INTERACTIONS OF HOST IMMUNE RESPONSES, IRON STATUS AND GENETIC BACKGROUNDS IN THE PATHOGENESIS OF MALARIAL ANAEMIA IN CHILDREN

BY

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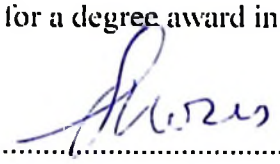
ABSTRACT

In search for pathophysiological mechanisms of malarial anaemia, this study investigated several serum soluble factors (cytokines, growth factors, iron markers) and mechanisms (red cell aging, complement regulation, genetic red cell disorders) that may explain why some children with malaria develop anaemia. Among anaemic malaria patients, children with inappropriate reticulocyte responses were compared to those with normal reticulocyte responses for the differences in serum soluble factor levels. Serum soluble factors were analyzed by multiplex bead-based platform with custom sandwich or competitive assay kits. Children with inappropriate reticulocyte responses had higher IL-1, IL-6, TNF-α, IL-10 and TNF-α/IL-10 ratio, and lower erythropoietin levels. In multivariate logistic regression analyses, only erythropoietin remained significantly associated and inversely related to poor reticulocyte response suggesting that erythropoietin influences reticulocytosis during malaria. RBC membrane surface molecules were measured by cytofluorometry and analyzed for their relationship with cytokine levels, RBC age and anaemia during acute malaria. Phosphatidylserine, IgG, CD35, CD55 and CD59 levels were not associated with cytokine levels, whereas TNF-α/IL-10 ratio associated positively with CD59 only. Loss of CD55 and CD59 occurs during erythrocyte ageing but this effect does not explain the changes occurring during malarial anaemia. CD55 levels were significantly lower in anaemic children and correlated positively with haemoglobin level, suggesting that the loss of CD55 may contribute to malarial anaemia. Immune responses to *P.falciparum* malaria in children with different genetic backgrounds were studied before and during first malaria episodes. Haemoglobin levels did not vary

according to the genetic backgrounds before malaria infection. Parasitemia, haemoglobin and cytokine levels (IL-1, IL-6, IFN-γ and IL-5) were significantly higher in age-matched children with normal haemoglobin than sickle cell carriers during malaria. Levels of TNF-α and ferritin varied on the basis of thalassemia status, and none of the serum soluble factors levels varied on the basis of G6PD genotypes. The results suggest that genetic red cell disorders vary in their effects to modulate immune response and these variations may be influencing disease outcomes. From these data it is concluded that several host factors interact to contribute to acute malarial anaemia including EPO response, CrP levels on RBC and genetic backgrounds.

DECLARATION

I, Moses Gwamaka do hereby declare to the senate of Sokoine University of Agriculture that this Thesis is my own original work and has not been submitted for a degree award in any other university.



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DEDICATION

In memory of my late father Rev. Moses Mbila Mwakyendelwa, who died on the 2nd November 2007, when I was finalizing writing this thesis.

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LIST OF ABBREVIATIONS

α	-	Alpha
Abs	-	Antibodies
AH	-	Haematin
AIDS	-	Acquired Immune Deficiency Syndrome
β	-	Beta
BC	-	Before Christ
BFU –E	-	Erythroid burst-forming units
BSA	-	Bovine serum albumin
Bp	-	Base pair
CR 1	-	Complement receptor type 1
C3	-	Complement fragment 3
C4	-	Complement fragment 4
C4d	-	Complement fragment 4d
CD	-	Cluster of differentiation
C3b	-	Complement fragment 3b
CI	-	Confidence Interval
CFU – E	-	Erythroid colony forming unit
CrP	-	Complement regulatory proteins
CRP	-	C-reactive protein
CSF	-	colony-stimulating factor
Cy5	-	Cynine 5
°C	-	Degree Celsius
DAF	-	Decay accelerating factor

DHEAS	-	Dehydroepiandrosterone Sulfate
DMTI	-	Divalent metal transporter 1
DNA	-	Deoxyribonucleic acid
EPO	-	Erythropoietin
FACS	-	Fluorescent activated cell sorter
FITC	-	Fluorescein Isocyinate
FL	-	Fluorescent detector
G6PD	-	Glucose 6 phosphate dehydrogenase
GM –CSF	-	Granulocyte-macrophage colony-stimulating factor
H_2O_2	-	Hydrogen peroxide
Hb	-	Haemoglobin
HbAA	-	Normal haemoglobin type
HbAS	-	Sickle cell trait haemoglobin
HbSS	-	Sickle cell haemoglobin
HIV	-	Human Immunodeficiency Virus
HZ	-	Hemozoin
IC	-	Immune complexes
IgG	-	Immunoglobulin G
IFN-γ	-	Interferon gamma
IL	-	Interleukin (1, 2, 3, 4, 5, 6, 10)
MAC	-	Membrane attack complex
MCV	-	Mean corpuscular volume
MCHC	-	Mean corpuscular haemoglobin concentration
MFI	-	Mean fluorescence intensity

MIF	-	Macrophage migration inhibitory factor
MOMS	-	Mother Offspring Malaria Studies
mRNA	-	Messenger ribonucleic acid
NO	-	Nitric Oxide
NADP	-	Nicotinamide adenine phosphate
NADPH	-	Nicotinamide adenine phosphate hydrogenase
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
PE	-	Phycoerythrin
pH	-	logarithm of the reciprocal hydrogen ion concentration
pO_2	-	Partial oxygen
PS	-	Phosphatidylserine
RBC	-	Red blood cells
RCD	-	Red cell deformability
RDW	-	Red cell distribution width
ROS	-	Reactive oxygen species
RPMI	-	Roswell Park Memorial Institute
SC5b9	-	C5b9/Protein S complexes (Inactive form)
SNP	-	Single nucleotide polymorphism
SR –PSOX	-	scavenger receptor for phosphatidylserine/oxidized lipoprotein
sTfR	-	soluble transferrin receptor
SUA	-	Sokoine University of Agriculture
Th1	-	T-helper 1

Th2	-	T- helper 2.
TGF-α	-	Transforming growth factor alpha
TfR	-	Transferrin receptor
TNF – RI	-	Tumour necrosis factor receptor 1
TNF – RII	-	Tumour necrosis factor receptor 2
TNF-α	-	Tumour Necrosis Factor- Alpha
UNICEF	-	United Nations Children's Fund
USA	-	United States of America
WHO	-	World Health Organization
Wt/Vol	-	Weight/Volume

CHAPTER ONE

1.0 INTRODUCTION

Malaria is an infectious febrile disease caused by protozoan parasites belonging to Plasmodium genus. Malaria is the most serious and wide spread protozoan infection of humans. An estimated 20 per cent of world's population mostly that living in developing countries is at risk of contracting malaria. Malaria causes more than three hundred million acute illnesses and kills at least one million people every year. About 90% of all malaria deaths in the world occur in Africa south of the Sahara, mostly in children under the age of five years due to their non-immune status. Majority of infections in Africa are caused by *Plasmodium falciparum*, the most dangerous of human malaria parasites (WHO/UNICEF REPORT 2003).

Malaria is characterized by cycles of chills, fever, and sweating which may advance to a complicated disease such as severe malarial anaemia and cerebral malaria. Case fatality rate of hospitalized children with cerebral malaria is higher than that of severe anaemia, but severe malarial anaemia claims more lives in endemic areas than cerebral malaria due to its high incidence (Biemba 2000a). Reportedly more than half of the malaria deaths result from severe anaemia (Murphy and Breman, 2001). Cases of anaemia are demonstrated to increase in times of peak malaria transmission (Koram *et al.*, 2000; Kurtzhals *et al.*, 2003), which suggest the relationship between malaria and anaemia.

Molecular pathogenesis of anaemia in *Plasmodium falciparum* infections is poorly understood as its etiological aspect is multifactorial (Ekvall, 2003). At least three established mechanisms can contribute to malarial anaemia: i) direct destruction of infected red blood cells (RBC) due to parasite invasion and rupture; ii) dyserythropoiesis and ineffective erythropoiesis; and iii) loss of uninfected RBC. However, direct destruction of infected RBC is considered a relatively minor contributing mechanism, because severe anaemia can occur in patients with low parasitemia and can persist for weeks after clearance of the parasite (Weatherall and Abdalla, 1982; Menendez *et al.*, 2000a). Thus, malarial anaemia is thought to arise mainly from insufficient erythropoiesis and removal of uninfected RBC.

The murkiness in the pathogenesis of malarial anaemia results from the fact that only a small proportion of malaria cases develop to a life-threatening anaemia whereas others retain normal haemoglobin levels regardless of similar causative agent and social status (Greenwood *et al.*, 1991; Biemba *et al.*, 2000a; Miller *et al.*, 2002). Such an observation can be explained on the basis of the differences in host factors, particularly the response to the pathogen (Clark and Cowden, 2003). Studies suggest that immunological responses vary among individuals and influence the clinical outcome of *P. falciparum* infections (Mc Guire *et al.*, 1999; Ubalee *et al.*, 2001). Most pathology seen in malaria is considered to result from the mechanisms designed to defend the body against the pathogen (Clark *et al.*, 1987; Clark *et al.*, 2004

Proinflammatory Th1 cytokines such as Tumour Necrosis Factor-Alpha (TNF-α), interleukin -1 (IL-1)-and interferon gamma (IFN) -γ play an important role in human immune response to malaria, but as for many acute illnesses, excessive production of these cytokines is associated with unfavourable outcome of the disease (Clark *et al.*, 2004). On the other hand it is widely accepted that anti-inflammatory (Th2) cytokines such as interleukin -10 (IL-10) regulate functional activity and production of Th1 cytokines particularly TNF-α (Ho *et al.*, 1995). Since both proinflammatory and anti-inflammatory cytokine concentrations increase during malaria (Wenisch *et al.*, 1995; Day *et al.*, 1999; Lyke *et al.*, 2004), the balance of these cytokines may play a key role to the outcome of *P. falciparum* infections (Grau and Behr 1994; Kurtzhals *et al.*, 1998; Day *et al.*, 1999; Othoro *et al.*, 1999). However, there is no comprehensive understanding on how the balance of cytokines contributes to malarial anaemia.

TNF-α, a prototype of inflammatory cytokines is involved in the mechanisms that can culminate into anaemia such as dyserythropoiesis (Roodman *et al.*, 1987; Johnson *et al.*, 1989), RBC membrane damages (Greve *et al.*, 2000; Griffiths *et al.*, 2001) and stimulation of erythrophagocytosis (Taverne *et al.*, 1994). Furthermore, inflammatory cytokines have been shown to suppress erythropoietin (EPO) production in anaemic patients (Faquin *et al.*, 1992; Jelkmann, 1998; Macdougall and Cooper 2002). On the other hand, high plasma level of IL-10 is associated with reduced risk of malarial anaemia, possibly by counteracting the effects of TNF-α (Ho *et al.*, 1995; van Vlasselaer *et al.*, 1995; Wang *et al.*, 1996; Othoro *et al.*, 1999). The development of malarial anaemia therefore appears to

involve many factors suggesting a heterogeneous anaemic population reflecting varied actiologies working singly or interactively to mediate anaemia. This study therefore aimed at investigating immune responses, iron status, RBC surface molecules and genetic backgrounds that may explain why some and not all children with malaria develop anaemia.

1.1 RESEARCH QUESTIONS

This study investigated possible interactive roles of immune responses, iron status, and RBC surface molecules changes in the pathophysiological mechanisms of malarial anaemia through studying the relationships between levels of serum soluble factors (cytokines, growth factors, and iron markers) and changes in the erythropoietic activity, and the mechanisms (red cell aging and complement regulation) during malaria. The following research questions were formulated to address the problem.

1. How serum soluble factor concentrations are related to erythropoiesis during *P. falciparum* infections?
2. What is the relationship between plasma cytokine concentration (IL-10 and TNF-α) and RBC surface membrane changes in malaria?
3. To what extent does RBC age contribute to the changes in the surface complement regulatory proteins during malarial anaemia?

Notably, genetic red cell disorders such as sickle cell, thalassemia and G6PD deficiency are widespread in various parts of the world. Their geographical distribution is closely related to the distribution of malaria because of the selective

advantage against severe malaria conferred to heterozygotes. In addition, most of these disorders are associated with anaemia of varying degrees. Therefore, this study involved screening all study participants for red cell genetic disorders in order to understand the effects of these on the haematological parameters, and the following questions were addressed in this study as well:-

1. What is the frequency of selected genetic red cell disorders in Muheza - Tanzania and how are they related to malarial anaemia?

2. What are the immune factors associated with protection against severe malaria in children with various RBC disorders?

1.2 Justification

This study provides an understanding of the roles of serum soluble factors (cytokines, growth factors and iron markers) on erythropoietic suppression and RBC membrane changes during *P. falciparum* infections which provide insight into the pathogenesis of malarial anaemia. Determining why some individuals develop anaemia and others do not is key to understanding the pathogenesis of this disease entity, and possibly the protective mechanisms responsible for non-severe cases. Elucidation of the pathogenesis of complicated malaria will lead to further development of new and more effective preventive and therapeutic measures.

1.3 OBJECTIVES

1.3.1 Broad objective

To study interactive roles of immune responses, iron status and genetic backgrounds on RBC turnover during *Plasmodium falciparum* malaria.

1.3.2 Specific objectives

1. To compare levels of serum soluble factors (cytokines, growth factors and iron markers) between children with appropriate and inappropriate reticulocyte response to anaemia during malaria, and to study the relationship between the levels of these factors with reticulocyte production in *P. falciparum* infected children with or without anaemia

2. To study the relationship between serum cytokine levels and TNF- α/IL-10 ratios with levels of RBC surface molecules including Phosphatidylserine, Immunoglobulin G and complement regulatory proteins (CrP) in children with malaria.

3. To determine the abundance of cells in different RBC age subpopulations, and the abundance of complement regulatory proteins: Decay accelerating factor (CD55) and Protectin (CD59) in different RBC age subpopulations, and relate these to risk for anaemia during malaria.

4. To determine the frequencies of hemoglobinopathies (Sickle cell and α - thalassemia) and Glucose 6 phosphate dehydrogenase deficiency in Muheza District in Tanzania, and relate these to the risk of severe malarial anaemia.

5. To determine host immune responses (particularly cytokine production) to *P. falciparum* infections in children with various genetic red cell disorders before and during *P. falciparum* infections

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 OVERVIEW

2.1.1 Malaria causative agents

Malaria is a febrile vector-borne disease caused by protozoan parasites of the genus *Plasmodium*. Four species of which infect human beings are *P. falciparum, P. vivax, P. ovale and P. malariae*. Of the four species, *Plasmodium falciparum* causes the most severe form of malaria and is responsible for the majority of infections in tropical Africa, parts of India and Pakistan, south East Asia and central and South America. The pathogenicity of this specie is linked to its ability to infect cells of all ages, and develop multiple ring form infections in one erythrocyte, which result into high parasitemia. Furthermore, *P. falciparum* infected cells adhere (cytoadherence) to vascular endothelium via formation of sticky knobs on RBC surface that bind to receptors on capillaries and venules causing sequestration of red cells in the microcirculation thus evading spleenic clearance and obstructing blood flow leading to multi-system dysfunction. *P. vivax* is the commonest cause of human malaria in Central America, North Africa, and Southern and Western Asia, causing over 80 million episodes of malaria each year, but only a small fraction of deaths. *P. malariae* has wide global distribution, found in South America, Asia, and Africa, but it is less frequent than *P. falciparum* in terms of association with cases of infection (Tobian *et al.*, 2000) whereas *Plasmodium ovale* is mostly limited to tropical Africa, the Middle East, Papua New Guinea, Indonesia and Southeast Asia.

2.1.2 Historical perspectives

History of malaria and its terrible effects is as ancient as the history of civilization. The mentions of malaria existence are found in the ancient Chinese, Egyptian, Indian and Roman manuscripts. The Ancient Chinese medical writing Nei Ching (The Canon of Medicine), dated 2,700 before Christ (BC), describes symptoms of malaria. Sumerian and Egyptian texts dating from 2,000 to 1,500 BC also refer to symptoms such as fever and splenomegaly, which are suggestive of malaria (Kakkilaya, 2006a). Malaria has shaped the course of history for millennia. In the 4th century BC, malaria was recognized in Greece as the primary determinant of the decline of many cities/nations and crushing military defeats, often having caused more casualties than the weapons themselves (Kakkilaya, 2006b). Generally malaria has had profound impact on human history, for centuries it prevented economic development in vast regions of the earth and continues to be a huge social, economical and health problem today.

2.1.3 Malaria distribution and burden

Malaria affects over 100 countries most of them tropical and subtropical (Figure 1). The pattern of disease distribution mostly coincides with the climatic factors such as temperature, humidity and rainfall, which favour multiplication and survival of the vector, female anopheline mosquitoes as well as growth of malaria parasite in the vector. Temperature above 20°C is critical for the perpetuation of *P. falciparum* infections, below which the parasite can not complete its growth cycle in the mosquito and thus can not be transmitted to man (CDC Malaria 2004).

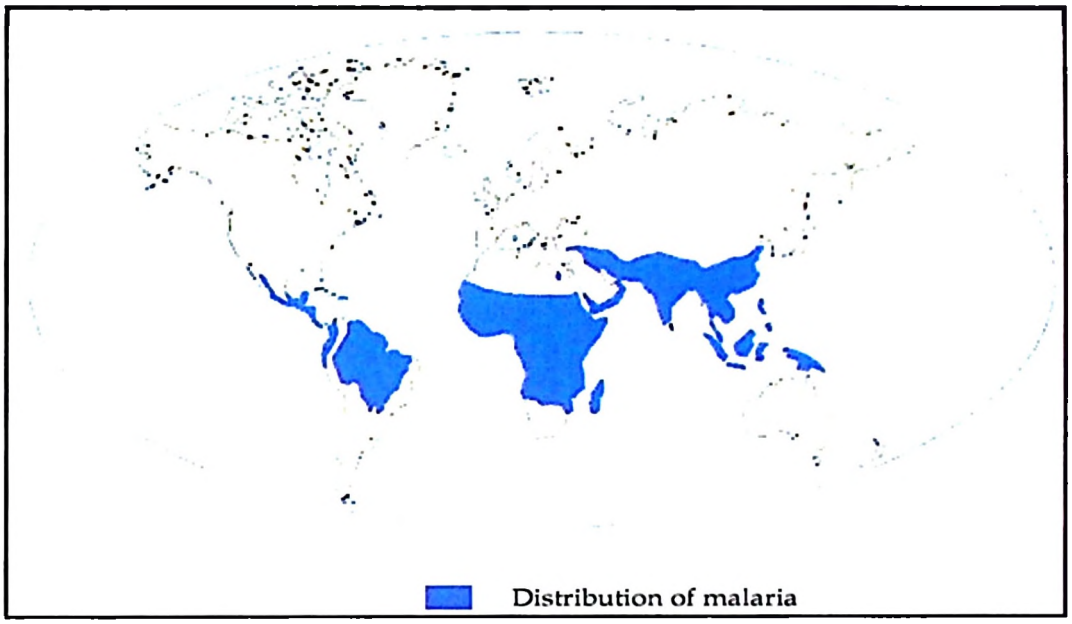

Figure 1: Geographic distribution of malaria
Source: CDC Malaria 2004

Carter and Mendis, (2002) estimated that 3.2 billion people (almost half of the world's population) live in areas where malaria is either endemic or epidemic). The morbidity of malaria has been estimated to be over 500 million each year (Breman, 2000) and the mortality is estimated at about 1 million people per year. However these figures may have increased in recent years due to many factors including increased resistance to chloroquine and sulfadoxine-pyrimethamine, which were the most commonly used, easily available and affordable antimalarial drugs in most regions (WHO/UNICEF REPORT 2003). Other factors leading to increased occurrences of malaria include changing agricultural practices, deforestation, and population movements into malarious areas (Mouchet *et al.*, 1998). These practices have resulted in the expansion of suitable habitat for the survival of the anopheline mosquitoes (Klinkenberg *et al.*, 2003).

Malaria causes more harm in Africa than in any other region in the world. About 90% of all malaria deaths in the world today occur in sub Saharan Africa. In this region 25 - 40% of all outpatient clinic visits are for malaria, and between 20% and 50% of all hospital admissions are related to malaria (WHO/UNICEF REPORT 2003). The devastating impact of malaria to Africa is mostly attributable to the fact that the majority of infections in Africa are caused by *Plasmodium falciparum*, the most dangerous of the four human malaria parasites. Also, high prevalence in this region of mosquito *Anopheles gambiae*, the most effective malaria vector plays a significant role in the spread of the disease. *A. gambiae* and *A. funestus* have long lifespan, breed in peridomestic habitats and focus their biting on people. Poor and declining health services in most African countries also escalate the disease impact to these communities.

In areas of stable transmission children under the age of five years suffer most from malaria due to their non-immune status. It is estimated that after every 30 seconds, malaria kills an African child. Effects of malaria on adults differ depending on whether the region is an endemic or epidemic zone. In endemic areas, people are exposed to the parasite regularly throughout their lives. Individuals who have survived childhood infections gradually build up resistance against the disease, and as a result the majority of malaria cases in adults are asymptomatic, with very few becoming fatal. In regions where malaria transmission is not stable and occasional epidemics occur, both adults and children are vulnerable due to their non-immune status.

Adult women in malaria endemic areas have a high level of immunity, but it is impaired during pregnancy, more so during the first pregnancy, when placental malaria due to *Plasmodium falciparum* is most frequent and severe. Malaria infection in the mother, especially in areas of low or unstable transmission, can result in abortion, stillbirth or congenital infection. More commonly, malaria infection, in combination with maternal anaemia, can interfere with foetal weight gain and contribute to intrauterine growth retardation resulting in low birth weight (Sullivan *et al.*, 1999; Menendez *et al.*, 2000b; Okoko *et al.*, 2002), which has negative impacts on health later in infancy and possibly into childhood.

These potentially fatal outcomes of pregnancy malaria can be prevented through implementation of chemoprophylaxix/chemosuppression strategies during pregnancy (Mutabingwa *et al.*, 2001). World Health Organization (WHO) recommends that suppressive doses of an effective antimalarial drug be given to women living in malaria endemic areas (WHO, 1996). However, current tools to detect malaria parasites in pregnant women are not sensitive enough to detect a low parasitemia. Furthermore, the kinetics, safety, and efficacy of available antimalarial drugs are poorly documented because pregnant women are systematically excluded from clinical trials (Steketee *et al.*, 1996; Nosten *et al.*, 2006). A considerable effort, involving clinical trials, is urgently required to improve the diagnosis and case management of malaria during pregnancy if the morbidity and mortality of maternal malaria is to be reduced.

2.1.4 Parasite growth in man

A malaria parasite undergoes a developmental cycle in the female anopheline mosquito. Infection in humans begins when an infected mosquito injects sporozoites that enter the peripheral circulation. The sporozoites in the blood quickly invade liver cells (hepatocytes). Sporozoites are cleared from the circulation within 30 minutes. During the next week the liver-stage parasites (pre-erythrocytic stage) differentiate and undergo asexual multiplication resulting in the release of tens of thousands of merozoites from the hepatoctye into the bloodstream, which subsequently invade RBC to initiate the intra -erythrocytic cycle. In RBC, parasites continue with the multiplicative cycle producing 16 -20 additional merozoites per RBC, which devour the RBC haemoglobin in the process. These merozoites are released by the lysis of the red blood cell and they immediately invade uninfected red cells. This repetitive cycle of invasion - multiplication - release - invasion continues. The intra erythrocytic cycle takes about 48 hours in *P. vivax*, *P. ovale* and *P. falciparum* infections and 72 hours in case of *P. malariae* infection. It occurs synchronously and the merozoites are released at approximately the same time of the day. Some invading merozoites do not divide, but differentiate into male (microgametocyte) and female (macrogametocyte) sexual forms. These sexual forms are taken from the bloodstream by a feeding *Anopheles* mosquito to initiate the mosquito stages of development (Kakkilaya, 2006c).

2.1.5 Clinical manifestations of malaria and complications

Clinical features of malaria appear during the erythrocytic schizogony in the blood. Malaria is characterized by periodic chills, fever, vomiting and sweats, which are associated with the synchronous rupture of the infected erythrocyte. Typical clinical features of malaria include three stages as follows: cold stage, hot stage and sweating stage. The cold stage is characterized by febrile episode beginning with shaking chills, which lasts from 15 minutes to 1 hour, followed by the hot stage which is characterized by high grade fever, reaching above 40, which lasts 2 to 6 hours. The cold stage then proceeds as profuse sweating and a gradual fall of fever over 2-4 hours (Kakkilaya, 2006d). The good predictability of clinical signs and symptoms for high-risk groups suggests that simple protocols may be applicable for the management of malaria in endemic areas (Gomes *et al.*, 1994). However, in an endemic area, diagnosis of malaria basing on clinical signs may be challenging and complicated. In such areas, malaria often presents with atypical manifestations such as headache, body ache, backache and joint pains, dizziness, vertigo, altered behavior, acute psychosis, chest pain, acute abdomen, vomiting and diarrhea, which are shared by other diseases (Ammah *et al.*, 1999; Kakkilaya, 2006d; Bronzan *et al.*, 2008).

Malaria causes death via complications such as cerebral malaria, more common in acute cases whereas repeated infections can kill via severe anaemia and hypoglycaemia (Breman *et al.*, 2004). Malaria also predisposes its victims to other potentially lethal complications such as acute respiratory diseases and organ

failure. If malarial anaemia results in blood transfusions, there is also a risk of HIV/AIDS, hepatitis B or hepatitis C infections.

2.1.6 Human immunodeficiency virus (HIV) infections and malaria risk

Conflicting reports exist regarding the impact of human immunodeficiency virus (HIV) infection on the risk of malaria infections. Nevertheless, it appears that HIV infections increase both the susceptibility to and the severity of malaria infection particularly *P. falciparum* malaria. HIV-infected patients are more susceptible to acquire malaria infection (Van Eijk *et al.*, 2003). Both the prevalence of malaria parasitemia and the incidence of clinical attacks of malaria are greater in patients with HIV-induced immunosuppression (Francesconi *et al.*, 2001; Cohen *et al.*, 2005). Furthermore, the risks of severe malaria and malaria-related death appear to increase significantly in HIV-infected patients of all ages who live in regions where malaria transmission is unstable (Grimwade *et al.*, 2004)

2.2 PATHOGENESIS OF MALARIAL ANAEMIA

Malaria triggers an imbalance between production and destruction of RBC, which subsequently leads to anaemia. Mechanisms behind the development of malarial anaemia can be put into two broad categories: (I) abnormal erythrocyte production and maturation in the bone marrow and (II) hemolysis (Ekvall, 2003). However the molecular mechanisms responsible for the development of malarial anaemia either at the production level or in the circulation are poorly understood.

2.2.1 Erythropoiesis

Erythropoiesis is the process by which red blood cells (erythrocytes) are produced. In humans, erythropoiesis occurs almost exclusively in the red bone marrow; but in response to a greater need for RBC production, the yellow bone marrow can turn to red marrow. RBC production is controlled by the tissue demand for oxygen.

2.2.1.1 Erythropoiesis during *P. falciparum* malaria

Inappropriately low reticulocytosis relative to the degree of anaemia is often observed in malaria patients suggesting that insufficient erythropoiesis causes or exacerbates malarial anaemia (Weatherall *et al.*, 1983; Kurtzhals *et al.*, 1997). Since reticulocytosis is the downstream event of erythropoiesis, studies on the possible changes in bone marrow cellularity of erythroid lineage in human malaria revealed two more-or-less distinct patterns of erythropoietic activity (Wickramasinghe and Abdalla, 2000). Firstly there is a reduced marrow cellularity, which is accompanied by reduced percentage of erythroblasts (hypo-proliferative erythropoiesis). This is particularly common in acute childhood malaria with high parasitemia. Dormer et al. (1983) demonstrated a marked loss of polychromatic erythroblasts during acute *P. falciparum* infection, which suggests that erythropoiesis, is halted during early developmental stages of erythroid progenitor cells. Furthermore, Knüttgen (1987) reported dysplastic features of erythroid precursors in the bone marrow of non-immune individuals with acute malaria. The second pattern is observed in chronic malaria in which there is increased marrow cellularity and percentage of erythroblasts (hyper-proliferative)

but the erythropoiesis is ineffective suggesting that cells of erythroid lineage are lost during later stages of development (Abdalla *et al.*, 1980; Wickramasinghe and Abdalla, 2000).

Ineffective erythropoiesis is often associated with intramedullary destruction of erythroid precursors by mechanisms such as erythrophagocytosis and morphological abnormalities of erythroblasts. Abnormalities are recognized by light microscope as deformed nuclei, bi and multiple nuclearity, megaloblastic changes, intranuclear chromatin bridges and karyorrhexis. Electron microscopic studies show many abnormalities of the erythroblasts including abnormally long intranuclear clefts, irregular nuclear shapes, myelination or loss of parts of the nuclear membrane, abnormally large autophagic vacuole and varying degrees of iron-loading mitochondria (Abdalla *et al.*, 1980). These dysplastic features of erythroblasts (dyserythropoiesis) and the lack of reticulocytosis appropriate to the degree of anaemia suggest that apoptosis may be the cause of insufficient erythropoiesis in malaria. Additionally, cell-cycle distribution of early polychromatic erythroblasts shows increased proportion of cells during a period of rapid growth to prepare for mitosis (G_2 phase) phase and an arrest during the progress of some cells through the DNA synthesis (S) phase.

Malarial anaemia is associated with a shift of iron distribution from functional compartments comprising metabolically active iron that is required for normal functions towards storage compartments that constitute an iron reserve (Das *et al.*, 1997). Ferrokinetic studies show an increase in plasma iron turnover and a

decrease in red cell iron utilization. This suggests that insufficient erythropoiesis results from either sub optimal response of erythroid progenitors to erythropoietin (EPO) stimulation or inadequate EPO production.

2.2.1.2 Soluble transferrin receptor levels as a marker of erythropoiesis during malaria

Active malaria is associated with changes in erythropoietic activity brought about by hemolysis, which would tend to increase sTfR levels but on the other hand is associated with inflammation which would rather decrease sTfR levels. Several investigators have studied sTfR levels in malaria as a marker of erythropoiesis however contradicting observations have been reported.

The commonly reported observation in acute malaria is the decreased sTfR levels which reflect suppression of erythropoiesis (Williams *et al.*, 1999; Beesley *et al.*, 2000) but other investigators did not observe any differences (Kuvibidila *et al.*, 1995) or an increased sTfR levels during clinical malaria (Menendez *et al.*, 2001). Mild or asymptomatic malaria infections show increased sTfR level indicating appropriate marrow response during malaria (Mockenhaupt *et al.*, 1999; Verhoef *et al.*, 2001). However, the interpretation of sTfR values in malaria can be difficult because this disease is associated with changes in erythropoietic activity triggered by hemolysis, and inflammation which may disturb iron homeostasis (Beguin, 2003). Moreover, iron deficiency is common in most of the areas where malaria is endemic such as Sub-Saharan Africa, and appears to modify the levels of sTfR (Ahluwalia 1998; Choi and Pai 2003). Iron deficiency protected mice against

infection with *P. chabaudi* (Harvey *et al.*, 1985) and was associated with the protection of children from mild clinical malaria in the coast of Kenya (Nyakeriga *et al.*, 2004). Thus, in most areas malaria and iron deficiency co-exist making the interpretation of sTfR results complicated and misleading if both factors are not taken into consideration. In iron deficiency, erythropoietic activity has a secondary role as predictor factor of sTfR levels (Sipahi *et al.*, 2004; Angeles Vázquez López *et al.*, 2006). It has been recommended that sTfR should be regarded as a marker of erythropoiesis only when iron stores are adequate, and becomes a marker of iron deficiency only when tissue iron deficiency (with or without adequate iron stores) occurs (Beguin 2003).

2.2.1.3 Erythropoietin production during malaria

Clinical studies provide conflicting data concerning the production of erythropoietin during malaria (Burgmann *et al.*, 1996). Experimental animal models and *P. falciparum* infected children show that erythropoietin (EPO) is increased to levels seemingly appropriate to the degree of anaemia (Yap and Stevenson, 1992; Burchard *et al.*, 1995; Kurtzhals *et al.*, 1997; Nussenblatt *et al.*, 2001; Chang *et al.*, 2004a). Additionally Kurtzhals *et al.* (1997) suggested that EPO production is sufficient during malaria but *Plasmodium falciparum* infections cause reversible suppression of the bone marrow response to EPO. This was a general finding occurring in all malaria cases irrespective of initial haemoglobin levels. On the other hand few studies show that EPO level in malaria patients is inadequate for the level of anaemia (Burgmann *et al.*, 1996; el Hassan

et al., 1997). Thus the status of optimal EPO production during malaria is unclear and therefore warrants further investigation.

The mechanistic basis for the suppression of erythropoiesis in the presence of adequate production of functional EPO is not fully understood. Researchers suggest that both parasite and host factors are responsible for the suppression of erythropoiesis. Among parasite factors, the malaria pigment (hemozoin) and malaria toxin (glycosylphosphatidylinositol) are known to suppress erythropoiesis (Rudin *et al.*, 1997; Giribaldi *et al.*, 2004). However, these effects are mediated indirectly through release of host factors including the pro inflammatory cytokines. Malaria pigment and toxins stimulate macrophages to produce TNF-α, which, together with IFN-γ and IL-1 are the major pro-inflammatory cytokines produced during acute response to blood stage malaria in humans and mice (Sherry *et al.*, 1995; Stevenson and Riley, 2004). Additionally, phagocytosis of RBC and parasitized RBC by monocytes and macrophages stimulates these phagocytes to increase the production of TNF-α and IL-1.

2.2.1.4 Cytokines production during malaria

During *P. falciparum* malaria there is an increase in concentration of both proinflammatory and anti-inflammatory cytokines (Wenisch *et al.*, 1995; Day *et al.*, 1999; Lyke *et al.*, 2004). Elevated plasma cytokines in malaria are associated with systemic pathologic abnormalities causing severe disease (Day *et al.*, 1999). The balance between pro- and anti-inflammatory cytokines appears to be an important factor in determining malaria presentation and outcome. In particular

high plasma concentrations of TNF-α over IL-10 are associated with the development of severe anaemia the mechanism of which is still unknown (Kurtzhals *et al.*, 1998; Othoro *et al.*, 1999; Perkins *et al.*, 2000). Cytokines and cells of monocyte/macrophage lineage induce changes in iron homeostasis, proliferation of erythroid progenitor cells, the production of erythropoietin, and the life span of RBC, all of which may contribute to the development of anaemia.

2.2.1.5 Cytokines induce dysregulation of iron Homeostasis

Effects of cytokines on erythropoiesis during malaria are considered similar to that of anaemia of inflammation. Chronic inflammatory conditions such as rheumatoid arthritis are associated with anaemia (termed as anaemia of inflammation). A hallmark of anaemia of inflammation is the development of disturbances of iron homeostasis, with increased uptake and retention of iron within macrophages/monocytes. This leads to a diversion of iron from the circulation into storage sites of the macrophages/monocytes phagocytic system, with subsequent limitation of the availability of iron for erythroid progenitor cells, and iron-restricted erythropoiesis resulting in anaemia.

Cytokines play a central role in the disturbances of iron homeostasis during inflammation. For example, mice injected with IL-1 and TNF-α develop hypoferremia and anaemia (Alvarez-Hermandez *et al.*, 1989). These conditions are linked to cytokine-inducible synthesis of ferritin, the major protein associated with iron storage, by macrophages and hepatocytes (Torti and Torti 2002). Furthermore, interferon-γ and TNF-α up-regulate the expression of divalent metal

transporter (DMT1), with an increased uptake of iron into activated macrophages. Proinflammatory cytokines stimuli also induce the retention of iron in macrophages by down regulating the expression of ferroportin, thus blocking the release of iron from these cells (Ludwiczek *et al.*, 2003). Ferroportin is a transmembrane exporter of iron, which mediates a process believed to be responsible for the transfer of absorbed ferrous iron from duodenal enterocytes to the circulation (Pietrangelo, 2002). Interleukin-6 induces hypoforremia by stimulating hepatic expression of the acute-phase protein hepcidin that inhibits duodenal absorption of iron. The induction of hypoferremia by interleukin-6 and hepcidin occurs within few hours and is not observed in interleukin-6–knockout mice (Andrews, 2004) suggesting that IL-6 plays an important role in the development of anaemia. In summary, high levels of these cytokines lead to a decreased iron concentration in the circulation and thus to a limited availability of iron for erythroid cells. In malaria, the impact of *P. falciparum* infection on iron metabolism could be a combination of the effects of hemolysis and of the infection. Parasites may affect iron status either directly by means such as release of iron into the circulation during intravascular hemolysis, sequestration of iron in malarial pigment (hemozoin) and consumption of iron for its own metabolism or indirectly by reducing intestinal iron absorption and promoting iron mobilization into body stores mediated by host cytokines.

2.2.1.6 Inhibition of erythropoiesis by cytokines

Up-regulation of cytokines such as TNF-α in human is associated with hematologic disorders including different forms of anaemia (Allen *et al.*, 1999;

Hara *et al.*, 2004) and several other studies show that cytokines modulate hematologic processes in the bone marrow. Proinflammatory cytokines such as TNF-α, IFN-γ, and interleukin-1 directly inhibit the differentiation and proliferation of erythroid progenitor cells. TNF-α mediates erythropoietic suppression in mice and humans by decreasing the responsiveness of erythroid precursors to erythropoietin (Rusten and Jacobsen, 1995, Issifou *et al.*, 2003; Chang *et al.*, 2004b; McDevitt *et al.*, 2004). TNF-α also indirectly inhibits erythropoiesis by inducing IFN-γ production by accessory cells (Means *et al.*, 1990; Means and Krantz, 1993). TNF-α has also been observed to induce apoptosis of erythroid precursors (Papadaki *et al.*, 2002a, 2002b). However, other investigators argue against TNF-α, IFN-γ and IL-1 as the host derived inhibitors of erythropoiesis. Yap and Stevenson (1994) demonstrated that neutralizing antibodies to TNF-α, IL-1 and IFN-γ were not effective in reversing suppressed erythropoiesis of splenic erythroid cells obtained from *P.chabaudi* infected mice. Moreover, Giribaldi *et al* (2004) demonstrated that malaria pigment, hemozoin (HZ) and 4-hydroxynonenal, a final product of lipid peroxidation generated by HZ inhibited growth of erythroid cells, therefore could be one of the factors for the development of anaemia during malaria.

2.2.2 HEMOLYSIS AND MALARIAL ANAEMIA

Hemolysis is the prime cause of anaemia seen in acute *P. falciparum* malaria (Das *et al.*, 1999; Jakeman *et al.*, 1999; Price *et al.*, 2001). The essential feature of hemolysis is a shortened RBC lifespan, thus haemolytic anaemia occurs when bone marrow function can no longer compensate for the shortened RBC survival.

Most RBC parasitized with *P. falciparum* die during parasite growth but the loss of infected cells does not correlate with the level of anaemia seen in most malaria cases. In many cases the decrease in haemoglobin has been greater than could be accounted for by the destruction of infected RBC alone (Menendez *et al.*, 2000a). Studies show that loss of uninfected RBC also occurs during malaria (Jakeman *et al.*, 1999; Price *et al.*, 2001), but the mechanism behind is not well understood.

Asexual development of *P. falciparum* in the erythrocyte is the pathogenic stage and the only gateway to the clinical disease. The repeated cycling growth, release and reinvasion leads to an exponential increase of parasites and destruction of RBC. This episode initiates a series of events which play an important role in the pathogenesis of malarial anaemia. Several mechanisms have been proposed to explain the RBC loss occurring during malaria including complement-mediated lysis, autoimmune hemolysis and phagocytosis, which indiscriminately affect infected and non-infected erythrocytes. The summary of these mechanisms may schematically be illustrated as portrayed below in Fig. 2.

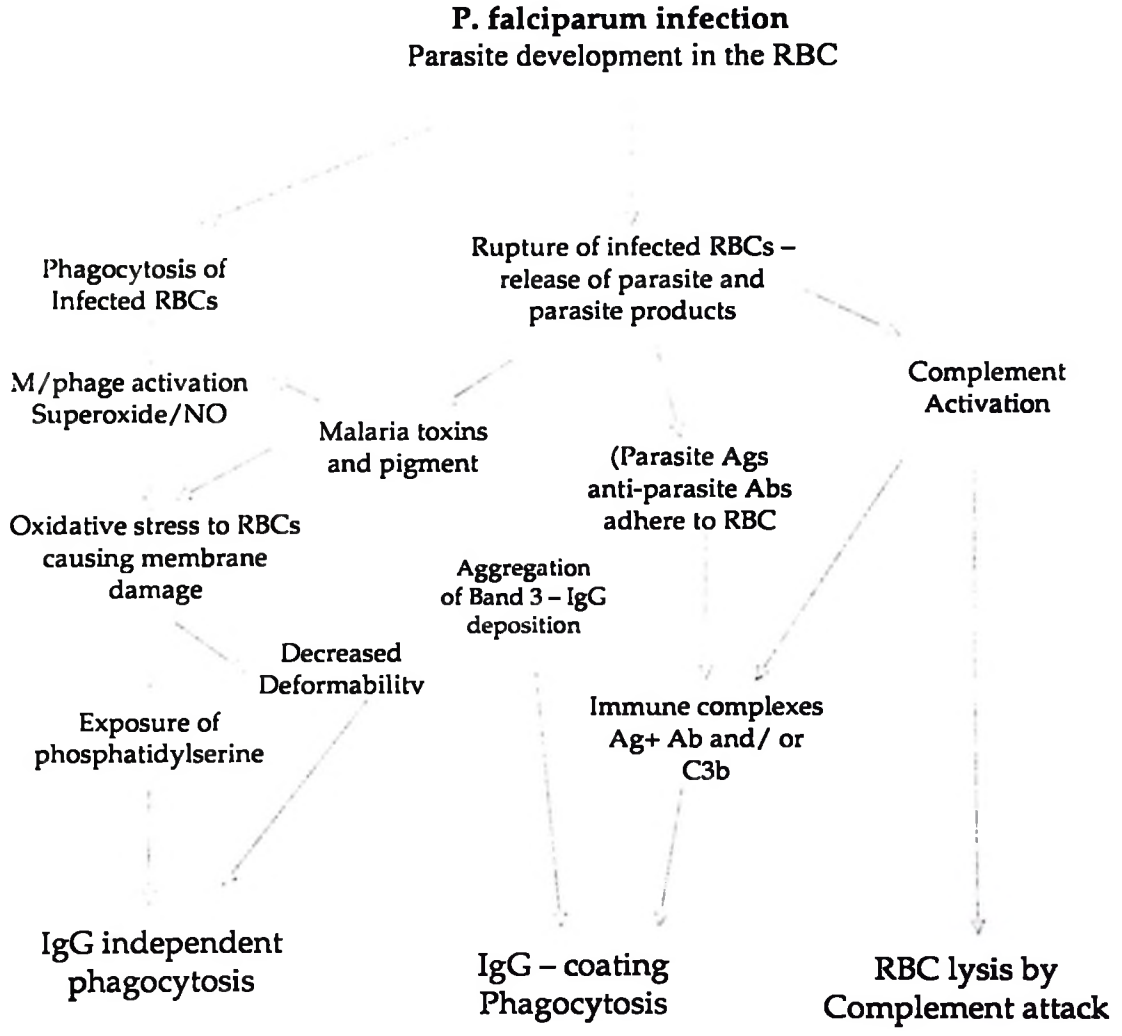

Figure 2. Summary of the proposed mechanisms for RBC destruction during *P. falciparum* malaria

2.2.2.1 Direct destruction of infected cells

Inside RBC, parasites grow and divide to form merozoites; thereafter the host cell bursts releasing merozoites into the circulation. Intravascular hemolysis is associated with low plasma haptoglobin (body's main tool for removing toxic free Hb) and sometimes haemoglobinuria. Low plasma haptoglobin is commonly reported in children with severe malaria anaemia (Newton *et al.*, 1997; Das *et al.*, 1999), suggesting a recent haemolytic crisis. Sudden appearance of haemoglobin in urine (haemoglobinuria) indicates severe intravascular hemolysis of which the haemoglobin release exceeded the binding capacity of plasma haptoglobin. However, it seems unlikely that haemoglobinuria occurring in some malaria cases originates exclusively from cell rupture consequential to parasite growth (Ekvall *et al.*, 2001), because during the intra-erythrocytic development the parasite, *Plasmodium falciparum* digests up to 75% of erythrocyte haemoglobin (Francis *et al.*, 1997).

The presence of detectable free haemoglobin in the plasma may also be linked to host genetic factors and drug effects. For example, studies have shown that sudden intravascular hemolysis which is characterized by haemoglobinuria during malaria is common in South Asia and Papua New Guinea. This is associated with the use of drugs such as quinine, halofrantrine and mefloquine (van den Ende *et al.*, 1998; Price *et al.*, 1999; Bruneel *et al.*, 2002) and genetic red cell disorders such as Glucose-6-Phosphate Dehydrogenase Deficiency (G6PD). Drugs act as haptens and stimulate production of drug dependent complement fixing antibody that causes RBC lysis. G6PD deficiency results into hemolysis when oxidative drugs

are administered or during stress induced by infection (Sarkar *et al.*, 1993; Sukumar *et al.*, 2002).

2.2.2.2 Effect of complement activation on red blood cells during malaria

It is hypothesized that activation of complement system results into the deposition of complement components on RBC which causes the destruction of RBC by either direct complement lysis (formation of Membrane Attack Complex, MAC) or indirectly through phagocytosis triggered by complement - dependent processes. Indices of plasma complement activation such as C3, C4, Bb, C4d, iC3b, and SC5b-9 show that both the classical and the alternative pathways of the complement system are activated during complicated malaria (Wenisch *et al.*, 1997).

Under normal circumstances erythrocytes are protected from autologous complement lysis by the complement regulatory proteins (CrPs) such as complement receptor type 1(CR1), decay accelerating factor (DAF or CD55) and membrane inhibitor of reactive lysis (CD59). Acquired or inherited deficiencies in the complement regulatory proteins play a key role in the pathogenesis of immune haemolytic anaemia such as paroxymal nocturnal haemoglobinuria (Nishimura *et al.*, 1998; Chrobak, 2000).

Parallel to the complement activation, the levels of red cell surface CrPs are altered in children with severe anaemia, including the decrease of CR1 and DAF/CD55 (Waitumbi *et al.*, 2000). Such changes would leave the RBC

unprotected against autologous complement attack (Molina *et al.*, 2002). However, the membrane inhibitor of reactive lysis, CD59 increases in children with malarial anaemia (Waitumbi *et al.*, 2000) which suggests that direct complement attack is unlikely to occur during malaria. CD59 plays a pivotal role in inhibiting complement lysis through preventing formation of polymeric C9 complex during MAC assembly (Rollins *et al.*, 1991). Holguin et al. (1992) demonstrated that no significant erythrocyte lysis could take place in the presence of functional CD59. Nevertheless, the reasons for the simultaneous decrease of complement regulatory proteins (CR1 and CD55) with haemoglobin level in malaria are not well understood.

Decrease of CrP may be linked to the removal of immune complexes by tissue macrophages. Parasite proliferation is associated with the generation of soluble plasmodial antigens (McGregor *et al.*, 1968), which may adhere to the surface of both infected and uninfected RBC. These antigens may react with antibodies to form immune complexes, ICs (Mohammed, 1982) on RBC surfaces. In such situations erythrocyte CR1 and CD55 could be adsorbing excessive amount of ICs, and these are transferred to the activated macrophages in spleen and liver in the process called transfer reaction. As a result significant amount of CrP may be lost along with ICs to the phagocytes in the process. When erythrocytes reach a certain level of CR1 and CD55 deficiency probably lose the ability to control complement activation and the removal of immune complexes from the circulation. As a result complement factor C3b and ICs may be deposited on the RBC leading to increased destruction of erythrocyte by the phagocytes in the

reticuloendothelial tissues escalating anaemia. It is expected therefore that the rate of uptake of complement opsonised cells will be directly associated with the level of activation of the phagocytes (Biemba *et al.*, 2000b) which might be influenced by cytokines. The persistence of the Th1 mediated immune response and macrophage activation may be involved in the lingering anaemia in children treated for malaria (Biemba *et al.*, 1998).

Also, surface complement regulatory proteins decrease as part of the normal red cell aging process (Fishelson and Marikowsky, 1993; Bratosin *et al.*, 1998; Bratosin *et al.*, 2002; Miot *et al.*, 2002). How this physiological phenomenon contributes to the changes in the erythrocyte surface molecules during malaria is unknown. Apparently, reduced erythrocyte production due to ineffective erythropoiesis as well as dyserythropoiesis in the bone marrow occurs in children with malaria (Wickramasinghe and Abdalla 2000). This would alter the red cell age profile so that older cells may dominate in the circulation. Thus, it remains unclear whether changes in RBC surface molecules contribute to the development of malarial anaemia, or whether they result from an altered RBC age profile. This paucity of data could be resolved only if the RBC populations were subjected to age fractionations prior to analysis to see if the changes occur in cells of all ages.

2.2.2.3 Autoimmune hemolysis (Immune dependent pathway)

There is a considerable variation in the literature on the role of autoimmune hemolysis in the pathogenesis of malaria anaemia. Many researchers suggest that the interaction between RBC and macrophages is immune dependent based on the

observation that in *P. falciparum* infections both infected and uninfected RBC bind large amounts of immunoglobulins (Facer *et al.*, 1979; Goka *et al.*, 2001). RBC coated with antibodies are targeted by macrophages/monocytes. It has been hypothesized that immunoglobulin G (IgG) is deposited on the surface of RBC during the course of the disease resulting from several major mechanisms. Firstly, the deposition of IgG results from oxidative damage due to the effect of reactive oxygen species (ROS), which are produced owing to the effect of proinflammatory cytokines, on macrophages. Exposure of RBC to ROS may cause oxidative injuries to membranes that become target for autoantibodies (Kremsner *et al.*, 2000). Also the development of parasite inside the red cell causes oxidative stress that induce stage dependent membrane modifications on the infected cell that lead to phagocytic recognition. These changes occur in the following sequence: deposition of hemichromes and oxidative aggregation of band 3; deposition of autologous IgG and complement; and final recognition by phagocytes (Giribaldi *et al.*, 2001). Secondly, host IgG may be directed to the malaria parasite antigens attached to the infected RBC surfaces, although it is equally true that autoantibodies detected may be part of immune complexes present on these surfaces. Thirdly, IgG may be deposited on the surface of infected RBC following the expression of *Plasmodium falciparum* variant surface antigen *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a family of parasite-encoded cytoadherence receptors localized to knobs on the surface of parasitized erythrocytes (Bull *et al.*, 1998; Kinyanjui *et al.*, 2004).

On the other hand, the idea of autoimmune involvement in the development of anaemia has not been greatly supported by other investigators due to the following reasons: (i) RBC surface antibodies are not consistently observed in all studies (Facer *et al.*, 1980a; 1980b; Merry *et al.*, 1986; Newton *et al.*, 1997), (ii) the anaemia occurs before a substantial antibody response to parasites or erythrocytes is generated (Jakeman *et al.*, 1999) and (iii) the number of IgG molecules bound to RBC are very small to serve as the sole signals to promote erythrophagocytosis by macrophages in acute malaria. Similar arguments have been put forward to elucidate the role of autoimmunity in the physiological removal of aged RBC (Bratosin *et al.*, 1998).

2.2.2.4 RBC membrane structural changes

2.2.2.4.1 RBC membrane alteration

Physiological removal of old RBC is basically an immunological process resulting from the generation of non-self identification sites on the RBC membrane and their recognition by phagocytes (Clark 1988; Bratosin *et al.*, 1998; Kiefer and Snyder, 2000). Similar mechanisms have been proposed to take place in falciparum malaria, in which both infected and uninfected RBC have been reported to develop lesions, which would serve as signals for macrophages to capture and engulf them (Joshi *et al.*, 1986; Giribaldi *et al.*, 2001; Eda and Sherman, 2002). Damages on the RBC membrane have been attributed to the host immune cells producing reactive oxygen species (Kharazmi *et al.*, 1987) and the products of parasite metabolism, haematin (AH) resulting from haemoglobin

digestion and hemozoin originating from crystallization process of AH in the parasite food vacuole (Francis *et al.*, 1997).

Proinflammatory cytokines such as TNF-α produced in response to malaria parasite infection, may be in excess amount thus stimulating macrophages to release excessive amounts of ROS. This being a non-specific immune response causes damages not only to the parasites but also to the uninfected RBC. Several studies have demonstrated that the generation of ROS increases during *P. falciparum* infections (Das *et al.*, 1993; Greve *et al.*, 2000). However its contribution to anaemia was incomprehensible because there was no correlation between the ROS plasma concentration and the degree of anaemia. Later, the study by Griffiths *et al.* (2001) may have resolved this ambiguity by providing direct evidence on ROS contribution to the damages of RBC membrane during malaria, and that ROS effects were mediated locally through the reduction of α-tocopheral reserve in the erythrocyte membrane.

Also, there is evidence that parasite cultures in the absence of immunological factors induce membrane changes on the erythrocytes similar to that seen in senescent cells. Omodeo-Sale *et al.* (2003) demonstrated in an *in vitro* study that the presence of parasite alone induces changes on RBC membrane that could serve as signals to attract phagocytic cells. This suggests that parasite factors are directly involved in damaging erythrocyte membrane during malaria. Uninfected RBC, which share the same environment with *P. falciparum* infected cells acquire membrane changes similar to those seen in senescent erythrocytes (Omodeo-Sale

et al., 2003; Bratosin *et al.*, 1998). Decreases in acids such as arachidonic and docosahexaenoic, and increases in palmitic acid are prominent features. Also, there is great alteration in the organization of membrane phospholipids including the exposure of phosphatidylserine (PS) on the outer surface. Exposure of phosphatidylserine has also been reported in both infected and non-infected RBC obtained from individuals with malaria (Joshi *et al.*, 1986; Eda and Sherman 2002). Such changes may be linked to the accelerated removal of RBC from circulation through IgG independent phagocytosis.

An increase in PS content of the surface of cell membranes, especially in membranes of aged, oxidatively damaged and sickled RBC is the basis for their recognition by macrophages (Sambrano and Steinberg, 1995; Wood *et al.*, 1996). Recognition of cells via exposed PS residues is attributed to a scavenger receptor that binds phosphatidylserine and oxidised lipoprotein, SR-PSOX (Shimaoka *et al.*, 2000; Minami *et al.*, 2001a& 2001b).

The process of hemozoin formation in the parasite and its release into the circulation during schizont rupture generates toxic radicals, which may be associated with the cell membrane damages through the oxidative processes (Dondorp *et al.*, 2003; Schwarzer *et al.*, 2003; Keller *et al.*, 2004). Hemozoin also catalyzes peroxidative process in cell cultures and cell free systems (Omodeo-Sale *et al.*, 1998; Omodeo-Sale *et al.*, 2001) that are capable of causing oxidative damage. Despite these observations, however it may not be pertinent to attribute effects of parasite products as the main cause of RBC membrane damage since no

correlation exists between parasite count and either membrane changes or haemolytic indices (Das and Nanda 1999; Griffiths *et al.*, 2001).

The lack of clear relationship between the level of anaemia encountered in malaria with proposed mechanisms of the RBC membrane damages such as immunological responses or parasite products could mean that these mechanisms play a minor role to promote IgG independent RBC clearance. On the other hand the possibility that the two mechanisms have a summing effect on causing the oxidative stress that damages the RBC membrane (Griffithis *et al.*, 2001) should be considered.

2.2.2.4.2 Reduced RBC deformability and splenic sequestration

RBC in severe malaria are characterized by reduced red cell deformability (RCD); this appears to affect mainly the uninfected RBC (Dondorp *et al.*, 1997; 1999a; 2000). This rigidity shortens the RBC life span due to premature removal. In addition the RCD of uninfected RBC in falciparum malaria is proportional to the severity of anaemia (Dondorp *et al.*, 2000). Reduced RBC deformability in severe malaria may contribute to impaired microcirculatory flow. Under normal circumstances the RBC will have to deform considerably in order to squeeze through the microcirculation, but in case of increased membrane rigidity the cells are likely to be trapped in the microvasculature and sinusoids of the spleen and eventually taken up by phagocytic cells. Impaired RCD is also considered to be the main determinant of anaemia in thalassemia (Dondorp *et al.*, 1999b).

The biochemical and structural changes in the uninfected RBC, which reduce deformability during acute malaria, have not been well characterized. Naumann *et al.* (1991) suggested that binding of exoantigens alters erythrocyte deformability in *P. falciparum* infections but this has not been confirmed by other studies. As stated above, for RBC membrane structural alteration, increased oxidation of uninfected RBC membranes resulting either from reactive oxygen radicals produced by activated macrophages or from parasite metabolism products have been proposed to be responsible for RCD. Oxidized RBC membrane become rigid thus unable to pass through microvasculature and spleen sinusoids (Dondorp *et al.*, 2003).

Splenomegaly is consistently observed in malaria and is considered to play an important role in host defence (Oster, 1980). Ho *et al.* (1990) found that splenic Fc receptor function may be important both in the control of infection and the development of anaemia in *P. falciparum* malaria. Splenomegaly is associated with increased filtration and phagocytic activities, the primary mechanism being mechanical sieve–like action resulting in RBC sequestration. There is evidence suggesting that the splenic clearance of RBC increases during malaria infection (Looareesuwan, 1987), probably making a substantial contribution to the severity of malarial anaemia.

2.3 RED CELL GENETIC DISORDERS AND MALARIAL ANAEMIA

There is a complex relationship between red cell genetic disorders, malaria and anaemia. Genetic red cell disorders such as sickle cell, thalassemias and G6PD deficiency are wide spread in various parts of the world including Africa, Middle East and Asia. Their geographical distribution is closely related to the past and present distribution of malaria because of the selective advantage against severe malaria conferred to heterozygotes. Individuals with some genetic red cell disorders are protected from severe malaria (Aidoo *et al.*, 2002; Agarwal *et al.*, 2000; Ruwende and Hill, 1998; Mockenhaupt *et al.*, 2004a) but to a large extent the basis of protection is not fully known. Limiting parasite invasion and growth in the erythrocyte has been proposed to be behind the resistance conferred by most of these genetic red cell disorders against severe malaria. However, little is known on the role played by host immune response of various genetic red cell disorders on protecting individuals against malaria. Additionally, most of these disorders are associated with anaemia of varying degree. As a result these can be potential confounding factors in studies dealing with malarial anaemia if not taken into account.

2.3.1 Sickle cell anaemia

Sickle cell anaemia is a chronic haemolytic anaemia occurring almost exclusively in black people and characterized by sickle shaped RBC caused by homozygous inheritance of HbSS. The sickle cell gene occurs widely throughout Africa and in countries with African immigrant populations, some Mediterranean countries, the Middle East, and parts of India

Sickle cell gene results from a mutation in a single amino acid substitution at position 6 of the β- globin chain where valine is substituted for glutamic acid. This decreases its electrical charge and it moves towards the anode more slowly than normal haemoglobin HbAA on electrophoresis. Deoxy – Hb S is much less soluble than Deoxy-HbAA therefore it polymerises to form a semi solid gel of rod like fibres that cause RBC to sickle in sites of low oxygen tension. HbSS molecules tend to clump together, making RBC sticky, stiff, and more fragile, and causing them to form into a curved, sickle shape. RBC containing HbSS can go back and forth between being shaped normally and being sickle shaped until they eventually become sickle shaped permanently. Instead of moving through the bloodstream easily, these sickle cells can clog small arterioles and capillaries which lead to occlusion and infarction. Unlike normal RBC that last about 4 months in the bloodstream, sickled RBC are too fragile to withstand the mechanical trauma of circulation. They break down after about 10 to 20 days, which usually causes anaemia. Patients with sickle cell anaemia have haemolytic anaemia of varying severity. The cells are normocytic, a low MCV and in many cases provides evidence of concomitant α-thalassemia. The blood film shows polychromasia and sickled erythrocytes often with elongated or pointed ends (Fig. 3). Nucleated cells are frequently seen and high reticulocyte counts >10 to 20 % is common. Heterozygotes or sickle cell trait (HbAS) subjects appear normal and do not experience hemolysis and other clinical abnormalities.

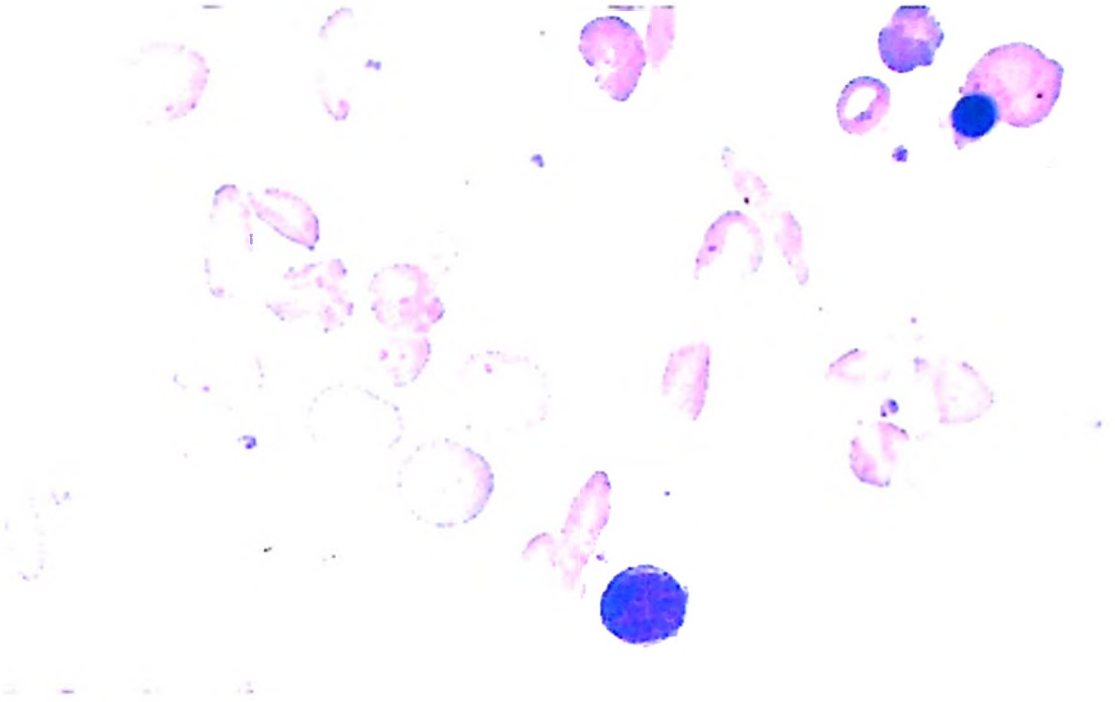

Figure 3: Photomicrograph of human peripheral blood film showing polychromasia and thin/sickled RBC during sickle cell crisis. Source: Jorde *et al.*, 2001

2.3.1.1 Sickle cell trait and malaria resistance

It is generally accepted that sickle cell trait (HbAS) confers a high degree of resistance to severe and complicated malaria during early life (Allison, 1964; Willcox *et al.*, 1983; Aidoo *et al.*, 2002), which explains the relatively high prevalence of this mutation in some areas reaching 30% in sub-Saharan African communities exposed to high rates of infection with *P. falciparum*. Nevertheless, the underlying protective mechanisms are poorly known. Various protective mechanisms have been proposed but to a large extent it appears that physical or biochemical properties of HbAS erythrocytes are behind this attribute. Studies show that invasion, growth, and development of *P. falciparum* parasites in cells

containing sickle haemoglobin (HbSS) is restricted, but only under conditions of low oxygen tensions (Friedman, 1978; Pasvol *et al.*, 1978; 1980). Parasite-infected HbAS/HbSS erythrocytes also tend to sickle (Friedman, 1978; Roth *et al.*, 1978; Hebbel, 2003), a process that may result in premature removal by macrophages/monocytes (Friedman, 1978; Shear *et al.*, 1993).

Additionally, it is suggested that the protective mechanisms of HbAS are not restricted at the erythrocyte level but might also involve an immune component. For example, in one study conducted in Gambia, it was found that immune recognition of *P. falciparum*–infected RBC was enhanced in HbAS children (Marsh *et al.*, 1989), and up regulation of malaria-specific cell-mediated immune responses has also been observed in HbAS individuals (Bayoumi *et al.*, 1990). However the role of host cytokine response has not received much attention with regard to the HbAS protection against severe malaria and there is scanty literature regarding cytokine production in association with haemoglobin type (HbAA or HbAS) before and during malaria infection. Excessive cytokine responses play a big role in the initiation, perpetuation and worsening of *P. falciparum* infections but how these cytokines may influence the clinical outcome of *P. falciparum* infection in people with different haemoglobin genotypes is unknown.

2.3.2 Thalassemias

Thalassemia is an inherited haemolytic disorder which results from unbalanced Hb synthesis caused by decreased production of at least one globin polypeptide chain. The thalassemias are classified into α-thalassemia and β -thalassemia

depending on which pair of globin chain is affected. The thalassemias are widely distributed throughout parts of Africa, Mediterranean region, Middle East, South East Asia and Indian subcontinent.

The β-thalassemias result from more than 180 different mutations in the β -globin gene which cause the output of globin to either decrease (β^+- thalassemia) or to be completely absent (β^0- thalassemia). Similarly for α-thalassemia if all α-globin genes are lost (α^0-thalassemia) no α-globin chains are synthesized and if only one pair is lost (α+- thalassemia) there is a reduced output of the α-globin chains. The inheritance of α-thalassemia is more complicated than β - thalassemia because genetic control of α -chain synthesis involves two pairs of structural genes on chromosome 16.

Table 1: Common thalassemia syndromes

	Genotype	Phenotype
α – thalassemia		
- -/αα	Heterozygous α^0 - thalassemia 1	Thalassemia minor
- α/αα	Heterozygous α^+- thalassemia 2	silent carrier
- -/- -	Homozygous α^0-thalassemia 1	Hb Bart's syndrome
- α/-α	Homozygous α^+- thalassemia 2	Thalassemia minor
- -/-α	α-thalassemia 1/α-thalassemia 2	Hb H disease
β- thalassemia		
	Heterozygous β^0 – thalassemia	Thalassemia minor
	Heterozygous β^+ - thalassemia	Thalassemia minor
	Homozygous β^0 –thalassemia	Thalassemia major
	B^0 –thalassemia/β^+-thalassemia	Thalassemia intermedia

β-thalassemia homozygote (thalassemia major) show symptoms of severe anaemia, expanded marrow space and absorptive iron overload whereas β-thalassemia heterozygotes (thalassemia minor) are carriers and have asymptomatic mild to moderate hypochromic microcytic anaemia with a low mean corpuscular haemoglobin (MCH) and mean corpuscular volume (MCV). Heterozygotes for a single gene defect (α-thalassemia-2; silent carriers) are usually free of clinical abnormalities whereas heterozygotes for double gene defect or homozygotes for a single gene defect(α-thalassemia-1; trait) show mild haemolytic anaemia similar to that observed in heterozygous β-thalassemia. Inheritance of both single gene defect and a double gene defect more severely impairs α –chain production. Impaired α-globin production leads to excess γ and β chains that form unstable and physiologically useless tetramers (causing Bart's Hb and Hb H respectively). The homozygous state for α^0-thalassemia results in the haemoglobin Bart's hydrops fetalis syndrome characterized by the stillbirth of severely hydropic infants in the second half of pregnancy. Patients with Hb H disease often have symptomatic haemolytic anaemia and splenomegaly.

2.3.2.1 Thalassemias and malaria resistance

The thalassaemias are some of the best-recognized malaria-protective polymorphisms having risen to such high frequencies in many populations (as high as 80%) that they are now considered the commonest monogenic disorders of humans (Weatherall and Clegg, 2001). The coinciding geographical distributions of malaria transmission and the thalassemias led Haldane almost sixty years ago to propose that red cell abnormalities have been selected owing to the fitness

advantage conferred against malaria: "malaria hypothesis" (Haldane, 1948). Evidence for protection has come principally from population studies, epidemiology and some case control studies (Allen *et al.*, 1997; Weatherall and Clegg, 2002; Mockenhaupt *et al.*, 2004b). However, the nature of the thalassemia protective mechanism against malaria is not fully understood. Although α+-thalassemia is strongly protective against severe and fatal malaria; its effects are not detectable at the level of any other malaria outcome including parasitisation, symptoms and density of parasites in peripheral blood (Wambua *et al.*, 2006). Earlier studies have pointed out that there is an increase in the expression of parasite derived antigens and binding of IgG on the surface of infected thalassemic erythrocytes (Luzzi *et al.*, 1991). This may lead to better immune recognition and clearance of infected cells and thus a better control of the blood stage infection including less severe pathology (Luzzi *et al.*, 1991; Williams *et al.*, 2002). Other mechanisms include increased susceptibility of infected thalassemic erythrocytes to erythrophagocytosis (Yuthavong *et al.*, 1988; Yuthavong *et al.*, 1990; Ayi *et al.*, 2004), and decreased rosette formation, a property which is associated with severe forms of malaria (Carlson *et al.*, 1994).

2.3.3 Glucose -6-phosphate dehydrogenase (G6PD) deficiency

Glucose -6-phosphate dehydrogenase (G6PD) is a cytoplasmic enzyme that is essential for cell's capacity to withstand oxidative stress (Ruwende and Hill, 1998). The G6PD enzyme functions in catalyzing the oxidation of glucose-6-phosphate to 6-phosphogluconate, while concomitantly reducing nicotinamide adenine dinucleotide phosphate ($NADP^+$) to NADPH. The NADPH produced is a

cofactor of many biosynthetic reactions and also is used to keep glutathione in its reduced form which acts as a scavenger for oxidative metabolites in the cell. There are other metabolic pathways that can generate NADPH in all cells except in the RBC where other NADPH-producing enzymes are lacking (Luzzatto and Mehta, 1989). This has a profound effect on the stability of RBC since they are sensitive to oxidative stresses in addition to having only one NADPH-producing enzyme to remove harmful oxidants. RBC of G6PD deficient individuals are particularly sensitive to oxidative stress and consequently hemolysis occurs to cause haemolytic anaemia.

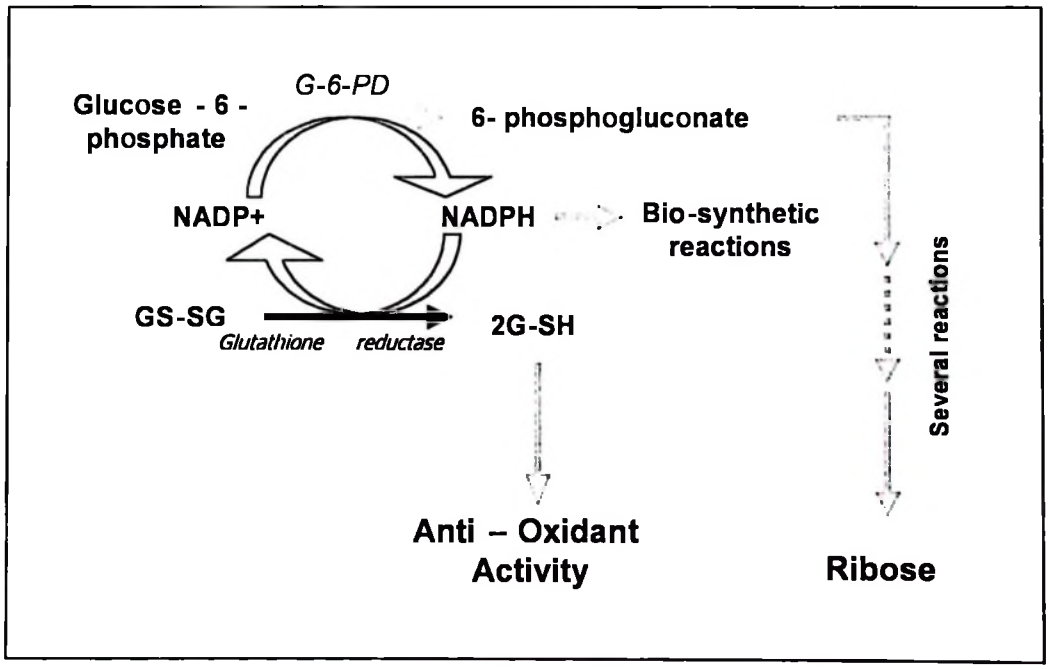

Figure 4: Functions of G6PD enzyme in the oxidation reaction of glucose-6-phosphate to 6-phosphogluconate

The G6PD gene is located in the long arm of the X-chromosome and is inherited as an X-linked recessive trait (Beutler, 1991). The G6PD gene is highly polymorphic and over 300 variants resulting from single point mutations are known to exist in different populations in the world (Beutler *et al.*, 1996). Several variants have significantly reduced activity of the enzyme and results in a condition known as Glucose -6-phosphate dehydrogenase (G6PD) deficiency. G6PD deficiency is the most common human enzyme deficiency affecting over 400 million persons worldwide (Beutler, 1991). G6PD variants are characterized biochemically based primarily on their differences in the residual enzyme activity and the associated clinical symptoms. From the quantitative point of view, variants may have normal or decreased activity (or very rarely, increased activity) with reference to that of the normal G6PD B variant with 100% enzyme activity as follows:

Class 1: Severely deficient associated with chronic non-spherocytic anaemia; Class II: Severely deficient, <10% residual enzyme activity; Class III: Moderately deficient, 10 -60% enzyme activity of normal variant; Class IV: Near normal or normal enzyme activity 60 -150% enzyme activity; Class V: Increased enzyme activity, >150%

In Africa G6PD is essentially a tri allelic polymorphism as shown in Table 2

Table 2: G6PD Alleles in Africa and their enzyme activities

Allele	Class	Enzyme activity	Frequency
G6PD B	IV	100%	0.60 – 0.80
G6PD A	IV	80%	0.15 – 0.40
G6PD A-	III	12%	0.00 – 0.25

The G6PD B is the normal (Wild) variant associated with normal or 100% enzyme activity and is the commonest allele in Africa. G6PD A allele results from a single nucleotide polymorphism at nucleotide 376, an adenine to Guanine substitution that results in an asparagine to aspartate amino acid substitution. A third allele G6PD A- is unique in that it contains two mutations, the first occurs at nucleotide 376 (G6PD A), and the second mutation occurs at nucleotide 202; at this point guanine is substituted by adenine leading to valine to methionine amino acid substitution (Hirono and Beutler, 1988).

In unstressed normal cells, the G6PD activity is only 2% of the total capacity. Therefore it is not surprising that most individuals with class II and III G6PD deficiency are usually asymptomatic. For class I G6PD deficiency variant the enzyme activity is very poor to extent that the life span is shortened even in the absence of stress and therefore class I individuals show splenomegaly and chronic non-spherocytic haemolytic anaemia ranging from mild to moderate anaemia (Luzzatto and Maehta, 1989). Clinical manifestations of other G6PD deficiency classes are mostly dependent upon the interaction with other factors such as infections, use of oxidant drugs and foods, and additional genetic factors

(Luzzatto and Maehta, 1989). The most common clinical symptom associated with G6PD deficiency is acute haemolytic anaemia. This occurs as a manifestation of this disorder on mature erythrocytes. The condition can be precipitated by infections or ingestion of some drugs and food (Fava beans) with oxidant properties. Drugs like primaquine, sulphonamides, nitrofurantoin and several anti-inflammatory agents are the most common drugs associated with hemolysis in G6PD deficient individuals (Luzzatto and Mehta, 1989; Green, 1993).

2.3.3.1 G6PD Deficiency and malaria protection

The concept of G6PD A- allele protection against malaria is derived from the geographical correlation between the prevalence of the trait and malaria endemicity (Ruwende and Hill, 1998). Early studies on G6PD deficiency in females showed higher levels of parasites in normal compared to G6PD deficient RBC. Although malaria invasion in the cells was similar, the growth of the cells in the G6PD deficient was impaired (Luzzatto *et al.*, 1983; Roth *et al.*, 1983). Results on whether hemizygous G6PD deficient males protect against malaria have been conflicting for many years. Several studies suggested that only the G6PD deficient heterozygotes are protected against *P. falciparum* parasitization (Luzzatto *et al.*, 1983; Luzzatto and Mehta, 1989) while G6PD hemizygotes and homozygotes are not. However, data from the largest African case control study carried out in malaria endemic regions strongly suggest that G6PD A- allele is associated with substantial resistance to severe malaria in both hemizygous males as well as in homozygous females (Ruwende *et al.*, 1995).

The mechanisms behind the impaired growth of *P. falciparum* in G6PD deficient individuals are not known. However it is generally accepted that the degree of enzyme deficiency is central to the protective effect on malaria (Figure 5) G6PD deficient cells fail to remove toxic oxidized substances such as disulphide glutathione and hemozoin which build up in the cell and alter the normal environment required for the parasite growth. As a result the multiplication and growth of the parasite is greatly impaired and fails to proceed with development process (abortive infection). Also there is evidence that infected deficient cells are haemolysed as a result of increased methaemoglobin and release of ferriheme, a known cytolytic agent. This cytolytic agent also exerts oxidative stress to cells causing membrane damages. The damaged cells are phagocytosed by cells of reticuloendothelial cells (Turrini *et al.*, 1993), these mechanisms result into a suicidal infection.

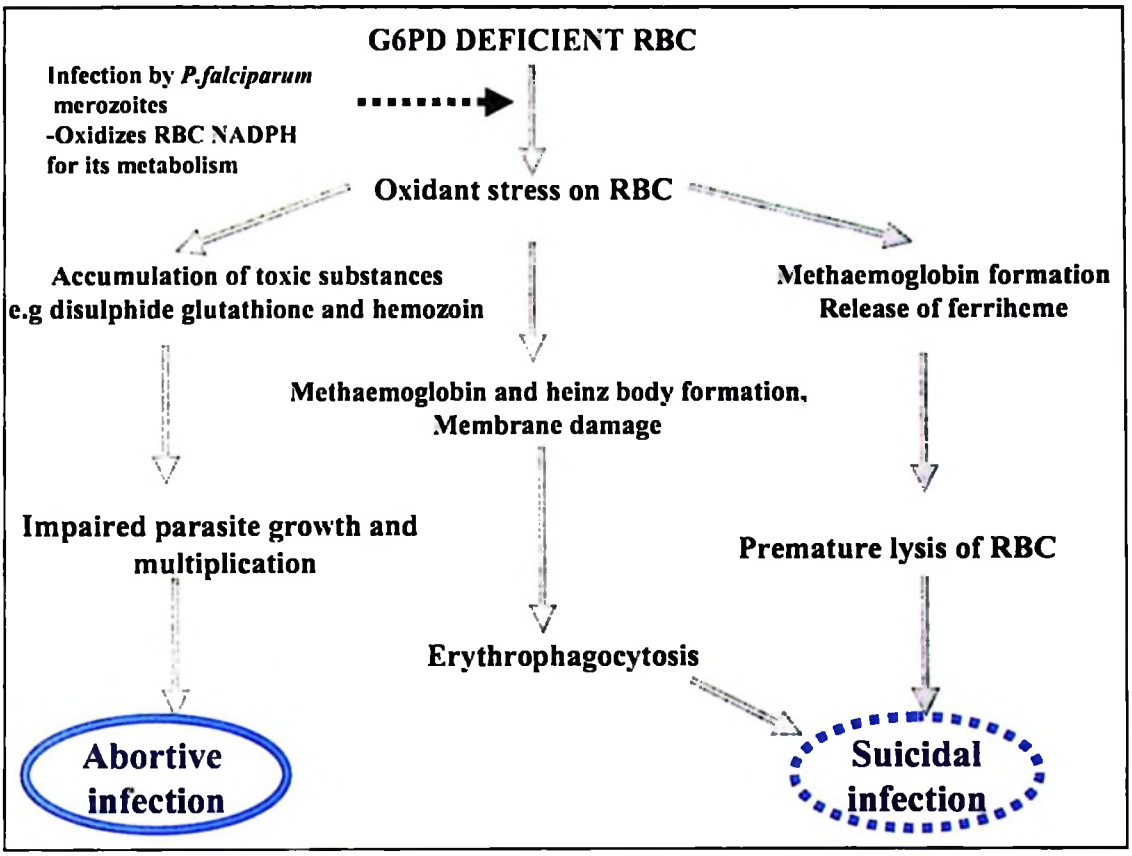

Figure 5: Proposed protective mechanism of G6PD deficiency against malaria. Source: Ruwende and Hill (1998).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 GENERAL STUDY DESIGN

3.1.1 Study population

Study population comprised of a sample of children participating in the Mother Offspring Malaria Studies (MOMS) project, which is based at Muheza Designated District Hospital (DDH), Northeast coast of Tanzania. This project is undertaken in collaboration between Seattle Biomedical Research Institute, USA and Muheza DDH. The project uses an approach of recruiting mothers among women presenting at Muheza DDH or its satellite clinics for antenatal care in the third trimester or for delivery. Recruited mothers provided informed signed consent prior to participation of their newborns in the study.

3.1.2 Ethical approval

Protocols for procedures used in this study were approved by the International Clinical Studies Review Committee of the Division of Microbiology and Infectious Diseases at the US National Institutes of Health, and ethical clearance was obtained from the Institutional Review Boards of Seattle Biomedical Research Institute and the National Institute for Medical Research in Tanzania.

3.1.3 Case monitoring, sampling and definitions

We monitored children for *P. falciparum* infection from birth up to 3 years of age. Blood smears were prepared monthly and blood samples were collected at 2 weeks, 3, 6 and 12 months of age, then once after every 6 months in the second

and third years of life. Blood was also collected any time the child fell sick. The children were seen by the medical officer upon presentation to the Hospital. A malaria case was defined as a child with asexual *P. falciparum* parasitemia by blood smear coupled with symptoms suggestive of malaria such as body temperature higher than 37.5 °C, nausea/vomiting, irritability and poor feeding.

Anaemia was classified with reference to the level of haemoglobin concentration as follows: haemoglobin level ≤ 5g/dl as severe anaemia; haemoglobin level between 5 and up to 8g/dl was classified as moderate; Hb >8 to 10g/dl as mild anaemia and haemoglobin level ≥ 10 g/dl as normal. Prompt treatment was made to the sick cases according to the guidelines set by Tanzania government. Children with sickle cell disease were excluded from the analysis because of the insufficiency in number.

Iron deficiency was defined as ferritin concentration of less than 30 ng/ml when CRP was less than 8.2 ng/ml (Iron deficiency in the absence of inflammation) or ferritin concentration of less than 70 ng/ml when CRP was equal or more than 8.2 ng/ml (Iron deficiency in the presence of inflammation) (Leenstra *et al.*, 2006).

3.2 STUDY DESIGN FOR EACH OBJECTIVE

3.2.1 Roles of serum soluble factors on erythropoiesis

A cross sectional study was carried out to ascertain roles of serum soluble factors concentrations in the pathogenesis of anaemia during malaria, with reference to the erythropoiesis. Samples were collected from children periodically during

routine visits, or during clinical malaria episodes. Reticulocyte count was used as a measure of erythropoiesis and was classified as appropriate or inappropriate reticulocyte response to anaemia based on reticulocyte production index, which is the corrected reticulocyte count after considering the hematocrit level as detailed in Appendix I. Levels of serum soluble factors (TNF-α, TNF-RI, TNF-RII, IL 1, IL 4, IL 5, IL 6, IL 10, EPO, Hepcidin, CRP, Ferritin and sTfR) were evaluated by multiplex cytokine assay as described by Coutinho *et al.* (2005), and later were compared between children with appropriate reticulocyte response and those with inappropriate reticulocyte responses.

3.2.2 Red cell membrane changes studies

3.2.2.1 Plasma cytokine concentrations and RBC surface molecules changes

The relationship between plasma cytokine concentrations and RBC surface membrane changes in malaria patients was studied in children with malarial anaemia and children having malaria without anaemia served as age and season-matched controls. The aim was to detect and quantify the exposure of phosphatidylserine (PS) and the abundance of IgG, CD 35, CD55 and CD 59 on RBC surface. The cohort under study was stratified according to the ratios of plasma cytokine concentrations (TNF-α: IL-10) as follows: Group 1 = patients having cytokine plasma ratio of TNF-α: IL-10 $\geq$ 1; Group 2 = Ratio of TNF-α: IL-10 <1. The cut-off value is based on the findings by Othoro et al. (1999), with slight adjustment i.e. from a ratio of 1.77 to 1.0. The quantification of PS and IgG was accomplished by the use of a Flow cytometer. The comparison on each

variable was made between samples obtained from individuals with malaria expressing different levels of cytokines ratio.

3.2.2.2 RBC age and surface molecules changes in children with malarial anaemia

The changes in the RBC surface molecules in malaria patients were studied in children with malarial anaemia, and children having malaria without anaemia served as controls. Red blood cells were fractionated according to age by Percoll density gradient separation. Flow cytometry was used to determine the abundance of CD55 and CD59 on red blood cells in each RBC fraction. The comparison was made between anaemic and non anaemic malarial cases matched by RBC age (fractions/subpopulations).

3.2.3 RBC genetic disorders and cytokine production monitoring

3.2.3.1 Screening for RBC genetic disorders

All children participating in the MOMS project were screened for the genetic red cell disorders including hemoglobinopathies (HbSS, HbC, HbE, and α-thalassemia) and Glucose-6-phosphate dehydrogenase variants in the first year of study.

3.2.3.2 Monitoring production of cytokines with regard to the RBC genetic disorders

All children were monitored for cytokine production during routine visits and illness for 3 years in a prospective cohort study (as detailed in section 3.1.2). The

cohort under this study was stratified according to the haemoglobin types and G6PD variants.

3.3 SAMPLE COLLECTION, PROCESSING AND ASSAYS

3.3.1 Blood collection and processing

Venipuncture was performed and 5 ml of blood was collected using a sterile disposable syringe. Blood was mixed thoroughly with appropriate amount of anticoagulant Citrate Phosphate Dextrose (CPD). Samples were stored at 4°C before further analysis. Plasma samples intended for analysis of soluble factors were stored at –80°C. Clinical and laboratory staff strictly adhered to the sterility and precautions required for handling bioharzadous material.

3.3.2 Parasite detection

The presence of parasite was determined by finger prick Giemsa-stained thick blood smears prepared from capillary blood. Parasite density was expressed as the number of asexual stage parasites per 200 white blood cells in the thick smear. Parasite species were determined by examination of the thin smears

3.3.3 Determination of haematological parameters

Haematological parameters were determined by haematology analyzer (Abbott Cell Dyne® 1200).

3.3.4 Determination of reticulocyte count

Reticulocyte count was determined using the following procedure:

1. 5µl of blood was mixed with 5µl of reticulocyte stain (Unopette, BD FRANKLIN LAKES NJ, USA) in the microcentrifuge tube (Eppendorf).
2. The mixture was allowed to stand for 10 minutes at room temperature
3. Two slides were prepared and allowed to dry
4. A total of 1000 red cells were examined and counted using oil immersion lens (1000X Objective). The proportion of reticulocytes in the circulation was calculated as follows:

   Reticulocytes count X100 (percent) / Total Number of RBC (1000) = Uncorrected Reticulocyte Count

3.3.5 Multiplex cytokines assays

Each serum sample was analyzed using a multiplexed bead based platform (BioPlex, BioRad, Irvine, CA) and custom assay kits. The details of all analytes included in these assay kits are shown in Appendix II. For each serum sample, all analytes were assayed in a single day, thus eliminating freeze/thaw cycles. All pipetting and sample identification was performed with a barcode enabled high speed pipetting robot (Megaflex, Tecan, Research Triangle Park, NC).

3.3.6 Determination of Red blood cell fractions

RBC were separated from other blood components (plasma and leukocytes) by centrifugation at 600 x g for 5 minutes. After removing the plasma and leukocytes, the RBC were washed twice in phosphate buffered saline buffer (PBS). RBC were fractionated onto Percoll/5% Sorbital (Wt/Vol) gradient into 4 fractions as shown in section 3.3.7.2. Each fraction of cells were aspirated and

transferred to a new tube, washed in RPMI 1640, then pelleted by centrifugation at 600x g for 5 minutes. The supernatant was removed leaving not more than 25µl on the top of pellet. Then the cells were resuspended by adding 975µl of RPMI 1640. Enumeration of cells was performed on a haematology analyzer - Cell Dyne® 1200.

3.3.7 Percoll/5% Sorbitol and density gradient preparation

3.3.7.1 Principle of Percoll cells separation method.

The Percoll method utilizes a density gradient. Percoll solution is made of small colloidal silica beads 15-30 nm diameter (23% w/v in water); these are coated with a chemical that does not interact with cells. Depending on the concentration of Percoll particles in the solution they sediment (and diffuse) at different rates in a gravitational field, thereby creating a density gradient. Biological materials (in this case RBC) are put at the top of Percoll gradient; upon centrifugation they diffuse in Percoll and reach a position where their densities and that of the surrounding Percoll medium are equal.

3.3.7.2 Preparation of Percoll density gradient

Percoll 5% Sorbitol was prepared as follows:

Dissolve 5gm of Sorbitol in 10ml RPMI 1640 then mix with 90ml Percoll to make a solution 5% Sorbitol in Percoll, then the solution was filtered. To prepare a density gradient, the above solution was diluted with RPMI 1640 as follows:

9 ml Percoll /Sorbitol + 1 ml RPMI (fraction 90%)

8 ml Percoll /Sorbitol + 2 ml RPMI (80%)

7 ml " + 3 ml" (70%)

6 ml " + 4ml " (60%)

4 ml " + 6 ml" (40%)

The gradients were made in 15 ml plastic tubes (2 ml of each fraction, starting with that of 90% density at the bottom of the tube). Each single fraction was added slowly using the automatic pipette. Thereafter, 0.1 ml packed RBC was diluted to 5 parts of RPMI 1640 then added at the top of the Percoll gradient. Centrifuge at 1075 x g for 20 minutes. Each fraction of RBC was aspirated and transferred to a new tube then washed twice in RPMI 1640 at 400 x g for 5 minutes.

3.3.8 Detection of IgG

Red blood cells were washed twice in FACS buffer (PBS supplemented with 1% BSA and 0.1% NaN_3). The cells were resuspended in FACS buffer at $2x10^6$ cells/ml. Appropriate amount of antibody was added in the sample then incubated at room temperature in dark for 30 minutes. The RBC were washed twice in FACS buffer then resuspended in the buffer followed by staining with secondary antibodies conjugated to a fluorescent dye Fluorescein isothiocyanate (FITC) then incubated in dark at room temperature for 30 minutes. The RBC were washed twice in FACS buffer then resuspended in the buffer ready for flow cytometry analysis. Negative control for IgG was prepared by using irrelevant monoclonal antibody of the same isotype (Molecular Probes, USA).

3.3.9 Detection of Phosphatidylserine

Annexin V-FITC apoptosis detection kit (Sigma-Aldrich) was used for detection of phosphatidylserine on RBC surfaces. The cells were washed twice in PBS and then resuspend in 1X binding buffer (100 mM HEPES/NaOH, pH 7.5 containing 1.4 M NaCl and 25 mM CaCl2) at a concentration of approximately 1 x 10^6 cells/ml then 1µl of Annexin V-FITC (Sigma) was added in each 100µl of cell suspension then mixed thoroughly by gently vortex. The tubes were incubated at room temperature for exactly 10 minutes in dark. Thereafter 400ml of binding buffer was added to each tube. Analysis by flow cytometer (Flomax –Partec® - Germany) was performed within one hour. The negative control was prepared each time by following all steps for PS staining except that the Annexin V-FITC was not added to the sample.

3.3.10 Detection of CD35, CD55 and CD59

RBC were washed twice in FACS buffer. The cells were suspended in FACS buffer at 2x10^6cells/ml. Appropriate amounts of antibodies against human CD35, CD55 and CD59 coupled to fluorescent dyes Fluorescein isothiocyanate (FITC), Phycoerythrine (PE) and Cyanine-5 (Cy5) respectively were added to the sample then incubated at 4°C in dark for 30 minutes. Irrelevant monoclonal antibodies of the same isotype were used as negative controls (Molecular Probes, USA). The RBC were washed twice in buffer, resuspended in the PBS and analyzed immediately by flow cytometer (Flomax – Partec, German).

3.3.11 Flow cytometry analysis

Stained cells were analyzed by flow cytometer (Flomax – Partec, German). The cells were excited with 488nm argon ion laser, and the logarithmic green (FITC) orange (PE) and red (Cy5) fluorescences were measured through fluorescence detectors FL1, FL2 and FL3 respectively. Red blood cells were gated on the basis of their logarithmic amplification of the light scatter properties. One thousand events were acquired in replicate for each sample. The results were presented as mean fluorescence intensity (MFI) of RBC with specific immunofluroescence above the background fluorescence as determined by isotype controls.

3.3.12 Haemoglobin types determination

Haemoglobin types (HbAA HbAS and HbSS) were determined by Helena's haemoglobin electrophoresis procedures (Helena Laboratories; Texas - USA). Both cellulose acetate paper in alkaline buffer (8.2 to 8.6) and citrate agar plate in citrate buffer (PH 6.0 to 6.3) were used; thereafter the results were confirmed by Polymerase Chain Reaction- restriction enzyme analysis (PCR- REA) method as described by Ayatollahi et al. (2005).

3.3.13 Alpha thalassemia

Samples were screened for α-thalassemia common deletions (3.7 kb and 4.2 kb deletions) by DNA analysis. Genomic DNA was extracted from peripheral blood using standard protocols. PCR was carried out using the protocol described by Chong et al. (2000).

3.3.14 Glucose-6-phosphate dehydrogenase variants

Three variants of the gene encoding for Glucose 6-phosphate dehydrogenase enzyme (G6PD B, G6PD A, and G6PD A-) were screened by using the Multiplex PCR followed by restriction enzyme analysis. DNA was isolated from filter paper blood spot according to the standard protocol as outlined by Gentra systems (Generation® Capture Card Kit). A region spanning third to fifth exon was amplified by an outer PCR (Forward Primers 5'-GGTGGAGGATGATGTATGTAG-3'and reverse primer 5'-GCAACGCTGCCACCTTGTG-3') followed by nested multiplex PCR. Primers to detect a 202 single nucleotide polymorphism (SNP) were G6PD- 202 F = 5'-CCT TCT GCC CGA AAA CAC CTT CACC-3' and G6PD-202 R= 5'- GTC CCC GAA GCT GGC CAT GCT GG -3' and primers to detect 376 SNP were G6PD-376 F = 5'- TAC CAG CGC CTC AAC AGC CCC ATG –3' and G6PD-376 R =5'- GGA CTC GTG AAT GTT CTT GGT GAC G-3'. The forward primers were engineered by introducing mismatches in the G6PD sequences as follows: T and A were replaced by C (underlined nucleotides) for 202 F and 376 F primers respectively. Mismatching allowed the recognition of the two mutation sites simultaneously by restriction enzyme digestion using NCO I (New England Biolabs). The enzyme recognizes the CCATGG sequence and cuts as follows – C/CATG\G. The amplification with these primers produced a 166-bp and 120- bp products respectively. A 166-bp fragment produced 142-bp and 24-bp fragments for individual with A mutation and 120-bp fragment produced 100-bp and 20-bp fragments for individuals with A- mutation after NCO I. The PCR conditions for the first reaction were as follows: Initial denaturation at 94°C for 60 seconds 58°C

for 45 seconds and 72°C for 60 seconds followed by 7 minutes at 72°C. The nested multiplex PCR conditions were similar to the above with the exception of the annealing temperature (56°C for 45 seconds).

3.4 Statistical analysis of data

Continuous variables that followed a normal distribution were analyzed by unpaired Student's test (two categories) or analysis of variance (several categories). Skewed continuous variables were compared with Mann Whitney test (two categories) or the Kruskall Willis test (several categories). The relationships among variables were assessed by Spearman rank correlation, and a logistic linear regression model was used to study the relationship of variables categories and dependent outcomes. Proportions were compared with Pearsons chi-square (X^2), and a p-value of 0.05 was used as a cut-off point for a significant difference.

3.5 Study duration and location

This work was done over a period of 3 years (from June 2004 to July 2007) at four different locations as follows: 1) Muheza Designated District Hospital (Tanga – Tanzania) - Sample collection, sample analysis and data analysis; 2) Seattle Biomedical Research Institute (Washington - USA) - Training on laboratory techniques and genotyping on α^+ thalassemia, G6PD deficiency; 3) Brown University (Rhode Island –USA) - serum samples analysis and 4) Sokoine University of Agriculture (Morogoro-Tanzania) - Final data analysis and thesis writing.

Table 3. Summary of techniques used in this study

Study	Activity	Technique
Serum factors on Erythropoiesis	Determination of serum soluble factors (TNF-α, TNF-RI, TNF-RII, IL-1, IL-4, IL-5, IL-6, IL-10, IFN-γ, sTfR, EPO, Ferritin, Hepcidin, CRP)	multiplex bead-based platform with custom sandwich or competitive assay kits
RBC membrane changes	Detection of IgG, PS, CD35, CD55, CD59	Flow cytometry
Hemoglobinopathies	Hb types	Hb electrophoresis/PCR
	Alpha thalassemia	PCR
G6PD Genotypes	G6PD genotyping	PCR

CHAPTER FOUR

4.0 RESULTS

4.1 ROLES OF SERUM SOLUBLE FACTORS ON ERYTHROPOIESIS

4. 1.1 Demographic characteristics and RBC indices

The study included 199 children with the mean age of 18.63 months ranging from 2.82 to 36.3 months of age, mean haemoglobin concentration was 10.66 ± 2.27g/dl and the male: female ratio of 102:97 as depicted in Table 4. Blood for cytokines and haematological control values was obtained from 142 children; these children had no apparent illness, were free from *P. falciparum* by microscopic blood smear examination and had no record of malaria attack for at least 3 months prior to sampling. Fifty seven children qualified as malaria cases at the time of sampling based on the microscope blood smear results coupled with clinical signs.

4.1.1.1 Haemoglobin levels

One hundred and two (102) uninfected children with haemoglobin level above the reference range (11.91 ± 2.21 g/dl) served as normal controls. Forty uninfected children were anaemic with haemoglobin concentration 8.39 ± 1.22 g/dl (mean ± SD). Of the malaria cases, 29 were anaemic (Mean Hb = 8.41 ± 2.36 g/dl) and 28 had mean haemoglobin level of 11.98 ± 1.95g/dl. None of the *P. falciparum* infected children had severe anaemia but 24.14% (7/29) had moderate anaemia and 75.86 % (22/29) had mild anaemia. Infected anaemic children were significantly younger than the infected non anaemic children, p = 0.0119 but were not significantly different from the normal control children (p =0.6695).

Table 4. Demographic and haematological data of the study populations

	Uninfected children		*P. falciparum* infected children		
	Non anaemic	Anaemic	Non anaemic	Anaemic	Ref. value
N (Male/female)	102(52/50)	40 (21/19)	28 (16/12)	29(13/16)	
Age (Months)	18.23 ± 10.65	17.3 ± 10.05	22.67 ± 7.61	17.94 ± 6.6 [b]	
Haemoglobin (g/dl)	11.91 ± 1.62	8.39 ± 1.22[a]	11.98 ± 1.95	8.41 ± 0.99 [ab]	>10 g/dl*
MCV (fl)	75.35 ± 8.298	69.42 ± 12.73[a]	72.99 ±6.49	70.25 ±18.49	70.8 - 90 fl*
RDW (%)	15.78 ± 2.48	17.4 ± 3.28[a]	16.45 ± 2	18.39 ± 2.21[ab]	11 -15%
RBC10^6/mm^3	5.32 ± 1.11	4.15 ± 1.12[a]	4.99 ± 0.78	4.63 ± 1.52 [ab]	4 – 6 x10^6/mm^3
% Reticulocyte	1.14 ± 1.1	2.05 ± 3.35	1.67 ±1.68	2.45 ±2.29[a]	0.5 – 1.5%
Reticulocyte Index	1.45 ± 1.26	2.68 ± 4.62	2.24 ± 2.2 [a]	3.52 ± 2.99 [ab]	
Parasitemia/200 WBC	0	0	2772 ±3326	2117.28 ± 2522.93	

All data are presented as means ± standard deviation

[a] Significantly different from the control values (uninfected non anaemic children)

[b] Significantly different from *P. falciparum* infected non anaemic children

*Source: Lugada *et al.*, 2006

4.1.1.2 RBC indices in *P. falciparum* infected and uninfected children

The overall mean corpuscular volume (MCV) was within the reference range (73.22 fl). The lowest MCV was detected in the anaemic uninfected children. Comparisons of MCV between children with appropriate reticulocyte response and those with poor response revealed no significant differences. Red cell distribution width (RDW) was significantly higher in anaemic children for both infected and uninfected groups (Fig. 6). Uninfected anaemic children with normal reticulocyte response had higher RDW than children with poor reticulocyte response and the difference was significant ($p = 0.0422$). However this difference was not significant in the malaria group although the RDW was slightly higher in children with normal reticulocyte response. Red blood cells counts were significantly lower in the anaemic populations as compared to the uninfected non anaemic control children.

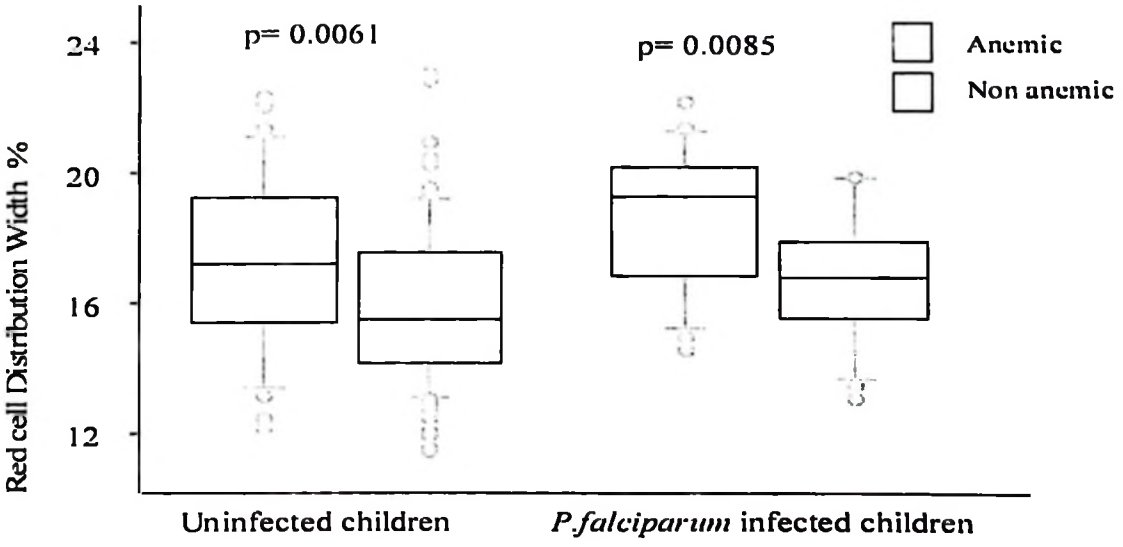

Figure 6. Box plots for red cell distribution width (RDW) in the *P. falciparum* infected and uninfected children stratified by level of haemoglobin (anaemia). Each box represents the interquartile range (25 -75%) of values, the whiskers represent 10% and 90% values and the middle line represents median value.

4.1.1.3 Reticulocyte response

Reticulocyte production increased during malaria and was highest in children with malarial anaemia, demonstrating increased production of cells in the bone marrow (Table 4). When *P. falciparum* infected anaemic children were stratified by reticulocyte response, 14 children had appropriate reticulocyte response to anaemia as indicated by high reticulocyte index (RI>2). In the other 15 children, the reticulocyte response was not appropriate to the level of anaemia (RI≤2). The mean age between children having inappropriate reticulocyte response (19.06 ± 6.95) and appropriate reticulocyte response (16.74 ± 6.27) was not significantly different (p=0.2949).

4.1.2 Serum soluble factors

4.1.2.1 Ferritin

There was a significant increase of serum ferritin during malaria in both anaemic and non anaemic individuals. However, the levels of ferritin were not significantly different between anaemic and non anaemic children (Figure 7) as well as children with different reticulocyte response status. Ferritin level correlated significantly with TNF-R1, TNF-RII, IL-1, IL -6, IL-4, IL-5 and, TNF-α in all infected children.

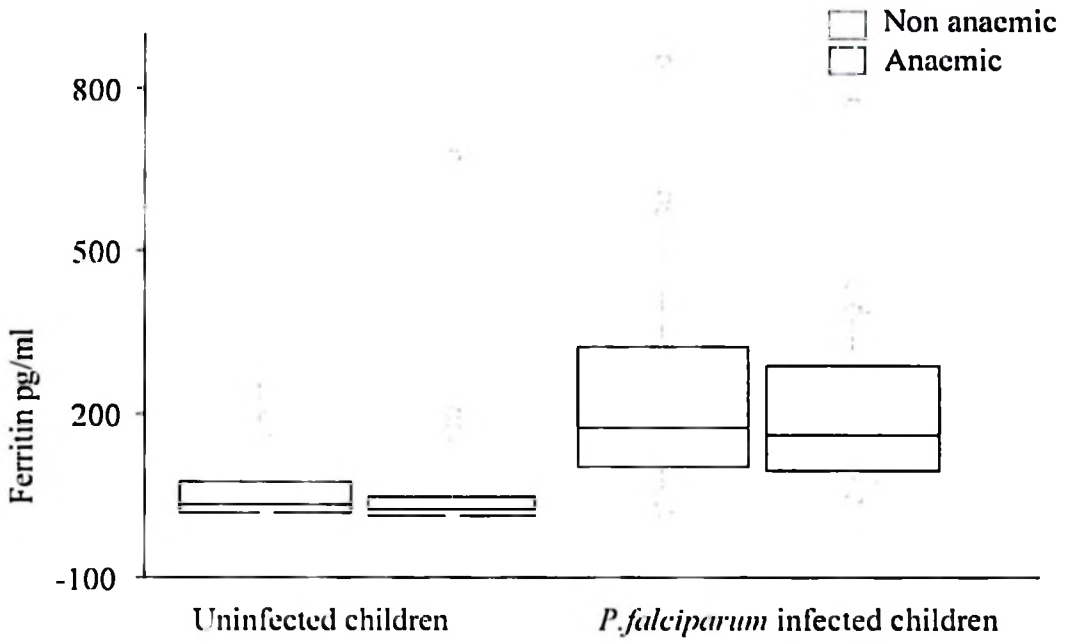

Figure 7. Serum ferritin level in the uninfected and *P. falciparum* infected children; the comparisons between anaemic and non anaemic donors. Each box represents the interquartile range (25 -75%) of values, the whiskers represent 10% and 90% values and the middle line represents median value.

4.1.2.2 Soluble transferrin receptor

Soluble transferrin receptor (sTfR) was markedly and significantly lower in children with malaria than in uninfected children. No significant differences were observed when the anaemic children were compared to non-anaemic children in both *P. falciparum* infected and uninfected children (Fig 8). Plasma sTfR level did not show a significant difference between children presenting different reticulocyte responses. The sTfR/log ferritin ratio assumed a similar trend as soluble transferrin receptor (Fig. 9). However, the sTfR/log Ferritin ratio which is indicative of iron deficiency was higher in the uninfected children (>2) than in the infected children (<1).

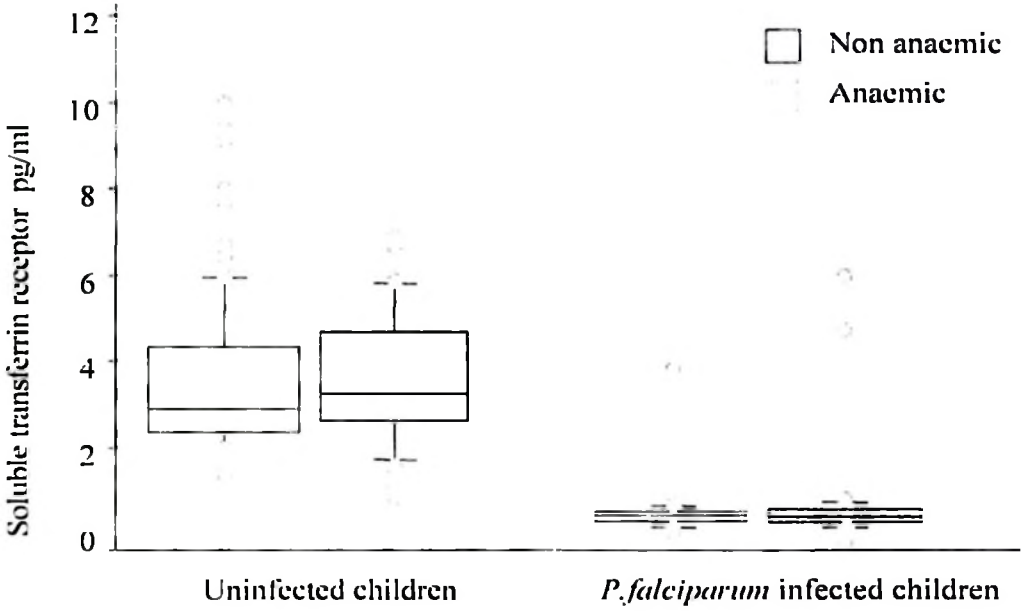

Figure 8. Soluble transferrin receptor (sTfR) in the *P. falciparum* infected and uninfected children; the comparisons between anaemic and non anaemic donors.

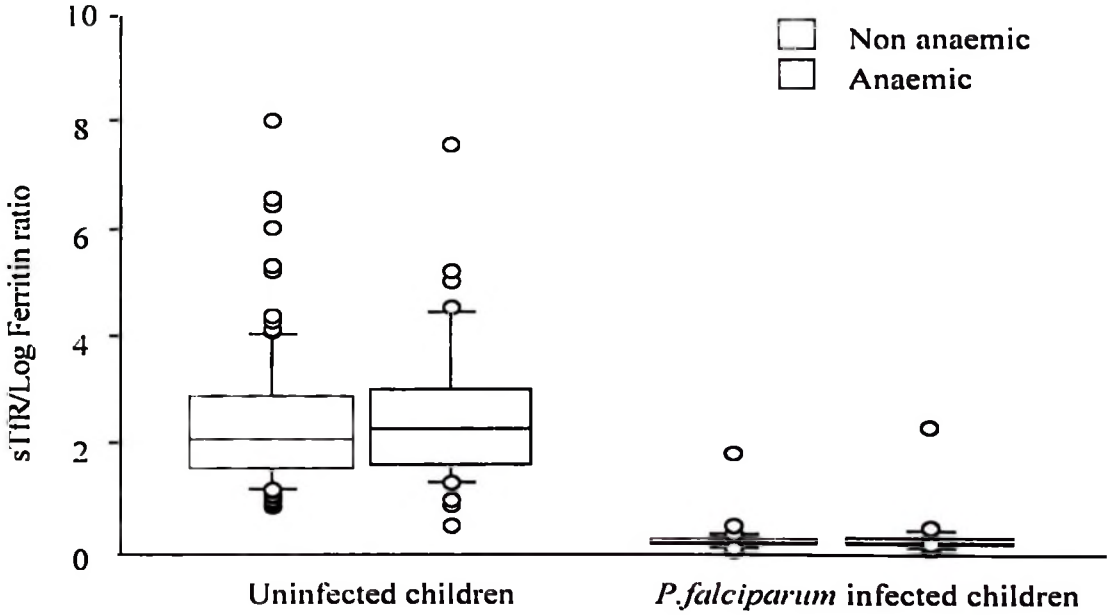

Figure 9. The sTfR /log ferritin ratio in the blood obtained from *P. falciparum* infected and uninfected children; the comparisons between anaemic and non anaemic donors. Each box represents the interquartile range (25 -75%) of values, the whiskers represent 10% and 90% values and the middle line represents median value.

4.1.2.3 Erythropoietin concentrations

Erythropoietin level increased during malaria but it was not significantly different from the control values as detected in uninfected non anaemic children. EPO was significantly lower in anaemic infected children with poor reticulocyte response versus those with normal reticulocyte response (Fig. 10).

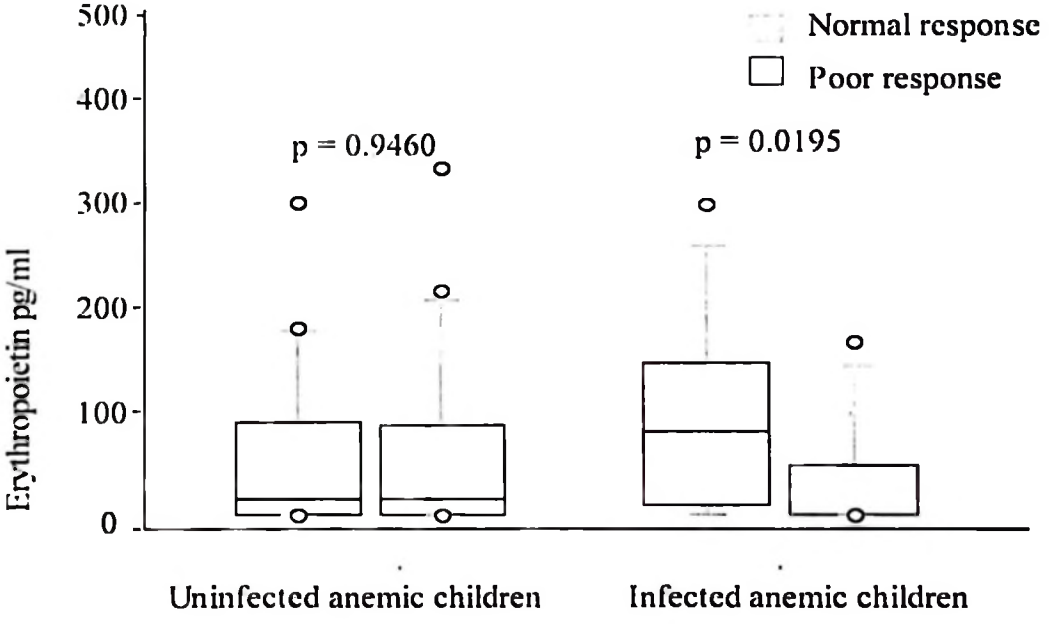

Figure 10. Box plots for erythropoietin level in the study groups' i.e. *P. falciparum* infected and uninfected children stratified by reticulocyte response to anaemia. Shown are interquartile range (25 -75%) of values, the whiskers represent 10% and 90% values and the middle line represents median value.

4.1.2.4 Cytokine concentrations

There was a significant increase in the concentrations of several cytokines (IL- 1, IL-6, IL-10, TNF-α and IFN-γ) during malaria. The TNF-α/IL-10 ratio was significantly higher in the uninfected children (Table 5). When the infected subjects were stratified based on the haemoglobin level as anaemic and non anaemic, there were no significant differences in the cytokine levels (Table 6). When infected anaemic children were stratified based on reticulocyte responses to anaemia, children with inappropriately low reticulocyte responses had significantly higher IL-1, IL- 6, IL- 4, TNF-α and IL -10 (Table 7).

Table 5. Serum soluble factor concentrations in the study subjects comparing uninfected control children and *P. falciparum* infected children

	Uninfected	*P. falciparum* infected children	p-value
N (Male/female)	57(30/27)	57 (29/28)	
TNF-α (pg/ml)	63.39 (93.71)	223.69(172.08)	<0.0001
TNF-RI (pg/ml)	1167.11(836.24)	2725.77 (2923.32)	<0.0001
TNF-RII (pg/ml)	0.00 (73.53)	261.57 (664.13)	<0.0001
IFN - γ(pg/ml)	1.14 (134.11)	207.73 (308.31)	<0.0001
IL-1 (pg/ml)	4.84(10.83)	24.11 (18.78)	<0.0001
IL-4 (pg/ml)	0 (0.0)	0.84 (8.64)	0.0317
IL-5(pg/ml)	4.45 (11.14)	8.89 (9.18)	0.0009
IL-6 (pg/ml)	0(7.01)	30.68(70.39)	<0.0001
IL-10 (pg/ml)	10.04 (35.26)	385.26 (1107.53)	<0.0001
EPO (pg/ml)	5.39 (74.73)	27.24 (82.57)	0.0603
Hepcidin (pg/ml)	331.67 (402.17)	72.15 (79.71)	<0.0001
Ferritin (pg/ml)	33.19 (48.29)	162.37 (186.77)	<0.0001
sTfR (pg/ml)	3.11(2.22)	0.39 (0.28)	<0.0001
CRP (pg/ml)	3.08 (10.82)	3284.72 (1505.74)	<0.0001
TNF- α /IL-10	4.15 (7.78)	0.69 (1.06)	<0.0001

Data are presented as median (Interquartile range=25 -75%)

Table 6. Serum soluble factor concentration in *P. falciparum* infected children comparing anaemic and non anaemic subjects

	Non anaemic	Anaemic	p value
N (Male/female)	28 (17/11)	29 (12/17)	
TNF-α (pg/ml)	236.82 (206.38)	218.36(154.49)	NS
TNF-RI (pg/ml)	2601.16 (2828.45)	3173.38(3073.99)	NS
TNF-RII (pg/ml)	319.25 (691.94)	177.64 (618.42)	NS
IFN (pg/ml)	211.19(329.14)	160.59 (327.86)	NS
IL-1 (pg/ml)	24.51 (19.29)	23.74 (19.8)	NS
IL-4(pg/ml)	1.78 (8.42)	0.81 (8.7)	NS
IL -5 (pg/ml)	12.7 (7.21)	8.36 (10.06)	NS
IL-6 (pg/ml)	28.71(97.77)	32.62 (70.48)	NS
IL-10 (pg/ml)	388.29(132846)	324.63 (530.03)	NS
EPO (pg/ml)	44.71 (81.67)	26.97 (89.44)	NS
Hepcidin (pg/ml)	68.88 (77.94)	80 (90.64)	NS
Ferritin (pg/ml)	174.94(218.680	161.49 (191.03)	NS
sTfR (pg/ml)	0.41 (0.24)	0.36 (0.3)	NS
CRP (pg/ml)	2735.92 (1180.47)	3772.44 (1331.91)	NS
TNF- α /IL-10	0.55 (1.32)	0.82 (0.88)	NS

Data are presented as median (Interquartile range=25 -75%)
NS - Non significant statistical difference (by Mann Whitney test)

Table 7. Comparisons of serum soluble factors between children with appropriate reticulocyte response to inappropriate reticulocyte response during malarial anaemia

N (Male/female)	Appropriate reticulocyte response 14(7/7)	Inappropriate reticulocyte response 15 (5/10)	p value
Age (months)	16.74 ± 6.27	19.06 ± 6.95	NS
Hb (g/dl)	8.36 ± 0.78	8.44 ± 1.17	NS
TNF- α (pg/ml)	181.31 (108.61)	240.82 (189.33)	0.0382
TNF-RI (pg/ml)	2474.14(2923.14)	3654.22 (3377.82)	NS
TNF-RII (pg/ml)	237.04 (428.85)	321.78(1114.10)	NS
IFN (pg/ml)	125.33 (318.37)	232.37 (234.37)	NS
IL-1 (pg/ml)	17.34 (13.89)	27.05 (16.13)	0.0325
IL-4 (pg/ml)	0 (8.62)	2.26 (8.86)	NS
IL -5 (pg/ml)	8.36 (4.59)	9.5 (9.24)	NS
IL-6 (pg/ml)	18.32 (40.59)	65.81 (68.22)	0.0402
IL-10 (pg/ml)	174.17 (267.49)	582.34 (931.05)	0.0232
EPO (pg/ml)	80 (137.94)	0 (47.07)	0.0195
Hepcidin (pg/ml)	62.15(55.21)	96.91 (224.37)	0.0256
Ferritin (pg/ml)	161.08 (195.22)	176.45 (168.78)	NS
sTfR (pg/ml)	0.38 (0.28)	0.33 (0.32)	NS
CRP (pg/ml)	3844.18 (1092.07)	3605.79 (2267.24)	NS
sTfR/log ferritin	0.16 (0.14)	0.15 (0.15)	NS
TNF- α /IL-10	0.94 (0.73)	0.41 (0.84)	0.0668
Parasite density-Trophozoites/ 200WBC	704 (3610)	1420 (2333.5)	NS

Age and Hb data are presented as means ± standard deviation (Unpaired t test)
Parasitemia and serum soluble factors are presented as median (Interquartile range=25 -75%) and tested by Mann Whitney test
NS - Non significant statistical difference

4.1.3 Iron deficiency based on ferritin and C-reactive protein

The overall prevalence of iron deficiency in the study population was 49.75% (99/199). Prevalence of iron deficiency in uninfected children was high 64% (91/142) as compared to the infected children, 10.67% (8/57). Iron deficiency significantly modified reticulocyte response in the uninfected children (X^2 = 5.09, p=0.0241) and not during malaria (X^2 = 1.31, p=0.2531) indicating that poor reticulocyte response during malaria was not related to iron deficiency. Univariate logistic regression showed that iron deficiency was significantly associated with an odds ratio (OR, 95% CI) for poor reticulocyte response of 0.427 (0.211 – 0.867), p=0.0184 in uninfected population but not in the infected children [2.417 (0.518 -11.28), p=0.2616].

4.1.4 Relationship between reticulocyte production index, erythropoietin, and cytokine concentrations in *P. falciparum* infected children

Erythropoietin concentrations positively correlated with the reticulocyte production index in the anaemic subjects (Table 8). The relationship between Erythropoietin with TNF- RII, ferritin, IL- 1, IL- 6, IL-10 and TNF-α was significantly positive in anaemic children with normal reticulocyte responses not with children with poor reticulocyte responses. There was no significant relationship between EPO and haemoglobin level in anaemic children. Plasma levels of IL -1, IL-6, IL-10 and TNF-α in the anaemic group were negatively correlated with the reticulocyte index (Table 8). This type of relationship was neither observed in children with normal haemoglobin level nor when the children were stratified by reticulocyte response. TNF-α/IL-10 ratio correlated positively

with reticulocyte production index in children with normal reticulocyte response (r=0.570, p=0.0483).

Table 8. Relationship between reticulocyte index and serum soluble factors in children infected with *P. falciparum*

	Non anaemic n = 28		Anaemic n = 29	
	Rho	p value	Rho	p value
TNF-α	-0.172	0.3718	-0.383	0.0428*
TNF-RI	-0.044	0.8211	-0.230	0.2245
TNF-RII	-0.099	0.6067	-0.287	0.1289
IFN	-0.086	0.6568	-0.113	0.5493
IL-1	-0.110	0.9004	-0.454	0.0162*
IL-4	0.131	0.4967	-0.243	0.1992
IL -5	-0.164	0. 3951	-0.112	0.5519
IL-6	-0.155	0.4197	-0.441	0.0198*
IL-10	-0.044	0.8178	-0.447	0.0180*
EPO	-0.214	0.2661	0.378	0.0455*
Hepcidin	-0.174	0.3673	-0.504	0.0102*
Ferritin	-0.300	0.1191	-0.231	0.2215
sTfR	0.162	0.3999	-0.033	0.8603
CRP	0.174	0.3658	0.307	0.1044
sTfR/log ferritin	0.199	0.3006	-0.02	0.9906
TNF- α /IL-10	-0.009	0.6947	0.399	0.0346*
Haemoglobin (g/dl)	0.008	0.9660	-0.190	0.3159

*Significant correlations by spearman rank correlation

4. 1.5 Parasite density and cytokine levels

Parasite density correlated positively with cytokine concentrations in all *P. falciparum* infected children regardless of haemoglobin status. However when the children were stratified by reticulocyte response, parasite density correlated positively with cytokines (IL- 1, IL- 4, IL-6, IL-10, TNF-α and IFN-γ) only in children with poor reticulocyte response (Table 9). As shown in Figure 11, *P. falciparum* infected children with poor reticulocyte responses had higher parasitemia than those with normal reticulocyte response though the difference was not statistically significant (p=0.0644).

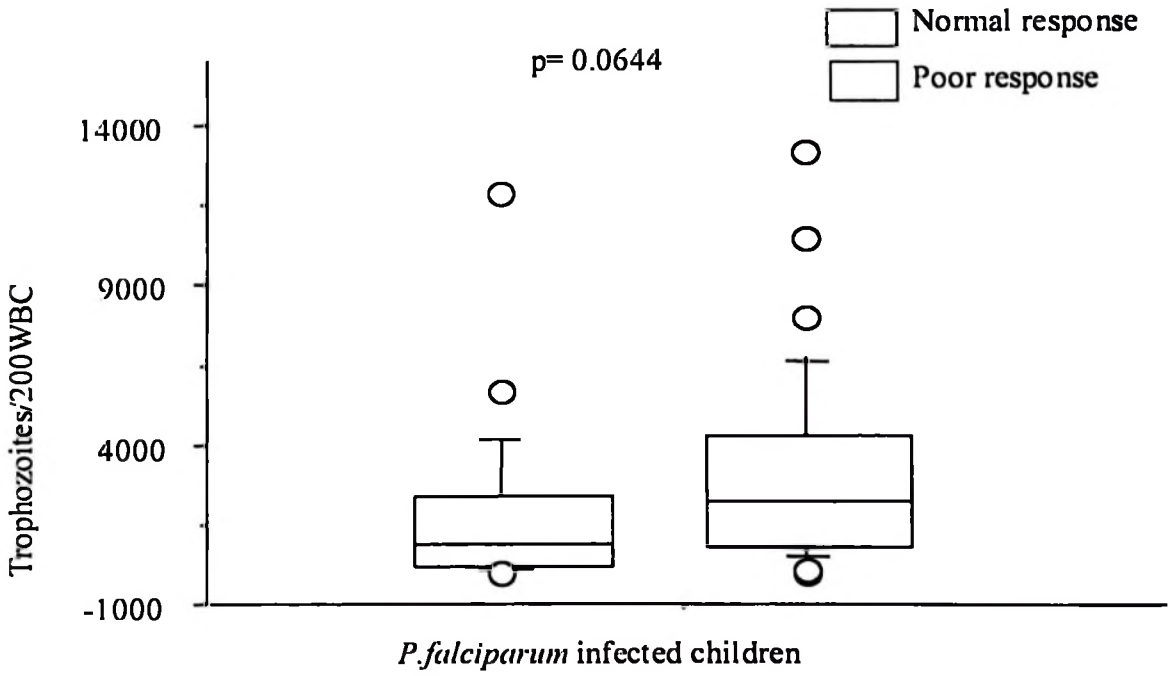

Figure 11: *P. falciparum* infected children with varying reticulocyte responses to anaemia were compared on the parasite density (Trophozoites/ 200WBC) (by Mann Whitney test).

Table 9. Relationship between parasite density and serum soluble factors in children infected with *P. falciparum*

	Appropriate reticulocyte response (n=14)		Inappropriate reticulocyte response (n=15)	
	Rho	p value	Rho	p value
TNF-α	-0.026	0.8980	0.547	0.0023*
TNF-RI	0.011	0.9579	0.395	0.0279*
TNF-RII	-0.090	0.6593	0.498	0.0056*
IFN	0.074	0.7182	0.414	0.0213*
IL-1	-0.146	0.4734	0.618	0.0006*
IL-4	0.194	0.3413	0.552	0.0021*
IL -5	-0.114	0.6803	0.144	0.5907
IL-6	0.017	0.9339	0.673	0.0002*
IL-10	0.239	0.2412	0.646	0.0003*
EPO	0.068	0.7388	0.240	0.1806
Hepcidin	-0.097	0.6434	0.150	0.4113
Ferritin	-0.005	0.9820	0.354	0.0484*
sTfR	-0.500	0.0143*	-0.095	0.5971
CRP	0.046	0.8211	-0.246	0.1715
sTfR/log ferritin	-0.449	0.0278*	-0.195	0.2776
TNF-α /IL-10	-0.365	0.0735*	-0.582	0.0012*

*Significant correlations by spearman rank correlation

4.1.6 Logistic regression analyses for reticulocyte response as dependent variables

Increases in IL-1, IL-6 or IL-10 level among the anaemic children was associated with the odds (OR, 95% CI) for poor reticulocyte response of 71.5 (1.2 – 4192), 3.7 (1.18 – 11.65) and 1.8 (1.1 – 1.6) respectively. On the contrary each log unit increase of EPO reduced the risk (OR, 95% CI) for poor reticulocyte response with OR, 0.38 (0.16 – 0.93).

To determine whether plasma concentrations of one cytokine were associated with poor reticulocyte response independent of the other cytokines in the cascade, a multiple logistic regression analysis was performed with outcome (reticulocyte response) as the dependent variable and cytokines as independent variables. None of the cytokine was independently associated with poor reticulocyte response. When EPO was included in the analysis its increase was associated with the odds (OR 95% CI) for poor reticulocyte response of 0.11 (0.018 – 0.75), indicating that EPO independently reduces the risk of poor reticulocyte response during malarial anaemia (Table 10).

Table 10. Logistic regression analysis to determine influence of serum soluble factors to poor reticulocyte response in malaria

	Anaemic children			Non-anaemic children		
Factor	OR	95%/CI	p-value	OR	95%/CI	p-value
Univariate logistic regression analysis						
TNF-α	30.51	0.682 – 1364	0.0779	1.295	0.07 – 23.86	0.8620
TNF-α RI	2.7	0.205 – 35.93	0.4488	0.58	0.071 – 4.5	0.5937
TNF-α RII	1.7	0.475 – 6.13	0.4132	1.31	0.607 – 2.8	0.4949
IL 1	71.52	1.22 – 4192	0.0398*	0.742	0.047 –11707	0.8322
IL-4	1.34	0.389 – 4.648	0.6392	0.937	0.271 – 3.248	0.9188
IL-5	0.793	0.0.085 – 7.377	0.8382	2.41	0.471 – 12.33	0.2908
IL-6	3.7	1.159 – 11.87	0.0272*	1.588	0.675 – 3.737	0.2896
IL-10	7.308	1.245 – 42.88	0.0276*	1.259	0.426 –3.719	0.6764
IFN-γ	1.138	0.531 – 2.437	0.7392	2.55	0.655 – 9.968	0.1769
EPO	0.379	0.155 – 0.926	0.0333*	2.319	0.285 – 18.85	0.4317
Hepcidin	18.46	0.799 – 42.656	0.0687	9.19	0.338 - 24.95	0.1879
Parasitemia	1.473	0.503 – 4.306	0.4786	1.941	0.776 – 4.853	0.1562
Multiple logistic regression analysis						
EPO	0.114	0.018 – 0.745	0.0234*			
IL -1	56.31	0.028 – 115185	0.3000			
IL-6	1.329	0.096- 18.41	0.8318			
IL-10	7.065	0.088 - 564	0.3817			

*Significant association; All data were log transformed before analysis.

4.2 RED CELL SURFACE MOLECULES CHANGES

Demographic characteristics of study population are presented in Table 11. Children were grouped according to the level of haemoglobin (Non-anaemic >10 g/dL and anaemic <10g/dl). Although there was no significant difference in child age and parasite density between the two groups, there was a tendency towards lower haemoglobin and parasitemia in younger children. Mean haemoglobin concentration for anaemic and non-anaemic children was 8.06g/dl and 11.42g/dl respectively and none of the anaemic children had severe anaemia.

Table 11. Demographic characteristics of study groups

	Anaemic children	Non anaemic	p value
Cases (Male/Female)	N = 50(32/18)	N = 34 (16/18)	
Hb (g/dl)	8.08 (1.51)	11.42 (1.68)	<0.0001
Age (Months)	17.62 (6.1)	19.45 (8.4)	0.0936
Parasite density (Trophozoites/200wbc)	2700.64 (3458)	2884.2(3122.5)	0.9715

Data are presented as Median (Interquartile range) values

4. 2.1 Red cell surface molecules changes during malaria

A total of 84 children that qualified as malaria cases were used in this study; of these, 50 were anaemic, and 34 non anaemic who served as age and season-matched controls. The mean fluorescence intensities for complement regulatory proteins (CD35, CD55 and CD59) and IgG was low in the anaemic subjects but significant differences were recorded for CD55 only. Phosphatidylserine level was higher in anaemic children though the difference to non anaemic subjects was not significant (Figs. 12a and 12b).

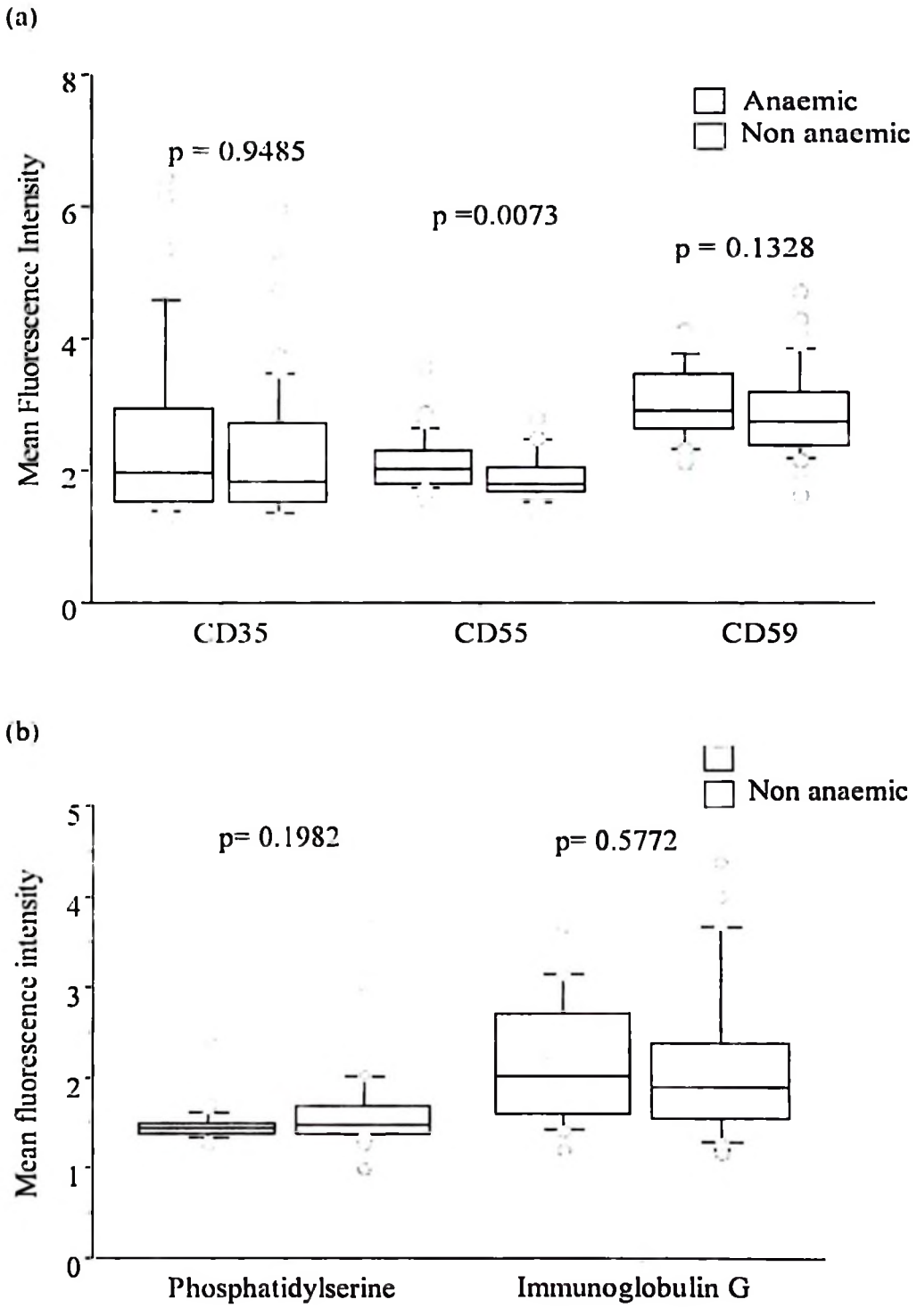

Figure 12. RBC surface molecules levels during malaria (a) CD35, CD55, CD59 and (b) Phosphatidylserine and Immunoglobulin G. Each box represents the interquartile range (25 -75%) of values, the whiskers represent 10% and 90% values and the middle line represents median value.

4. 2.2 Red cell surface molecules in relation to cytokine levels

A total of 63 children with malaria were studied for red cell surface molecules changes in relation to plasma cytokine concentration (TNF-α and IL-10). The ratio of TNF-α /IL-10 was used as a measure to determine the effect of the cytokines on the RBC surface molecules. Thirty eight children were classified as having low TNF-α/ IL-10 ratio (<1) and 25 children had high TNF-α/IL-10 ratio (≥1). The mean age (Months± SD) for the children with low TNF-α/IL -10 ratio was 17.34 ± 5.05, which was significantly younger (p=0.0167) than children with high TNF-α/IL-10 ratio (20.93±5.99). However the two groups were not significantly different in the haemoglobin level (p =0.4648).

Flow cytometric results for mean fluorescence intensities (MFI) of the RBC surface molecules are depicted in Table 12. There were no significant differences in the levels for CD 35, CD55, PS and IgG between children having different ratios of cytokines. CD59 was significantly different between the two groups. Furthermore correlation studies revealed that the TNF-α/IL-10 ratio was positively related to CD 59 (r = 0.412; p = 0.0036) but there were no significant relationship with other molecules. TNF-α/IL-10 ratio correlated negatively with parasitemia in the anaemic children (r = -0.704; p<0.0001) and the non anaemic children (r = -0.303; p =0.1456), but this was only significant in the anaemic group. On the contrary haemoglobin level showed a significant negative correlation with the TNF-α/IL-10 ratio in children with normal haemoglobin level but not with the anaemic children(r = -0.393; p = 0.0300). Among the RBC surface molecules IgG correlated positively with PS in anaemic individuals [(r=0.343), (p=0.0280)].

Table 12. Changes in the RBC surface molecules in children with different plasma TNF -α /IL-10 concentration ratios

Molecule	Mean fluorescence intensities		p value
	Low TNF-α/ IL -10	High TNF-α/ IL -10	
Phosphatidylserine	1.46 (0.29)	1.44 (0.27)	NS
IgG	2.37 (1.29)	1.88 (0.82)	NS
CD 35	1.93 (0.79)	1.99 (1.1)	NS
CD 55	1.95 (0.52)	1.98 (0.39)	NS
CD 59	2.61 (0.54)	3.37 (0.87)	0.0075

4. 2.3 Red cell age and red cell surface molecules in children with malarial anaemia

4.3.3.1 Abundance of cells in different age subpopulations

Separation of RBC by Percoll density centrifugation produced four distinct fractions of RBC (Figure 13), designated as A, B, C, and D for young, mature, old, and very old, respectively. An additional top most layer (O) of cells containing mostly leukocytes and red cell ghosts was observed in some samples, this pool of cells was not included in the analysis. Compared to other children, children with anaemia displayed significant increases in the proportion of young red cells (p=0.0087), and non-significant changes in the other subpopulations (Fig. 14).

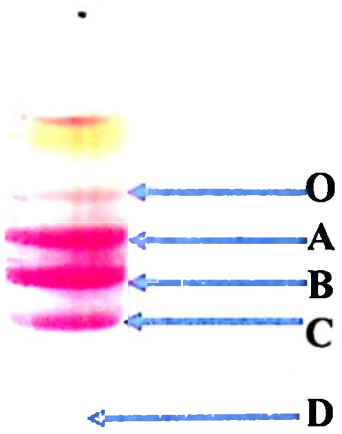

Figure 13. Red blood cells layers produced by Percoll density centrifugation method; each layer represents a distinct population of cells (RBC) of same density (age): (O) Mixture of white cells, cell debris and infected cells (A) Young (B) mature (C) Old (D) very old RBC.

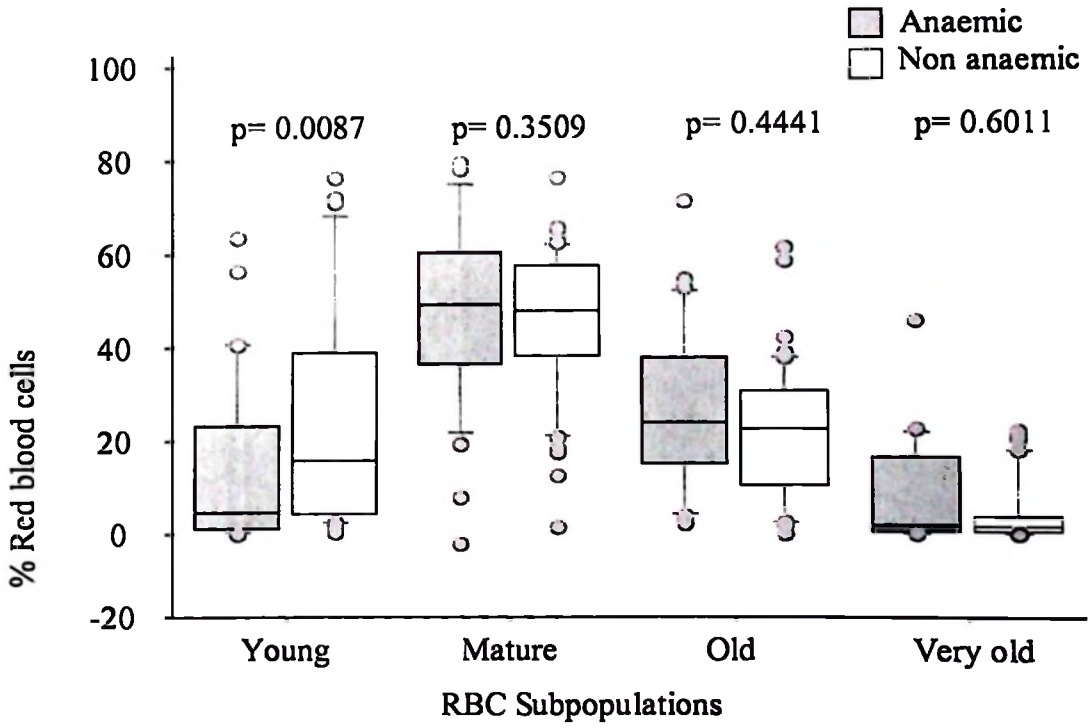

Figure14. Proportion of cells in each RBC subpopulation. The abundance of cells in each RBC subpopulation from children with malarial anaemia (Empty boxes) was compared to the non anaemic children (solid boxes) matched by RBC age.

It was also investigated whether child age and parasite density could be influencing the levels of haemoglobin and the proportion of cells in each subpopulation. There was no significant correlation between child age with either the Hb level or the proportion of cells in all subpopulations. Furthermore, there was no significant relationship between parasite density and Hb level as well as the proportion of cells in each subpopulation for either anaemic or non-anaemic donors.

4. 2.3.2 Surface molecules level in RBC of varying age

The mean fluorescence intensity for cells with detectable complement regulatory proteins (CD55 and CD59) decreased progressively from the youngest (A) to the oldest (D) RBC in all children regardless of the haemoglobin level, indicating that erythrocyte age is a determinant for erythrocyte surface CrP levels. The differences in MFI among subpopulations were highly significant for CD55 and not CD59 (Fig 15a & b). When split by the haemoglobin levels, the MFI of cells expressing CD55 (Fig 16a) were lower in all red cell subpopulations of anaemic children versus children with normal haemoglobin, and these differences were significant for all except the oldest RBC subpopulation. In contrast to the CD55, there were no significant differences in the level of CD59 between anaemic and non-anaemic children in all subpopulations (Fig.16b).

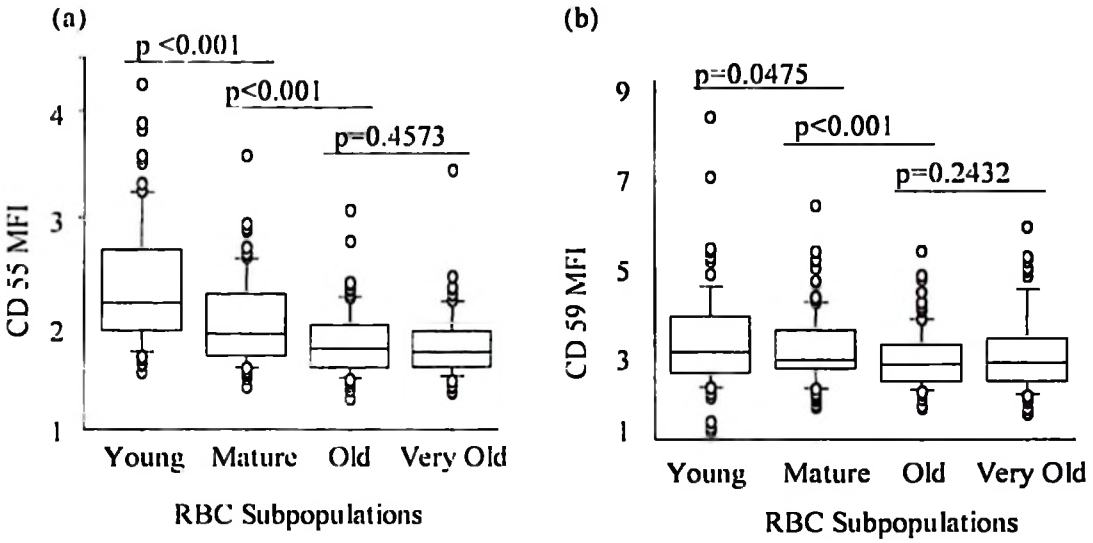

Figure 15. Mean fluorescent intensity of CrP on the surface of red blood cells obtained from *P. falciparum* infected children (a) CD 55 and (b) CD 59. Note a decreasing trend from young cells to the oldest subpopulation.

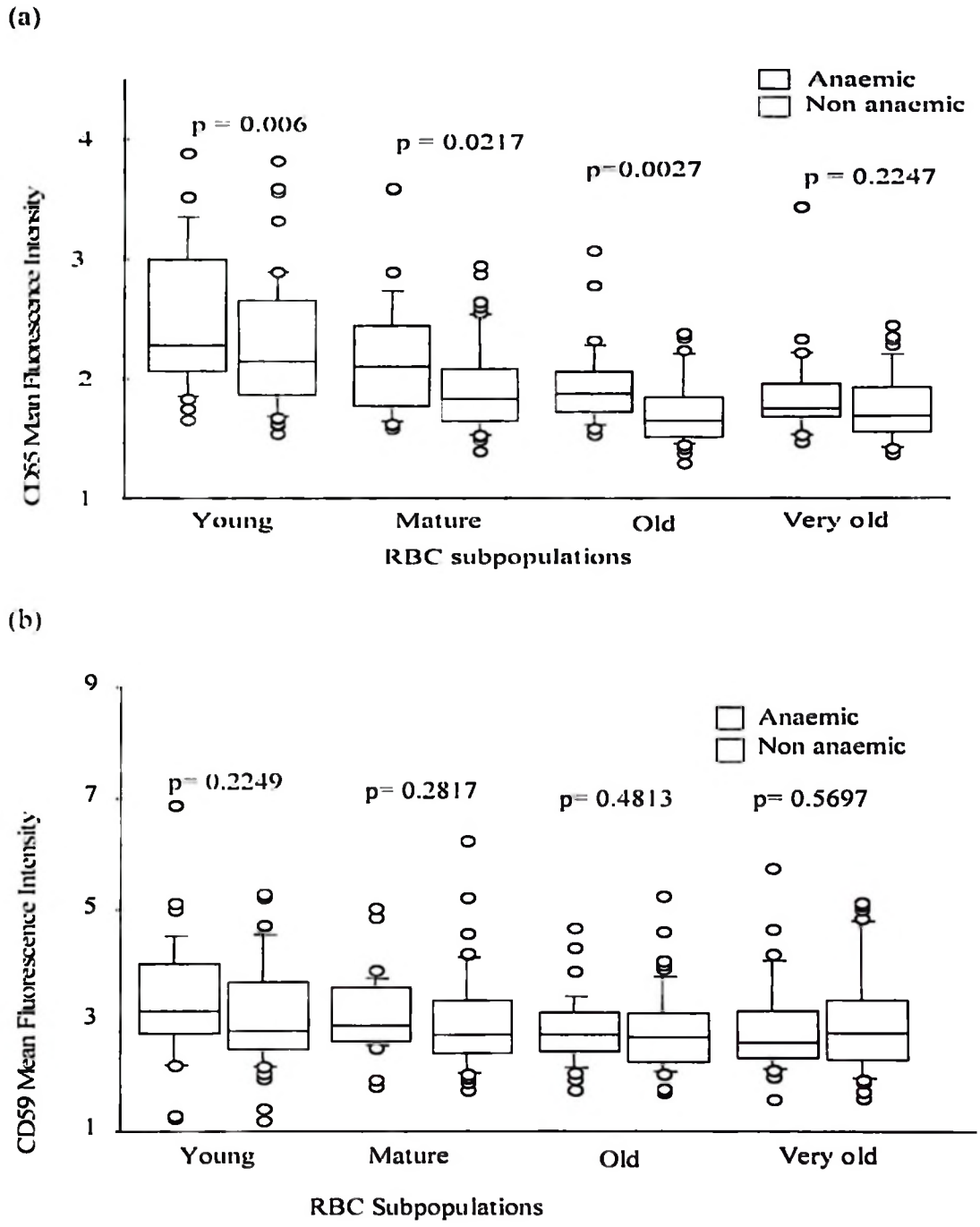

Figure 16. Changes in the Mean fluorescence intensity for CD555 (a) and CD 59(b) on the surface of red blood cells obtained from children infected with *P. falciparum*. Empty boxes (□) are for the anaemic children and solid boxes (■) represent non-anaemic control (solid boxes). The box represents the interquartile range that contains 50% of values, and the whiskers are lines that extend from the box to the highest and lowest values, excluding outliers (circles) and the medians (horizontal lines inside boxes).

The MFI for the cells expressing CD55 in anaemic groups correlated positively with the level of haemoglobin in all RBC subpopulations (A, r= 0.428, p=0.0174; B, r =0.453, p=0.0098; C, r =0.559, p=0.0008; D, r=0.210, p=0.0328) but this relationship was not observed in non-anaemic children nor with CD 59.

It was further assessed whether child age and parasite density could be influencing the levels of red cell surface CrP. There was no significant correlation between child age and MFI for either molecule. However parasite density was negatively associated with CD55 levels; this relationship was significant in non-anaemic children but not in anaemic children (Fig. 17).

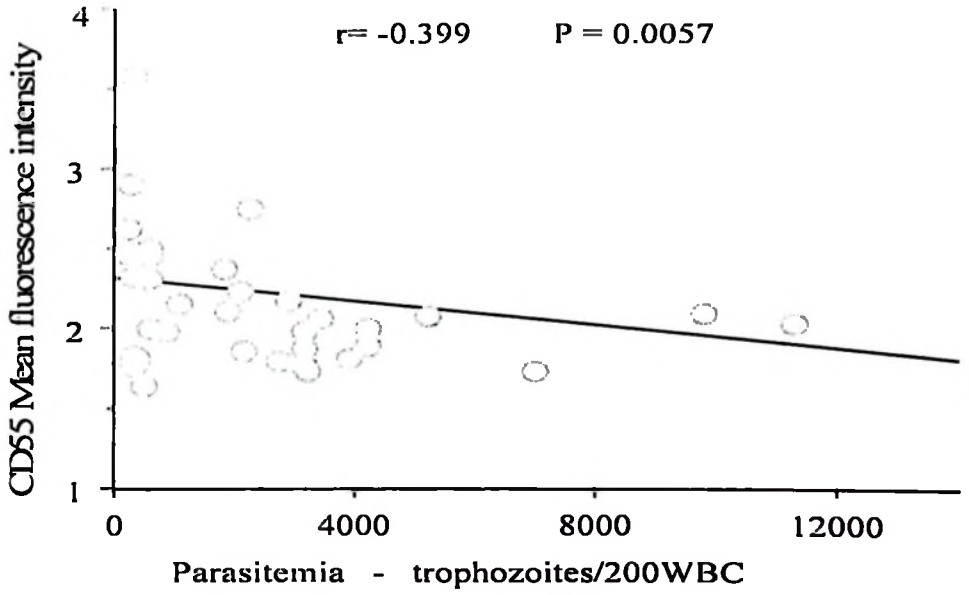

Figure 17. Associations between CD55 levels and parasitemia in non anaemic children.

4.3 HEMOGLOBINOPATHIES AND G6PD DEFICIENCY

4. 3.1 Sickle cell

4. 3.1.1 Sickle cell gene screening results

A total of 980 children were screened for sickle cell gene and the results are shown in Table 13. Figures 18 and 19 show images for Hb electrophoresis (citrate agar) and PCR results on electrophoresis gel respectively.

Table 13: Haemoglobin types of children participating in the MOMS project in Muheza -Tanzania.

Haemoglobin type	Male (%)	Female (%)	Total (%)
HbAA (Normal)	410 (41.84)	407 (41.53)	817 (83.37)
HbAS (Sickle cell trait)	86 (8.78)	70 (7.14)	156 (15.92)
HbSS (Sickle cell)	3 (0.31)	4 (0.41)	7 (0.71)
Total	499 (50.92)	481 (49.08)	980 (100)

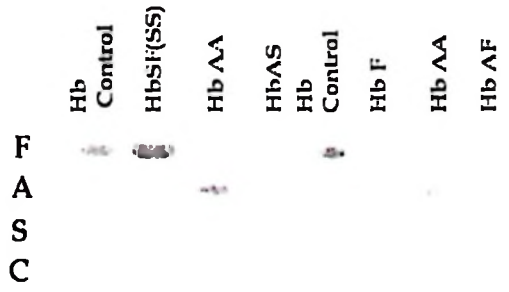

Figure 18. Haemoglobin electrophoresis images run on citrate agar plate showing different haemoglobin types.

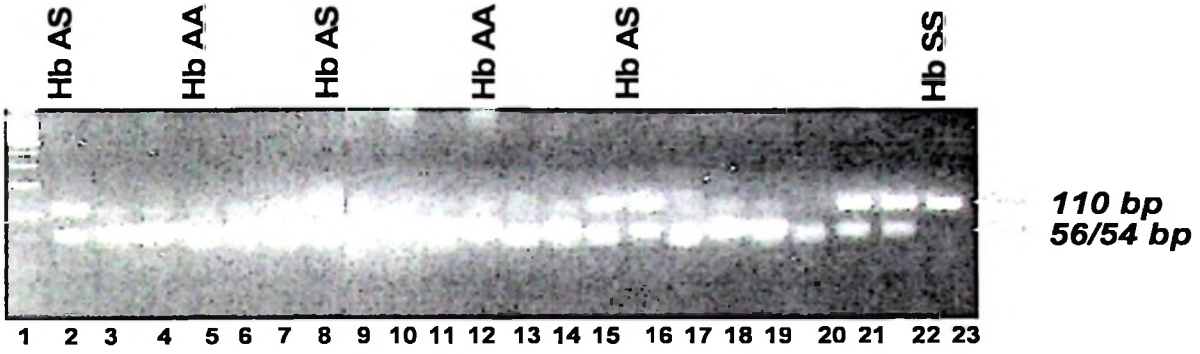

Figure 19. Gel electrophoresis image (1% Agarose). Lane 1 is the molecular marker (50–750 bp). Lanes 2, 8,15,16,21 and 22 show PCR products of the amplified DNA from children in the heterozygous state (HbAS), lane 23 show DNA product from a homozygous individual (HbSS) and other lanes show DNA from normal (HbAA) subjects.

4. 3.1.2 Sickle cell gene and cytokine elves before and during first malaria

Of the children with sickle cell trait (HbAS), 105 were compared to a similar number (105) of children having normal haemoglobin (HbAA) for differences in levels of plasma cytokine and other serum soluble factors before first malaria illness as shown in Table 14. Children in the two groups had comparable age and haemoglobin levels. There was a trend of producing more cytokines in children with normal haemoglobin than children with sickle cell trait though these differences were not statistically significant. Significantly high level was recorded for TNF-RI in children with normal haemoglobin (HbAA).

During malaria, 61 children with normal haemoglobin type qualified as age matched comparisons with similar number of sickle cell carriers on the levels of serum soluble factors. Children from the two groups had similar age at the time of analysis but haemoglobin concentration was higher in the HbAS children with the difference approaching significance level (0.0556). Children with normal

haemoglobin produced more cytokines than sickle cell gene carriers (Table 14). The differences were significant for IL-1, IL- 6, IFN-γ and IL-5. The plasma TNF-α level was higher in children with normal haemoglobin but the difference was not significant (p=0.0844). The concentration of sTfR during malaria was significantly higher in children carrying sickle cell trait than children with normal haemoglobin. Median parasite density was significantly higher (p=0.0030) in children with HbAA gene, and correlated positively with cytokine levels in both genotypes.

Table 14. Haematological data and cytokine concentrations in children stratified by haemoglobin types before and during first malaria infection

	Before malaria infection			First malaria episode		
Hb types	HbAA	HbAS	p-value	HbAA	HbAS	p-value
N (M/F)	105(51/54)	105 (57/48)	-	61(39/22)	61(34/27)	-
Age (Months)	6.31 ± 5.17	6.13 ± 5.18	0.3739	10.25 ± 6.53	10.27 ± 6.56	0.9816
Hb g/dl	10.98 ± 2.23	10.95 ± 2.92	0.8233	9.27 ± 2.17	10.25 ± 2.36	0.0559
Parasite density-Trophozoites/ 200WBC	0.0	0.0	-	3488(2126)	819(402.5)	0.0030*
TNF-α (pg/ml)	89.98 (120)	67.9(115)	0.1931	176.02(152.11)	129.55(160.98)	0.0844
TNF-α RI (pg/ml)	1378 (339.8)	1199.3(1145.98)	0.0113*	2883.49(2757)	2058.67(2470)	0.1008
TNF-α RII (pg/ml)	23.68(183.68)	45.91(149.49)	0.9891	245.27(575)	179.44(424.37)	0.2279
IFN-γ (pg/ml)	0.0 135.57)	0.0 (116.98)	0.6611	131.87(314.44)	35.22(212.06)	0.0338*
IL 1(pg/ml)	5.06(11.53)	4.61(7.52)	0.3384	22.82(17.53)	14.68(18.25)	0.0021*
IL-4(pg/ml)	0.0 (4.97)	0.0 (1.8)	0.2873	0.18(5.87)	0.0 (2.18)	0.1160
IL-5(pg/ml)	4.7(14.34)	4.28 (10.68)	0.6350	7.53(10.37)	4.21(9.49)	0.0365*
IL-6(pg/ml)	0.0 (10.21)	0.0 (4.56)	0.7231	20.35(48.19)	9.48 (36.27)	0.0400*
IL-10(pg/ml)	10.3(38.48)	8.18(20.44)	0.3154	334.02(1137.88)	203.55(598.98)	0.1978
STfR (pg/ml)	3.294 (2.105)	3.08 (2.76)	0.7608	0.446 (3.27)	2.32 (5.31)	0.0249*
Ferritin (pg/ml)	41.16(64.61)	41.13(68.23)	0.2887	116.26(135.38)	102.06(169.62)	0.4719
CRP (pg/ml)	5.31(2.102)	5.92(16.99)	0.7832	1799(2307)	1938(1989)	0.6783

Haemoglobin levels are presented as means ± Standard deviation

Age, Cytokine concentrations and parasite density are presented as median (Interquartile range=25 -75%) values

* indicate values which are significantly different from the other group

4. 3.2 Alpha thalassemia

4. 3.2.1 Alpha thalassemia genotyping results

Alpha thalassemia genotyping by PCR (Figure 20) demonstrated that the 3.7kb deletion is the commonest defect in the α-globin gene in this population (Table 15). The α^{-} thalassemia allele frequency is within the expected range as reported previously (Wellems and Fairhurst. 2005). The 4.2 kb deletion is rare; only one child (0.13%) in the study population was found to be a heterozygote of this genetic defect (α2/α4.2).

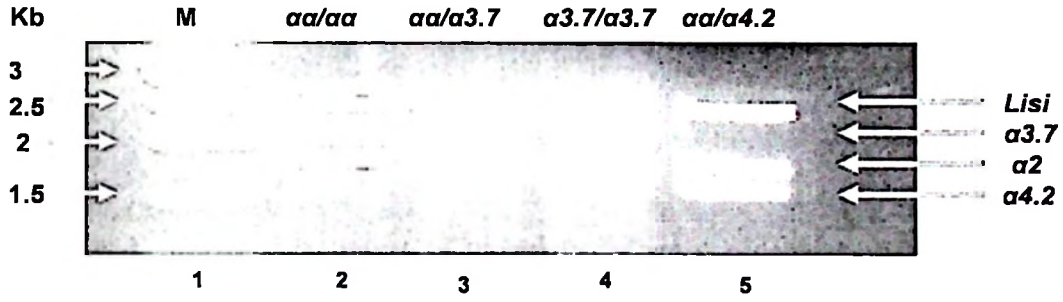

Figure 20. Multiplex-PCR genotype analysis of the α-globin gene. Electrophoresis gel image (1%Agarose) representing results from DNA samples of children with various alpha-globin genotypes. The M indicates the 1 kb-Plus molecular weight marker (Life Technologies), lane 2 shows a normal α-globin gene, lane 3 shows heterozygous state of 3.7 kb deletion, lane 4 shows a homozygous 3.7 kb deletion and lane 5 shows a heterozygous of 4.2kb deletion.

Table 15: Proportions of α-globin genotypes of children participating in the MOMS project in Muheza -Tanzania

Haemoglobin type	Male (%)	Female (%)	Total (%)
3.7kb deletion			
αα/αα	207 (26.54)	181 (23.21)	388 (49.74)
αα/α3.7	162 (20.77)	154 (19.74)	316 (40.51)
α3.7/α3.7	39 (5)	36 (4.62)	75 (9.62)
4.2kb deletion			
αα/α4.2	1 (0.13)	0 (0)	1 (0.13)
α4.2/α4.2	0 (0)	0 (0)	0 (0)
Total	409 (52.44)	371 (47.56)	780 (100)

4.3.2.2 Alpha thalassemia in relation to cytokine levels before and during malaria

A total of 135 children were used to study cytokine production before and during first malaria illness. Each group (genotype) comprised 45 children. The data on age (months), haemoglobin, cytokine level and parasite density of children enrolled in the study are shown in the Table 16. Alpha thalassemic (α3.7/α3.7) children had significantly higher ferritin and TNF-α than their normal counterpart children before infection. This trend was reversed during malaria at which normal children had significantly high levels of ferritin and TNF-α than α-thalassemic children. Moreover, the correlation between these two variables was significantly positive (rho=0.902, $p<0.0001$). Also children with normal α-globin gene (αα/αα) presented significantly higher levels of IL-5 during infection than the heterozygotes. Concentrations of other cytokines did not differ significantly among the groups.

Table 16: Haematological data and cytokine concentrations in children stratified by α-globin genotype before and during first malaria infection

	Before malaria infection			First malaria episode		
Hb types	αα/αα	αα/α3.7	α3.7/α3.7	αα/αα	αα/α3.7	α3.7/α3.7
N	45	45	45	45	45	45
Age (Months)	5.79 (2.9)	5.82 (2.93)	5.82 (3.17)	8 (5.58)	8.07 (5.77)	8.04 (5.85)
Hb g/dl	10.53±2.77	10.86±2.79	10.51±2.38	9.46 ±2.26	10.23 ± 2.98	8.87±2.07[b]
Parasite density-Trophs/ 200WBC	-	-	-	610 (2572)	706 (3064)	730 (3665)
TNF-α (pg/ml)	39.24 (86.76)	56.58 (95.91)	75.13 (101.33)[a]	249.97 (212.64)	137.81 (142.29)[a]	137.81 (185.62)[a]
TNF-α RI (pg/ml)	1375.6 (12030	1285.6(922.85)	1421.6 (864.73)	3621(2965.24)	2523.9(3046.59)	2939.5(2451.13)
TNF-α RII (pg/ml)	60.2 (195.03)	0.0 (161.47)	23.68 (135.89)	247.69 (1073.94)	279.37 (725.84)	246.03 (542.72)
IFN-γ (pg/ml)	0.0 (34.62)	0.0 (86.78)	0.0 (88.05)	120.24 (238.91)	113.28 (213.27)	58.18 (262.2)
IL -1(pg/ml)	3.98 (4.45)	5.54 (6.32)	5.6 (5.72)	22.09 (17.390)	15.74 (17.45)	16.3 (19.79)
IL-4(pg/ml)	0.0 (0.0)	0.0 (2.06)	0.0 (0.0)	0.02 (6.490	0.0 (1.66)	0.0 (2.08)
IL-5(pg/ml)	3.35 (5.45)	4.71 (8.59)	2.78 (6.69)	7.25 (6.99)	4.39 (6)[a]	6.43 (9.39)
IL-6(pg/ml)	0.0 (4.02)	0.0 (4.8)	0.0 (1.07)	28.6 (62.55)	15.32 (46.79)	18.27 (39.19)
IL-10(pg/ml)	9.73 (13.16)	10.38 (16.54)	8.27 (22.50)	306.65(738.72)	202.46(717.35)	314.87 (868.55)
sTfR(pg/ml)	3.57 (2.8)	3.05 (2.69)	3.35 (2.07)	0.58 (3.41)	1.93 (4.58)	1.59 (5.34)
Ferritin (pg/ml)	30.99 (64.93)	27.45 (59.61)	41.58 (80.19)[a]	256.22 (380.58)	102.79 (146.87)[a]	132.66 (157.73)[a]
CRP (pg/ml)	12(31.48)	5.97(12.588)[a]	8.61(30.49)	2263.42(18590)	2094.99(4584)	1663.91(2512)[a]

Haemoglobin levels are presented as means (Standard deviation)

Age, Cytokine concentrations and parasite density are presented as median (Interquartile range =25 -75%) values

[a] significantly different from the normal α-globin genotype; [b] significantly different from heterozygous alpha thalassemic group

4.3.3 G6PD deficiency

4. 3.3.1 Glucose - 6 phosphate dehydrogenase (G6PD) deficiency genotyping results

G6PD screening was performed in 1003 samples. A 1230bp fragment spanning the third to fifth exon of the G6PD gene was amplified in the first PCR, later 2 fragments (342 and 320bp) were amplified in the nested multiplex PCR and these were subjected to restriction enzyme (NCO I) digestion. Figure 21 shows the agarose gel electrophoresis of the amplified DNA along with the digested products of various polymorphic sites. Screening results are shown in Table 17; the frequency of G6PD alleles falls within the reported frequency range for Africans in Sub-Saharan Africa region (Luzzatto, 1973, Beutler, 1996, Ruwende and Hill, 1998).

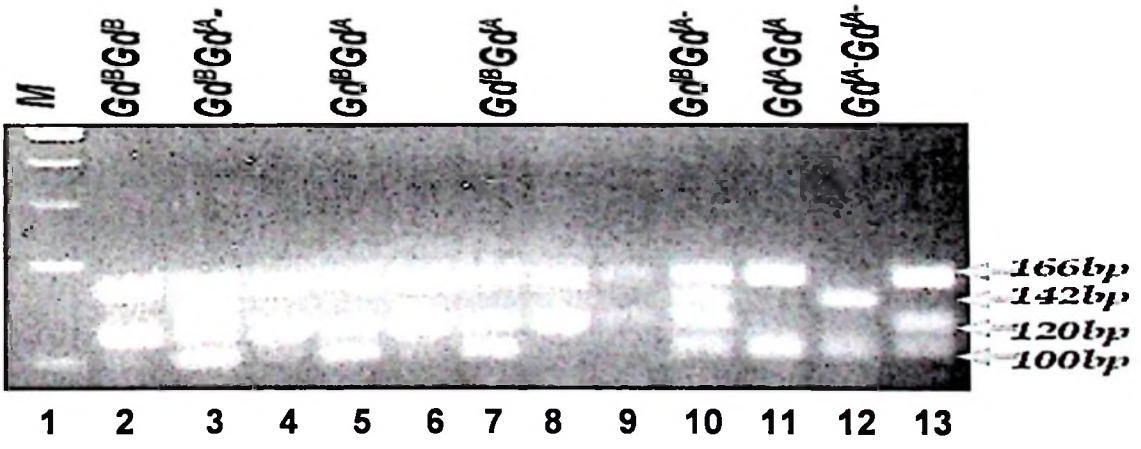

Figure 21. Multiplex PCR analysis of the G6PD gene. Gel electrophoresis image (4%Agarose) showing representative results of DNA samples obtained from children with various G6PD genotypes. The M indicates the 100bp molecular weight marker (Life Technologies). Lanes 2, 4, 6 and 8 show PCR products of the amplified G6PD gene in the homozygous state, lanes 3 and 10 show A-heterozygous ($Gd^{B}Gd^{A-}$), lanes 5,7 and 13 show A heterozygous ($Gd^{B}Gd^{A}$), lane 11 shows a mutation A (Gd^{A} or $Gd^{A}Gd^{A}$) and lane 12 shows a G6PD deficient variant A-(Gd^{A-} or $Gd^{A-}Gd^{A-}$).

Table 17: Proportions of G6PD genotypes of children participating in the MOMS project in Muheza -Tanzania.

G6PD Genotype	Male (%)	Female (%)	Total (%)
B *(Gd^B or Gd^BGd^B)*	348 (34.69)	282(28.12)	630 (62.81)
A *(Gd^A or Gd^AGd^A)*	78 (7.78)	51 (5.08)	129 (12.86)
A-*(Gd^{A-} or $Gd^{A-}Gd^{A-}$)*	86 (8.57)	17 (1.69)	103 (10.27)
A heterozygous *(Gd^BGd^A)*	-	79 (7.88)	79 (7.88)
A-heterozygous *(Gd^BGd^{A-})*	-	62 (6.18)	62 (6.18)
Total	512(51.05)	491(48.95)	1003(100)

4. 3.3.2 G6PD and cytokine levels before and during malaria

Forty six G6PD deficient children (Gd^{A-}) were compared to children of similar age having normal G6PD genotype (Gd^B and Gd^A) on plasma cytokine levels and other serum soluble factors before first malaria illness. Also 108 children qualified for age matched comparisons between Gd^{A-} and Gd^B/Gd^A during malaria (36 children in each group). The age profile, parasitemia, cytokines and Hb levels for males are shown in Table 18 and Table 19 for females.

Plasma cytokine levels were also compared between homozygous (Gd^BGd^B) and heterozygous G6PD deficient (Gd^BGd^{A-}) children before and during malaria. The two groups had equal numbers of subjects at each time point. The comparisons of cytokine levels in hemizygous deficient children (Gd^{A-}) with age matched children having normal G6PD gene (Gd^B or Gd^A) before exposure to malaria and during malaria revealed no differences. Similar findings were observed when homozygous normal children (Gd^BGd^B) were compared to heterozygotes G6PD deficient children (Gd^BGd^{A-}) at the same time point of sampling.

Table 18: Haematological data and serum soluble factors concentrations in male children stratified by G6PD genotypes before and during first malaria infection

	Before malaria infection			First malaria episode		
Hb types	Gd^{A}	Gd^{A-}	Gd^{B}	Gd^{A}	Gd^{A-}	Gd^{B}
N	46	46	46	36	36	36
Age (Months)	3.411(3.11)	3(3.11)	3.43(3)	8.143 (5.460	8.09(7.38)	8.19(5.250
Hb g/dl	10.24 ± (2.8)	9.78 ± (2.37)	10.31 ± (2.6)	9.55 ± 1.73	9.88 ± 1.63	9.54±1.7
Parasite density-Trophozoites/200WBC	-	-	-	3240 (8309)[ab]	962(2322)	724(2424)
TNF-α (pg/ml)	70.78(104.01)	80.81(120.62)	68.91(114.48)	208.82(281.06)	205.48(161.55)	169.03(143.25)
TNF-α RI (pg/ml)	331.75 (1194.2)	1531.29(874.82)	1384.12(1071.18)	3839.2(4451)	2709.44(2526.7)	2631.5(3098.9)
TNF-α RII (pg/ml)	33.23(180.19)	69.4 (77)	50.32(170.05)	271.15(680.49)	363.58(914.81)	198.52(767.29)
IFN-γ (pg/ml)	0.0 (82.92)	0.0 (86.86)	0.0 (98.35)	87.57(221.45)	76.79(213.17)	102.95(340.67)
IL 1(pg/ml)	4.93(8.72)	5.46(6.38)	5.09(9.08)	21.05(24.86)	21.23(16.92)	17.73(16.31)
IL-4(pg/ml)	0.0(3.2)	0.0(0.0)	0.0 (0.76)	0.0 (5.77)	0.0 (4.7)	0.56 (7.49)
IL-5(pg/ml)	3.87(6.31)	3.75(7.49)	3.75(7.49)	6.76 (9.21)	4.68(9.75)	7.57(7.28)
IL-6(pg/ml)	0.0 (16.18)	0.0 (2.63)	0.0 (5.47)	20.77(77.37)	14.98(40)	15.14(35.58)
IL-10(pg/ml)	9.5 (24.94)	6.29(14.56)	10.3(48.91)	520.02(1017.02)	301.39(946.68)	213.51(942.07)
sTfR (pg/ml)	3.14(2.5)	3.6 (3.29)	3.21(2.81)	1.53(7.26)	0.75(4.31)	2.14(4.29)
Ferritin (pg/ml)	34.55(71.42)	46.89(95)	44.99(67.64)	166.97(334.21)	156.99(134.67)	116.06(180.06)
CRP (pg/ml)	12.11(20.62)	11.85(26.48)	7.94(21.26)	2232(1945)	2366(4316)	1969(1985)

Haemoglobin levels are presented as means ± Standard deviation

Age, Cytokine concentrations and parasite density are presented as median (Interquartile range=25 -75%) values

[a] significantly different from the normal G6PD genotype (Gd^{B}), [b] significantly different from G6PD deficient genotype(Gd^{A-})

Table 19: Haematological data and serum soluble factors concentrations in female children stratified by G6PD genotypes before and during first malaria infection

	Before malaria infection			First malaria episode		
Hb types	$Gd^{B}Gd^{A-}$	$Gd^{B}Gd^{B}$	p-value	$Gd^{B}Gd^{A-}$	$Gd^{B}Gd^{B}$	p-value
N	39	39		28	28	
Age (Months)	5.82(4.55)	5.82(7.44)	NS	9.46 (13.16)	9.36 (13.77)	NS
Hb g/dl	11.06±1.58	10.78± 2.77	NS	9.42±2.07	9.88±2.69	NS
Parasite density-Trophozoites/ 200WBC	-	-	-	652(3878)	615 (2676)	NS
TNF-α (pg/ml)	87.16(89.29)	63.25(85.14)	NS	162 (129.49)	144.87(167.92)	NS
TNF-α RI (pg/ml)	1562.24(973)	1245.8(1019)	NS	2373(2719.6)	1979.4(2944.7)	NS
TNF-α RII (pg/ml)	0.0 (74.93)	0.0 (115.48)	NS	128.41 (748.71)	320.23(553.13)	NS
IFN-γ (pg/ml)	0.0 (95.16)	2.37(136.22)	NS	124.83 (348.32)	91.41 (192.54)	NS
IL-1 (pg/ml)	4.32(5.39)	4.37(8.05)	NS	14.17 (16.14)	18.25 (20 72)	NS
IL-4(pg/ml)	0.0 (1.54)	0.0 (3.66)	NS	0.0 (13.44)	0.0 (1)	NS
IL-5(pg/ml)	2.38 (6.45)	4.5 (5.83)	NS	5.71 (13.56)	2.86 (7.84)	NS
IL-6(pg/ml)	0.0 (1.46)	0.0 (2.640	NS	14.98 (47.050	12.48 (40.36)	NS
IL-10(pg/ml)	8.7(10.91)	6.76 (19.25)	NS	180.09 (808.07)	212.06 (685.5)	NS
STfR(pg/ml)	2.9(1.47)	2.4 (1.4)	NS	0.66 (6.58)	0.45 (1.05)	NS
Ferritin (pg/ml)	72.9(112.02)	50.04(91.01)	NS	130.17(127.49)	113.16(135.57)	NS
CRP (pg/ml)	33.7(2155)	19.08(1744)	NS	2255(7299)	997.36(13557)	NS

Haemoglobin levels are presented as means ± Standard deviation

Age, Cytokine concentrations and parasite density are presented as median (Interquartile range=25 -75%) values

NS - Non significant statistical difference

CHAPTER 5

5.0 DISCUSSION

5.1 ERYTHROPOIESIS

In search for pathophysiological basis of malarial anaemia, *Plasmodium falciparum* infected anaemic children with low or inappropriate reticulocyte responses were compared to those with normal reticulocyte responses for their levels of serum soluble factors including cytokines and erythropoietin. Our hypothesis was that causes of anaemia are multifactorial, and therefore the anaemic population may be heterogeneous, reflecting varied aetiological mechanisms for anaemia. Results from this study shows that among the serum soluble factors analyzed, EPO is the principal regulator of reticulocytosis, and the influence of cytokines on the production of reticulocytes is insignificant during malaria episodes according to multivariate analyses (Table 10).

5.1.1 Relationship between cytokine levels and reticulocyte responses

Cytokine levels in anaemic children were comparable to those of non anaemic children as shown in Table 5. The results are consistent with previous reports that cytokine levels in moderately or mildly anaemic individuals do not differ from asymptomatic malaria, whereas significantly higher levels of cytokines are detected in severely anaemic cases (Kurtzhals *et al.*, 1998). Interestingly, among anaemic patients, cytokine concentrations IL-1, 1L-6, TNF-α and IL-10 were significantly higher in children with poor reticulocyte response than those with normal reticulocyte response (Table 7) but correlation analyses indicated lack of relationship between cytokine levels and reticulocyte count in anaemic children

mechanisms not related to erythropoietic suppression. Cytokine stimulated erythrophagocytosis (Taverne *et al.*, 1994) and RBC surface membrane damages by reactive oxygen species (ROS) have been reported in malaria (Greve *et al.*, 1999; Griffiths *et al.*, 2001). Young RBC may suffer most because membranes of young cells are sensitive to oxidative stress and thus more vulnerable to oxidative damage than older cells (Omodeo-Sale *et al.*, 2003).

5.1.2 Low erythropoietin causes poor reticulocyte response during malaria

P. falciparum infected anaemic children with poor reticulocyte responses had lower levels of EPO compared to normal responders (Figure 10). It is plausible that low erythropoietin levels may explain the poor reticulocyte response observed in these children. This suggestion is based on the fact that EPO plays a significant role during the final stages of development of erythroid progenitor cells (Fisher, 2003) and hence making an important factor for regulating development of red blood cells. The level of EPO in plasma ultimately influences the rate of production of new erythrocytes by haematopoietic tissues. On the other hand, failure to increase the amount of circulating EPO in response to hypoxia interferes with the development of erythroid progenitor cells to fully functional cells thus causing anaemia. Inadequate in EPO production has been associated with anaemia in many chronic diseases such as rheumatoid arthritis, malignancies and immunodeficiency syndrome (Pincus *et al.*, 1990; Ludwig *et al.*, 1990; Fischl *et al.*, 1990).

Low levels of EPO may also cause a selective hemolysis of young circulating RBC (Neocytolysis), a physiologic process which takes place in circumstances of excess RBC (Alfrey *et al.*, 1997). Neocytolysis is initiated by a fall in erythropoietin levels and has been reported in physiological conditions such as emergence of newborns from hypoxic uterine environment, descent of polycythermic high altitude dwellers to sea level and return of astronauts from space (Rice *et al.*, 2001; Rice and Alfrey 2005). This process have been implicated in the pathogenesis of anaemia in haemolytic anaemias, type 2 diabetes mellitus and renal diseases (Rice *et al.*, 1999; Rice and Alfrey 2005; Alfrey and Fishbane 2007; Wittmann *et al.*, 2007), and other investigators have suggested to play a role in the pathogenesis of anaemia malaria and sickle cell anaemia (Trial and Rice, 2004).

Reasons for inadequate EPO responses in some malaria patients observed in this study as well as in other diseases are unknown. Many studies report that impaired erythropoietin production in different diseases occur by a common mechanism involving the production of inflammatory cytokines that not only suppress the production of erythropoietin but also interfere with the proliferation of erythroid progenitor cells (Faquin *et al.*, 1992; Vannucchi *et al.*, 1994; Rusten ad Jacobsen, 1995; Macdougall and Cooper 2002; Felli *et al.*, 2005; Jelkmann 1998; La Ferla *et al.*, 2002). However, correlation analyses in this study show that the overall contribution of cytokines to the variation in EPO levels in anaemic children with poor reticulocyte responses was insignificant. In addition to that, serum EPO levels correlated positively with the serum cytokine concentrations suggesting that

at least at this stage, cytokine levels did not interfere with EPO production, similar to the previous report by Chang and Stevenson (2002).

Low EPO levels in *P. falciparum* infections, probably resulted from the disruption of oxygen sensing mechanisms and EPO producing cells in the kidney. Damage to the renal tubules could upset sodium transport system, which is the major determinant of renal blood flow ((Eckardt and Kurtz 2005). Renal blood flow changes in turn influences oxygen supply to the kidneys, which is an important factor for the regulation of EPO production (Eckardt and Kurtz 2005). This mechanism has also been reported in renal diseases (Koury *et al.*, 1988; Maxwell *et al.*, 1997). Since *P. falciparum* infections are associated with systemic pathologic abnormalities including renal damage (Prakash *et al.*, 1996; Day *et al.*, 1999; Günther *et al.*, 2002; Patel *et al.*, 2003), we argue that renal damage may explain for the diminished EPO production during malaria. Our argument is further supported by the reports that impaired renal function is common in African children with *P. falciparum* malaria (Weber *et al.*, 1999; Burchard *et al.*, 2003). However, the relationship between the indicators of renal damage and EPO levels during malaria is yet to be determined.

Likewise the inhibitory effects of ROS to oxygen sensors in EPO producing cells could suppress EPO production during malaria. ROS such as hydrogen peroxide (H_2O_2) are part of the signalling chain of the intracellular oxygen sensing mechanisms that regulate EPO synthesis in kidneys (Canbolat *et al.*, 1998; Neumcke *et al.*, 1999). Elevated cellular H_2O_2 levels have been demonstrated to

inhibit EPO protein production and the hypoxia induced EPO mRNA expression (Fandrey *et al.*, 1994; Imagawa *et al.*, 1996). Increases in ROS and pro-oxidants levels have been reported in malaria cases (Greve *et al.*, 2000; Nanda and Das 2000; Griffiths *et al.*, 2001), thus we suggest that ROS may be among the factors that affect the production of EPO, and possibly the pathway to anaemia in *P. falciparum* infections. In our study children with low levels of EPO also tended to have high parasitemia, which suggests a link between parasite factors and suppression of EPO production in malaria. Parasite density may determine the amount of hemozoin produced (Jaramillo *et al.*, 2005) and consequently the amount of ROS generated, which would interfere with the process of EPO production.

5.1.3 No correlation between EPO and haemoglobin level during malaria

There was a positive correlation between EPO and reticulocyte count in anaemic subjects but this relationship was not reflected by the haemoglobin level (Table 8). This contrasts with the finding by Kurtzhals et al. (1999), who reported increases in erythropoietin levels in the face of decreasing haemoglobin concentrations suggesting sufficient EPO response to low haemoglobin. This may be related to the fact that long standing asymptomatic *P. falciparum* infections were recruited in that study unlike the current study which included acute malaria cases. In anaemia of acute causes, there may be insufficient time to compensate for the blood loss. Evidence shows that EPO can be detected in circulation within hours after exposure to hypoxia (Ge *et al.*, 2002) but it takes 2-3 days to detect an

increase in reticulocyte count and up to 5 days to observe an increase in RBC count following EPO stimulation (Chang *et al.*, 2004c).

5.1.4 Iron deficiency and poor reticulocyte response during malaria

Analyses of data indicate that poor reticulocyte response in children with malarial anaemia was not due to iron deficiency (section 4.1.3). Hepcidin levels (a protein which regulate iron homeostasis and plays a key role in anaemia of chronic diseases) were high in anaemic *P. falciparum* infected children with poor reticulocyte response but in multivariate logistic regression analysis its increase was not associated with the risk of poor reticulocyte response (Table 10). Furthermore at this time hepcidin had no obvious relationship with iron deficiency. This observation makes an important facet to support the unique regulatory effect of erythropoietin on reticulocyte production in malaria cases of acute course. The effects of hepcidin on iron kinetics as well as reticulocyte responses would probably be noticeable in advanced or chronic malaria cases.

5.1.5 Iron deficiency modifies the level of serum soluble Transferrin Receptor

Children with clinical malaria had significantly lower plasma sTfR levels as compared to uninfected children as depicted in Figure 8. This observation is in line with previous studies (Williams *et al.*, 1999; Beesley *et al.*, 2000) but contrasts with the findings reported by Kuvibidila *et al* (1995) who reported that sTfR levels do not vary with malaria infection status. The deviation of the latter study may be attributed to iron deficiency, which was reported to be high in the study area (Zaire). Iron deficiency has a much stronger impact than erythropoietic

activity on serum sTfR levels. Serum sTfR is regarded as a reliable indicator of iron deficiency even in inflammatory conditions (Ahluwalia, 1998; Choi and Pai, 2003).

In the current study, iron deficiency was highly prevalent in uninfected children reaching 64% which coincided with higher sTfR levels whereas in *P. falciparum* infected children the prevalence of iron deficiency was low (10.67%). Furthermore iron deficiency modified the reticulocyte response among uninfected children such that children with iron deficiency were more likely to have poor reticulocyte responses than those without iron deficiency. Our findings therefore suggest that iron deficiency is a stronger determinant of serum sTfR levels than erythropoietic activity and therefore sTfR should be used as a marker of erythropoiesis during malaria cautiously. This is also supported by the finding that plasma sTfR levels were far higher in the uninfected non anaemic children with low erythropoietic activity, and lower in *P. falciparum* infected anaemic children in whom reticulocyte count was significantly higher.

5.1.6 Soluble transferrin receptor as an indicator of reticulocytosis during malaria

In our study there was no significant difference in sTfR levels between children with poor reticulocyte response and children with normal reticulocyte response as shown in Table 7, and the sTfR did not correlate with the reticulocyte count (Table 8). This observation is intriguing because the sTfR level in the absence of iron deficiency normally reflects erythropoiesis (Kohgo *et al.*, 1987, Huebers *et*

al., 1990, Beguin 1992), and therefore it was projected that the changes in reticulocyte count would be paralleled by changes in sTfR levels (R'zik *et al.*, 2001). This observation therefore suggests that reticulocytosis during malaria may not necessarily reflect the level of erythropoietic activity taking place in haematopoietic tissues, which is indicative of ineffective erythropoiesis that has been reported in malaria (Wickramasinghe and Abdalla 2000). The possible explanation for this suggestion is that during malarial anaemia, erythroid progenitor cells may be produced in response to anaemia but most of them do not develop to reticulocytes. Thus sTfR levels detected may be reflecting level of erythroblasts formation but not the reticulocyte counts in the peripheral blood, probably because the developing erythroblasts die or are prematurely phagocytosed in the haematopoietic tissues before they develop into reticulocytes (Wickramasinghe and Abdalla 2000).

Inadequacy in EPO production and high levels of inflammatory cytokines may be behind the ineffective erythropoiesis during malaria. EPO decreases the rate of death of erythroid progenitor cells in the bone marrow by preventing apoptosis (Koury and Bondurant 1990), thus inadequacy in EPO most likely halts the growth of these cells. High levels of inflammatory cytokines also interfere with the later stages of erythropoiesis. Observations from murine experiments show that recombinant human TNF-α induce a marked erythroid hyperplasia of late normoblasts but only a slight reticulocytosis in the peripheral blood suggesting ineffective erythropoiesis (Ulich *et al.*, 1990). This implies that in anaemic conditions with low EPO and high cytokine concentrations such as those with

poor reticulocyte response, the erythroid progenitor cells do not develop to fully functional erythrocytes despite the erythroid hyperplasia triggered by anaemia.

5.2 RED BLOOD CELLS MEMBRANE CHANGES

5.2.1 Relationship between cytokine levels and RBC membrane changes

The relationship between cytokine concentrations and changes in RBC surface molecules during malaria was investigated. There were no differences in Phosphatidylserine (PS) and IgG levels among *P. falciparum* infected children with various cytokine concentrations (Figure 12b). This implies that plasma cytokine levels had no effect on the modifications of RBC surface molecules contrary to the original concept that, TNF-α stimulates macrophages to generate reactive oxygen species (Greve *et al.*, 1999) that are involved in scrambling the RBC membranes during malaria. Nevertheless these findings suggest that factors other than cytokine imbalance may be mediating RBC membrane changes, possibly the parasite factors. Several lines of experiments have shown that the presence of the parasite in the cell or parasite products induces changes in the RBC membrane (Joshi and Gupta, 1988; Omodeo -Sale *et al.*, 2003). Among the parasite factors, hemozoin likely modifies cell membranes because its formation as well as its release during schizont rupture generates toxic radicals that have been implicated in damaging cell membranes in malaria (Dondorp *et al.*, 2003; Schwarzer *et al.*, 2003; Keller *et al.*, 2004).

Noteworthy, the current study demonstrated increase in PS levels in the anaemic children suggesting that PS exposure on the RBC surface may be mediating

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Noteworthy, the current study demonstrated increase in PS levels in the anaemic children suggesting that PS exposure on the RBC surface may be mediating

malarial anaemia. This suggestion is further supported by a positive correlation between PS and IgG in the anaemic children. IgG may became deposited on the surface of RBC as a result of exposure of PS. Externalization of PS on RBC membrane surface initiates binding of naturally occurring autoantibodies (Lutz 2004). Several studies also show that the levels of surface PS correlate with the propensity of the RBC to be bound *in vitro* by autologous monocytes and to be rapidly cleared *in vivo* by the spleen (Allen *et al.*, 1988; Connor *et al.*, 1994; Bonomini *et al.*, 2001). Other mechanisms that could lead to the simultaneous deposition of IgG and PS exposure on the RBC include host immunoglobulin targeting *P. falciparum* infected cells or malaria antigens attached to the RBC surfaces (Abdalla, 1986; Waitumbi *et al.*, 2000; Goka *et al.*, 2001). IgG may also target other surface molecules such as band 3 that are exposed in senescent and damaged red blood cells (Arese *et al.*, 2005). Erythrocytes opsonized by IgG and complement are recognized and phagocytosed by macrophages (Turrini *et al.*, 2003; Ensinck *et al.*, 2006).

5.2.2 Relationship between cytokine levels and RBC surface complement regulatory proteins

It was also investigated whether cytokine interactions during malaria would have an effect on the levels of RBC surface complement regulatory proteins (CrP). Among the CrP studied only CD59 showed a positive correlation with the TNF-α/IL-10 ratio. This increase may be attributed to the effect of TNF-α in the regulation of synthesis of CrP in the erythroid precursor cells or hepatocytes. Inflammatory cytokines have been shown to induce the expression of CrP in many

cells including chondrocytes, hepatocytes and cancer cells (Hyc *et al.*, 2003; Spiller *et al.*, 2000; Varsano *et al.*, 1998). Why only CD59 levels increased and not CD55 levels is not clear. However, Moutabarrik *et al.* (1993) pointed out that the up-regulation of surface CD55 requires prolonged treatment of cells with the stimulating agent such as cytokines. Possibly prolonged exposure to cytokines would have an up regulating effect on the expression of CD55 on RBC membranes as well.

Another explanation for this observation could be due to the differences in the complement regulatory activities between the two proteins. CD55 protects cells from surface deposition of autologous C3b being the first factor in the complement cascade, whereas CD 59 regulates the third downstream event in the complement cascade preventing the assembly of membrane-attack complexes. Consequently, in diseases like malaria which are associated with complement activation, CD55 is more likely to be consumed than CD59. This may also explain why anaemic children had significantly lower CD55 levels than non anaemic children.

5.2.3 Relationship between cytokine levels and parasitemia

Disproportionate increase in IL-10 over TNF-α was associated with parasitemia. This observation is consistent with other studies which show that the balance between pro and anti-inflammatory cytokines may be an important determinant of malaria presentation and outcome (Kurtzhals *et al.*, 1998; Othoro *et al.*, 1999). During acute malaria attacks, large amounts of proinflammatory cytokines such as

TNF-α and IFN- γ are released into the circulation. This T-helper type-1 biased immune response contributes either directly or indirectly to the killing of parasites. Also, studies show that IL-10 (an anti-inflammatory cytokine) mediated by the T-helper type 2-like response, is elevated during malaria (Wang *et al.*, 1994; Ho *et al.*, 1998). Positive correlations between IL-10 and parasitemia have been reported previously (Jason *et al.*, 2001, Issifou *et al.*, 2003; Hugosson *et al.*, 2004). Therefore the current observation suggests further that dysregulation of the cytokine balance may lead to malaria complications including hyperparasitemia.

5.2.4 Red cell age and red cell surface molecules in children with malarial anaemia

Results of this study confirm earlier findings that CD55 levels decreased on the surface of RBC in children with malarial anaemia (Waitumbi *et al.*, 2000; Stoute *et al.*, 2003) and suggest that CD55 loss begins early in the disease. Altered RBC age profiles during *P. falciparum* infections could therefore modify CrP levels, but this effect does not account for the decrease in CrP during malarial anaemia. This study focused on acute episodes of mild to moderate anaemia. Children were followed intensively with routine blood smears and clinical examination, and therefore chronic malaria cases were unlikely. The increased number of young RBC in anaemic children (Figure 14) demonstrates that these were acute and not chronic episodes as reported by Abdalla *et al.* (1980). Earlier studies focused on hospitalized children with severe malarial anaemia and presumably many of these were chronic cases (Waitumbi *et al.*, 2000). Although CD55 levels were higher in young RBC; the increase in young RBC seen during malarial anaemia in this

cohort did not lead to an overall increase of CD55 levels. RBC of all ages had lower CD55 levels in anaemic versus non-anaemic children (Figure 16a). These results indicate that newly formed as well as mature RBC have low CrP levels in anaemic children with malaria.

5.2.4.1 CD55 and CD59 decrease as RBC age

To date this is the first study to show that CD55 and CD59 levels decrease throughout the RBC lifespan as depicted in figures 16a and 16b. Earlier studies had reported differences in CR1 levels between reticulocytes and normocytes (Fishelson and Marikovsky, 1993; Miot *et al.*, 2002) and among normocytes of various ages (Ripoche and Sim, 1986). The progressive reduction in CD55 and CD59 molecules as RBC age suggests that non-pathological mechanisms exist to mediate CrP loss throughout RBC life. These mechanisms are not fully understood, though several studies suggest that surface CrP molecules are lost from RBC through vesicle formation and extrusion from the cell surface (Abdalla *et al.*, 1980; Miot *et al.*, 2002). Additional mechanisms may include proteolytic cleavage during transport and clearance of immune complexes from the RBC surface in the liver and spleen (Ripoche and Sim, 1986)

5.2.4.2 CrP loss is a selective process

The loss of RBC surface molecules appears to be a selective process during malarial anaemia. CD55 levels were lower in anaemic donors, whereas CD59 levels were not significantly different. An active process separate from physiological cell ageing may be taking place during malaria that preferentially

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removes specific RBC surface molecules. Probably the same mechanism responsible for physiological loss may be taking place during malaria but at an accelerated rate for CD55. A process resembling transfer reaction of CR1 has been proposed to explain the loss of CD55 on RBC during malaria (Craig *et al.*, 2005). During transfer reaction immune complexes are removed from RBC surfaces by phagocytes. In healthy individuals, RBC act as passive shuttles for the transport of complement-coated immune complexes from the circulation to the reticuloendothelial phagocytes in the liver and spleen.

Also, the differential loss of CD55 and CD59 may be related to their different roles in protection from complement attack. Activation of complement occurs in a step - wise fashion, and each regulatory protein acts at a different step in the cascade. CD55 acts at the initial enzymatic step to prevent the activation of C3 to C3b by accelerating the dissociation of the C3 convertase C4-2a and C3bBb (Nicholson-Weller and Wang, 1994; Wiesner *et al.*, 1997; Kuttner-Kondo *et al.*, 2001). CD59 prevents formation of polymeric C9 complex at the final step of MAC assembly (Rollins *et al.*, 1991). At least during acute malarial anaemia, CR1 and CD55 may sufficiently regulate the complement cascade to limit formation of MAC, thereby consuming CD55 and sparing CD59.

5.2.4.3 CD55 correlates with haemoglobin in children with malarial anaemia

In this study, levels of CD55 but not CD59 correlated with haemoglobin levels in anaemic children. This may indicate that CD55 loss is contributing to RBC loss. Erythrophagocytosis resulting from C3b deposition on RBC could explain the

concomitant decrease of CD55 and haemoglobin. Severe malaria is associated with elevated levels of circulating immune complexes (Mibei *et al.*, 2005), and CD55 could be adsorbing excessive amounts of these, which are subsequently transferred to macrophages (Craig *et al.*, 2005). The loss of RBC CD55 during this process could compromise its regulatory function and allow the deposition of opsonin C3b on RBC, leading to increased RBC destruction by the phagocytes in the reticuloendothelial tissues. Complement binding to RBC has been reported to be associated with macrophage activation and reduced haemoglobin in *P. falciparum* malaria (Goka *et al.*, 2001). Alternatively, direct lysis of RBC by membrane attack complex (MAC) could explain the concomitant decrease of CD55 and haemoglobin, but this seems less likely because CD59 did not decrease significantly during anaemia episodes in this or earlier studies which implies that complement mediated lysis is not the likely mechanism for RBC loss. CD59 is a principal regulatory protein of complement attack (Mibei *et al.*, 2005). Wiesner *et al.* (1997) found that despite the activation of all lytic complement factors, no complement-mediated lysis of RBC occurred in the presence of functional intrinsic CD59. Because CD59 levels do not change significantly during malarial anaemia, this may limit complement mediated lysis.

5.3 HEMOGLOBINOPATHIES AND G6PD DEFICIENCY

5.3.1 Population genetics of hemoglobinopathies and G6PD deficiency

The current study demonstrates that hemoglobinopathies including sickle cell and α^+ thalassemia are prevalent in the study area, where malaria is endemic similar to previous reports (Wellems and Fairhurst 2005). Haemoglobin C, which is common in malarious areas of West Africa (Agarwal *et al.*, 2000; Modiano *et al.*, 2001; Mockenhaupt *et al.*, 2004a; May *et al.*, 2007) was not detected in any of the study participants, suggesting that this genetic disorder is not common in East African region. This study demonstrates further that, of the α^+ thalassemia genotypes, the 3.7kb deletion is the commonest in this area as reported earlier by other investigators (Flint *et al.*, 1998; Enevold, *et al.*, 2007). The allele frequency of α^+ thalassemia and sickle cell in the study area fall within the reported range for East African coast region (Wellems and Fairhurst 2005; Wambua *et al.*, 2006; Enevold, *et al.*, 2007) and in other malaria endemic areas (Aluoch, 1997; Aidoo *et al.*, 2002). Also, the frequency of G6PD deficient alleles in this population falls within the reported frequency range for sub-Saharan African population (Luzzatto, 1973; Beutler, 1996; Ruwende and Hill, 1998).

High frequencies of hemoglobinopathies (α^+ thalassemia and Sickle cell) and G6PD deficient alleles in this region may reflect a natural selection due to protection from potentially fatal and severe malaria (Ruwende *et al.*, 1995; Mockenhaupt *et al.*, 2004b; Williams *et al.*, 2005a, 2005b; Guindo *et al.*, 2007). However, the influences of these inherited traits on the outcome of *P. falciparum*

infections were not addressed in the current study because it was beyond the scope of the study.

5.3.2 Relationship between RBC disorders and levels of serum soluble factors

5.3.2.1 Sickle cell trait and serum soluble factors levels before and during malaria

Results show that children with normal haemoglobin (HbAA) produced more inflammatory cytokines than sickle cell carriers (HbAS) and the differences were significant during first malaria attack (Table 14). These results may explain why children with normal haemoglobin type (HbAA) often suffer severe malaria than sickle cell carriers.

This is the first study to show variations in cytokine levels among children with different haemoglobin phenotype (HbAA and HbAS). This observation is interesting because cytokines play significant role in the clinical presentation and outcome of malaria infections (Day *et al.*, 1999; Clark *et al.*, 2004; Prakash *et al.*, 2006). Proinflammatory cytokines may increase cytoadherence of infected erythrocytes to endothelium through up regulation of adhesion molecules (Ho and White, 1999; Weiser *et al.*, 2007). High plasma levels of proinflammatory cytokines in the presence of low levels of anti-inflammatory cytokines have been also associated with severe malarial anaemia (Kurtzhals *et al.*, 1998; Othoro *et al.*, 1999), and high plasma TNF-α concentrations are associated with cerebral malaria (Akanmori *et al.*, 2000). Based on the current observation, children having sickle cell trait may therefore be protected from severe malaria because they produce

less inflammatory cytokines during malaria episodes than children with normal haemoglobin (HbAA). This suggestion is further supported by the current observation that children with HbAA had lower haemoglobin levels than HbAS children during malaria (Table 14).

Variations in the levels of cytokines among children with different haemoglobin phenotypes may be associated with the ability of HbAS individuals to limit parasitemia. Parasite-infected HbAS red blood cells tend to sickle and cluster red cell membrane protein band 3 (Friedman, 1978; Roth *et al.*, 1978; Hebbel, 2003), a process that may result in their premature removal by the phagocytes and limiting parasite multiplication (Friedman, 1978; Shear *et al.*, 1993). Parasitemia could be a strong determinant of cytokine production in malaria since in the current study parasitemia correlated positively with cytokine levels regardless of the haemoglobin type. Parasite factors such as hemozoin have been demonstrated to induce production of proinflammatory cytokines (Jaramillo *et al.*, 2004; Jaramillo *et al.*, 2005) and therefore could be responsible for high cytokine levels in HbAA children as observed in this study.

Alternative explanation for this observation is that sickle cell carriers could have acquired immunity to malaria infection at a younger age. Studies show that individuals with acquired immunity to malaria produce low levels of inflammatory cytokines during clinical malaria (Rhee *et al.*, 2001) and sickle cell carriers are likely to develop immune response earlier than HbAA children (Cornille–Brogger *et al.*, 1979) due to early phagocytosis of infected cells that

could enhance antigen processing and hence early immune response. However, this explanation may not apply to the children participating in the current study because the children were closely monitored for parasitemia from birth and the results presented here are for the children matched by age before and during first clinical malaria episode.

5.3.2.2 Alpha thalassemia and serum soluble factors levels before and during malaria

Among the serum soluble factors measured in the present study, only TNF-α and ferritin results were intriguing with regard to α-globin genotypes. The levels of these factors were significantly different between α-globin genotypes; being higher in the alpha thalassemic individuals before infection and lower during first malaria episode (Table 16). This finding is interesting because suggests that two different pathophysiological mechanisms may be operating in thalassemic individual depending on the *P. falciparum* infection status, and may be responsible for the clinical presentation of the disease.

At a steady state, thalassemic individuals have higher absorption rate of dietary iron presumably as a result of erythroid hyperplasia and ineffective erythropoiesis (Pippard *et al.*, 1979; Raja *et al.*, 1994), this may end up with high total body iron stores and probably iron overload (Van Wyck *et al.*, 1984; Mazza *et al.*, 1995). Thus, it is likely that thalassemic individual demonstrated high ferritin level probably because of higher total body iron. Higher serum ferritin levels in thalassemic individuals at a steady state have been reported elsewhere (Rees *et al.*,

1998). Slight increase of TNF-α in thalassemic subject could be a result of immunomodulation which have been reported to take place (Wiener *et al.*, 1996; Kyriakou *et al.*, 2001). In thalassemic patients, TNF-α increases along with IL-1 and IFN-γ (Wanachiwanawin *et al.*, 1999, Butthep *et al.*, 2002) most likely as part of a low grade inflammation that is demonstrated by the presence of activated endothelial cells (Kyriakou *et al.*, 2001; Butthep *et al.*, 1997; Butthep *et al.*, 2002).

An opposite trend observed during first malaria episode such that TNF-α and ferritin levels were lower in thalassemic individuals suggests that another mechanism which is different from that occurring during steady state may be taking place. It is possible that high levels of iron in thalassemic children may be influencing TNF-α production during infection. Excess iron is thought to have a pro-oxidant activity in promoting hydroxyl radical formation (Rosen *et al.*, 1995) and interferes with TNF-α factors, such as NF-κB which is sensitive to cellular redox status (Vlahopoulos *et al.*, 1999). Hydroxyl radical scavengers inhibit TNF-α production in lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells (Vulcano *et al.*, 1998) and in alveolar macrophages (O'Brien-Ladner *et al.*, 1998). Possibly, this mechanism occurs in thalassemic individuals during malaria because iron levels may be exceedingly higher in thalassemic patients being a result combined effect of hemolysis due to infection (Das *et al.*, 1999; Jakeman *et al.*, 1999; Price *et al.*, 2001) and thalassemia. In α-thalassemic individuals hemolysis occurs as a consequence of accumulation of excess unmatched β-globin in red cells (Weatherall, 1998). The results of this study are also interesting because during malaria episode, thalassemic individuals produced

lower TNF-α than their counterpart children having normal haemoglobin. This observation may explain the protective advantage against a range of clinical manifestations of severe malaria that is enjoyed by thalassemic individuals. We propose that lower levels of TNF-α and ferritin detected in thalassemic individual may contribute to lessen malaria severity. This is particularly important because local and systemic release of TNF-α has been implicated in the pathogenesis of severe malaria (Clark and Chaudhri, 1988; Clark and Cowden, 2004).

5.3.2.3 G6PD deficiency and serum factors levels before and during malaria

None of the serum soluble factors measured in this study varied in their levels on the basis of G6PD alleles before and during first malaria episode. This finding suggests two important things: Firstly G6PD alleles found in Muheza Tanzania do not differ in their ability to produce cytokines contrary to other studies (Liese *et al.*, 2002; Upperman *et al.*, 2005) which demonstrated variation in cytokines levels among G6PD alleles, the reason for this discrepancy is not known. Secondly, the basis of protection against malaria provided by G6PD deficient alleles (Ruwende *et al.*, 1995; Guindo *et al.*, 2007) is not associated with the varying capacity to produce cytokines and other serum factors measured in this study. Other mechanisms such as reduced rosetting which have been reported in other red cell polymorphisms including thalassemia, sickle cell and HbC may be considered instead (Carlson *et al.*, 1994; Fairhurst *et al.*, 2005).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Based on the observations of this study, it is concluded that poor reticulocyte response to malarial anaemia is a consequence of inadequate levels of erythropoietin in the circulation, and that the role of cytokines on suppressing reticulocyte production is insignificant according to multivariate logistic regression analyses. However, it would be prudent to defer affirmative conclusion on these observations pending to further verification that must be based on a larger sample size. Also, there is a need for a larger study that will elucidate which factors underlie inadequate EPO production in children with malarial anaemia. A better understanding of the cellular and molecular mechanism(s) behind inadequate EPO levels in some children with malarial anaemia will provide novel information for the development of effective preventive and/or treatment of malarial anaemia.

6.2 It has been demonstrated that cytokine levels are not associated with the RBC membrane surface changes during malarial anaemia with the exception of TNF-α/IL-10 ratio that was positively associated with CD59. Therefore it is concluded that RBC membrane surface changes are mediated by mechanisms not related to increased cytokine levels. Parasite factors such as formation and release of hemozoin have been proposed to explain the changes observed on RBC surfaces during malaria.

It is also concluded that RBC ageing is accompanied by loss of RBC surface complement regulatory proteins (CD55 and CD59), but this physiological process does not account for the selective CD55 loss seen in children with malarial anaemia. CD55 loss occurs early and is extensive in red cells of all ages. Furthermore, significant correlation between CD55 levels and haemoglobin levels demonstrated in children with malarial anaemia suggests that loss of CD55 is pathological and may contribute to the development of anaemia.

6.3 It has been demonstrated that genetic red cell disorders vary in their effects to modulate immune response in particular cytokine production. Therefore it is proposed that dysregulation of immune response by some genotypes may be influencing the outcome of *P. falciparum* infections and may explain for the protective advantage against severe malaria enjoyed by individual with genetic red cell disorders e.g. HbAS. However, it is not pertinent to draw a conclusive remarks based exclusively on the current observations, because none of the disease outcomes apart from haemoglobin level were addressed in this study. Further studies are therefore required to determine the relationship between cytokine levels and malaria outcomes in individuals with various genetic red cell disorders that are known to confer protection against severe malaria.

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APPENDICES

APPENDIX I: RETICULOCYTE PRODUCTION INDEX

The **Reticulocyte production index** is a calculation used in the diagnosis of anaemia.
The idea of the RPI is to assess whether the bone marrow is producing an appropriate response to an anaemic state.
The calculation is as follows:

Reticulocyte index = (reticulocyte count x corrected reticulocyte count)/ maturation correction

Corrected reticulocyte count = Hematocrit/Normal hematocrit

The normal ranges for hematocrit in children are dependent on age. The normal ranges are as follows:
Newborns: 55-68%
One week of age: 47-65%
One month of age: 37-49%
Three months of age: 30-36%
One year of age: 29-41%
Up to ten (10) years of age: 36-40%

Maturation correction: is to correct for the longer life span of prematurely released reticulocytes in the blood, a phenomenon of increased red blood cell production. This relies on a table:
Hematocrit (%).....Retic survival (days)/maturation correction
36-45.....1.0
26-35.....1.5
16-25.....2.0
15 and below.....2.5

Interpretation
The reticulocyte index (RI) should be between 1.0 and 2.0 for a healthy individual.
RI<2 with anaemia indicates decreased production of reticulocytes (Poor or inappropriate reticulocyte production)
RI>2 with anaemia indicates increased compensatory production of reticulocytes to replace the lost red blood cells (Normal reticulocyte production).

APPENDIX II: MULTIPLEX CYTOKINES ASSAYS

A 12-plex sandwich capture based assay kit and a 3-plex competitive binding based assay kit were used. For the 12-plex sandwich assay, 500ug of each detection antibody (IL-1, IL-4, IL-5, IL-6, IL-10, IFN-g and TNF-a from BD Pharmingen, San Diego, CA; IL-1, TNF-a Receptor I (TNF-RI), TNF-a Receptor II (TNF-RII) and leptin from R&D Systems, Minneapolis, MN; EPO from StemCell Technologies, Inc., Vancouver, British Columbia; ferritin from Maine Biotechnology Services, Portland, ME) was coupled to 6.25 x 10^7 micro-spheres (Luminex, Austin, TX) from unique bead regions using a carbido-amide linkage and standard coupling chemistries according to manufacturers instructions (Pierce, IL). Coupled beads for each of the analytes were pooled and aliquoted (200,000 beads/analyte/tube) into single use aliquots and stored in light tight boxes at 4 deg C. Standards (IL-1 IL-4, IL-5, IL-6, IFN-g and TNF-a from BD Pharmingen, San Diego, CA; IL-1, TNF-RI, TNF-RII, EPO and Leptin from R&D Systems, Minneapolis, MN; Ferritin from Fitzgerald Industries International, Concord, MA) were prepared at appropriate concentration as a single lot, aliquoted into single use tubes, and stored at -80 deg C. Detection antibodies were prepared by pooling biotinylated antibody for each individual analyte, aliquoting into single use aliquots and storing at -80 deg C. Controls were prepared by pooling purified standard for each individual analyte, diluting to an appropriate concentration (typically 500 pg/ml for cytokines), aliquoting into single use aliquots and storing at -80 deg C. Commercial controls for ferritin, and DHEAS (Bio-Rad, Irvine, CA) and EPO (Nichols Institute, San Clemente, CA) were stored in single-use aliquots at -80 deg C.

The 4 -plex competitive assay kit for DHEAS, sTfR, CRP and Hepcidin was developed in a similar fashion. However instead of coupling capture antibody to the beads; the analyte (DHEAS from Steraloids, Newport, RI; sTfR from Hytest, Turku, Finland, and CRP from Scripps Laboratories, San Diego, CA) was coupled. Biotinylated detection antibodies (DHEAS from Imochem, Christchurch, NZ; sTfR and CRP from Hytest, Turku, Finland) and controls were prepared as above.

All plates included a custom, pooled control containing all analytes as well as commercial controls with expected values for ferritin, EPO, and DHEAS. The assay kits demonstrated less than 2% inter-analyte interference and the median inter-assay CV was 15% as assessed with 48 replicate controls on consecutive plates. Assay runs were accepted as valid if: 1) results for commercial controls were within the proscribed range, 2) results for custom controls obeyed Westguard rules.

APPENDIX III: PCR PROTOCOL FOR ALPHA THALASSEMIA GENOTYPING

Each 50 µL reaction contained 20 mmol/L Tris-HCl pH 8.4, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mol/L betaine (SIGMA, St. Louis, MO), 0.2 µL of each primer, 0.2 mmol/L of each dNTP, 2.5 units of polymerase (Platinum *Taq*; Life Technologies, Gaithersburg, MD), and 100 ng of genomic DNA. Reactions were carried out on a thermal cycler (Genius; Techne, Cambridge, UK), with an initial 5-minute denaturation at 95°C, 30 cycles of 97°C for 45 seconds, 60°C for 1 minute 15 seconds, 72°C for 2 minutes 30 seconds, and a final extension at 72°C for 5 minutes. Following amplification, 10 µL of product was electrophoresed through a 1% agarose, 1 3 TBE gel at 5-6 volts/cm for 1 hour, stained in ethidium bromide, and visualized on an ultraviolet transilluminator.