoiding the problems associated with fluorescent stains and epifluorescence microscopy. The silver staining kit is designed specifically for use with comet slides to minimize unwanted background and the amount of hazardous waste generated by silver nitrate. It is used for research only, not for use in diagnostic procedures.

REAGENT PREPARATION

Fixation Solution

Prepare immediately before fixation. Mix per sample:

10µl 10x fixation Additive

30µl de-ionised water

50µl methanol

10µl glacial acetic acid

2X Staining Reagent #4

Before first use, add 12ml of de-ionised water to bottle, stir until dissolved. Store at 4°c, pre-warm to room temperature before each use.

Staining Solution

Prepare immediately before staining. The staining reagents 1, 2 and 3 are ready to use in the staining solution as described here:

Per sample, mix in a microfuge tube:

35µl de-ionised water
5µl 20x staining reagent #1
5µl 20x staining reagent #2
5µl 20x staining reagent #3
Mix by tapping tube then add 50µl 2x staining reagent #4(at room temperature)

Stop Solution

Prepare a 5% acetic acid solution, 100µl per sample area.

ASSAY PROTOCOL

To reduce assay-to-assay variability, slides are dried, fixed and then silver stained.

1) Drying: slides should be dried completely before the fixation step. To accelerate the drying step, simply immerse the slides into cold 80% ethanol for 5 minutes, gently tap off excess and air dry.

2) Fixing: fixation is recommended as it improves repeatability of staining between assays. After electrophoresis and drying, samples are covered in fixation solution.

   a) Cover the sample area with 100µl of fixation solution

   b) Incubate for 20 minutes at room temperature.

   c) Rinse in de-ionised for 30 minutes.

3) Staining Reaction

   a) Cover sample area with 100µl of staining solution.

   b) Incubate at room temperature for 5 to 20 minutes. (Intensity of staining can be visualized under the microscope using 10x objective and reaction stopped when comets are easily visible).

   c) Stop reaction by covering samples with 100µl of 5% acetic acid and incubate for 15 minutes.

   d) Rinse in de-ionised water.

   e) Air dry.

   f) Store in the dark.

APPENDIX C

SUPEROXIDE DISMUTASE (SOD) PROTOCOL FOR CELL LYSATE ASSAY

Reagents

WST solution

Enzyme solution

Buffer solution

Dilution buffer

Preparation of reagents

1. WST working solution; prepare 1:19 dilution of WST solution using buffer solution
2. Enzyme working solution; prepare 3:500 dilution of enzyme solution using dilution buffer
3. SOD solution; prepare two-fold serial dilution of SOD solution using dilution buffer

Preparation of sample

1. Spin the blood down at 3000rpm for 10mins at 4ºC
2. Take off the supernatant (serum/plasma) and the buffy coat
3. Take a known volume of the RBC and dilute 5X using ddH_2O
4. Allow the cells to lyse while standing on ice for 5-10mins
5. Spin the lysed cells at 12000rpm for 15mins at 4 ºC
6. Take off the supernatant and use for the assay or store at -80ºC

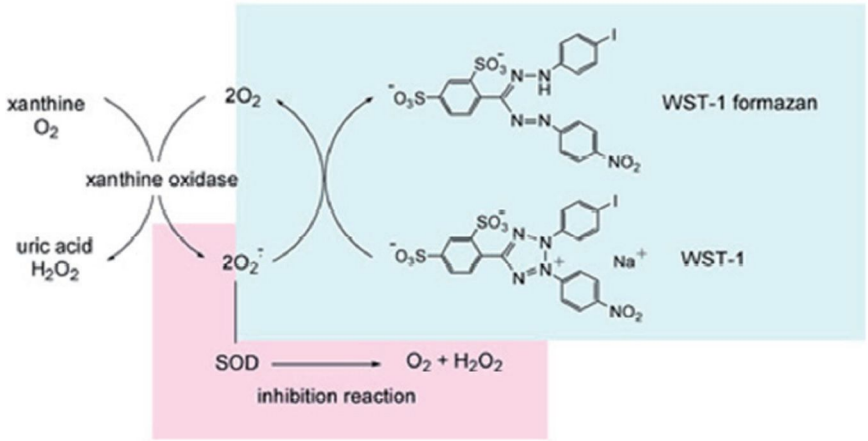
SOD assay

1. Dilute the supernatant 100X using ddH_2O
2. Add 20µl of samples to each well including blank 2 except blanks 1 and 3
3. Add 200µl of WST working solution to each well and mix well
4. Add 20µl of dilution buffer to blanks 2 and 3 wells
5. Add 20µl of enzyme working solution to samples and blank 1 except 2 and 3
6. Mix thoroughly
7. Incubate at 37ºC for 20mins
8. Read absorbance at 450nm

SOD activity is calculated as follows:

$$\text{SOD activity (inhibition rate, \%)} = \frac{\{(A_{blank1} - A_{blank3}) - (A_{sample} - A_{blank2})\} \times 100}{(A_{blank1} - A_{blank3})}$$

SOD Analysis



Principle of the determination of SOD activity using SOD Assay Kit – WST