ANALYSES OF CLASS AND SUBCLASS ANTIBODY OF CIRCULATING IMMUNE COMPLEXES IN CHILDREN WITH SEVERE *PLASMODIUM FALCIPARUM* MALARIA IN ENDEMIC REGIONS OF WESTERN KENYA

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DECLARATION

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

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DEDICATION

To my family; loving wife **Agnes**, our children **Percy, Ramsay**, **Purity and Jabez.**

To my parents, **Mr. Samwel Temuge** and **Mrs. Martha Temuge** for their care, concern, understanding, support and encouragement throughout my studies.

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ACRONYMS AND ABBREVIATIONS

IC – Immune complex

CIC – Circulating Immune Complex

Ig – Immunoglobulin

IgG – Immunoglobulin class G (Gamma)

IgM – Immunoglobulin class M (mu)

IgA – Immunoglobulin class A (alpha)

IgE – Immunoglobulin class E (epsilon)

CM – Cerebral malaria

SA, SMA – Severe malarial anaemia

C1q – Complement component 1q

PEG – Polyethylene glycol

WHO – World health organization

TNF – Tumour necrosis factor

RBCs – Red blood cells/erythrocytes

NK – Natural killer cells

NO – Nitric oxide

IFN – Inteferon

IL – Interleukin

Th1 – T helper subset 1

Th2 – T helper subset 2

VSA – Variant surface antigen

CSP – Circumsporozoite protein

MSP – Merozoite surface protein

LSA-1 –Liver stage antigen-1

SERA 1 – Serine rich antigen

PfEMP-1 - *Plasmodium falciparum* erythrocyte membrane protein-1

AMA-1 – Apical merozoite antigen

EBA-175 – Erythrocyte binding antigen-175

SSP-2 – Sporozoite surface protein-2

RAP – Rhoptry-associated antigen

RESA - Ring-infected erythrocyte surface antigen

CR1 – Complement receptor 1

ADCC – Antibody-dependent cell cytotoxicity

FcRs – Fragment cristalizable receptors, antibody receptors on cell surfaces

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

2-DE – 2-Dimensional gel electrophoresis

IEF – Isoelectric focussing

KDH – Kisii District Hospital

NPGH – Nyanza Provincial General Hospital

Ag – Antigen

ABSTRACT

Plasmodium falciparum infection is characterized by deadly complications such as severe malaria-associated anaemia (SMA) and cerebral malaria (CM).The exact mechanisms underlying pathogenesis of these severe forms of *Plasmodium falciparum* malaria are not fully understood yet they are associated with a lot of morbidity and mortality. Studies have shown a link between severe *P. falciparum* malaria and levels of circulating immune complexes (CIC) but the exact role of these CICs in the pathogenesis of severe *P. falciparum* malaria is still unclear. This study aimed to investigate the quantitative and qualitative differences in antibody classes and subclasses in serum immune complexes (ICs) between children with the severe forms of *P. falciparum* malaria and those with uncomplicated malaria as well as identifying the predominant *P. falciparum* antigens that contribute to IC formation in these clinical groups. A total of 75 children with SMA and 32 children with CM were enrolled from hospitals in western Kenya and matched with 74 and 52 control children respectively with uncomplicated symptomatic malaria. IC levels were measured using solid phase ELISA protocols and antibody classes and subclasses were identified using polyspecific sera for the classes or monoclonal antibodies for the subclasses. ICs were purified using polyethylene glycol (PEG) precipitation. The isolated ICs were dissociated by using an acidic buffer (Glycine-HCL pH 2.0). These were then electrophoresed on one-dimensional and two-dimensional polyacrylamide gel blotted by Western transfer and probed using human anti-*P. falciparum* antibodies. The study showed a general increase in levels of ICs as a result of *P. falciparum* infection in severe malaria cases and their symptomatic controls. Although IgG IC levels were elevated in children with severe malaria upon enrolment, children with CM had the highest levels of ICs for all the antibody classes. Conditional logistic regression showed a borderline association between IgG4-containing ICs and increased risk of SMA (OR = 3.11, 95% CI 1.01 to 9.56, P = 0.05). Total IgG-containing ICs (OR = 2.58, 95% CI 1.20 to 5.53, P < 0.02) and IgE-containing ICs (OR = 3.27, OR 1.38 to 7.78, P < 0.01) were associated with increased risk of CM. Six specific *P. falciparum* antigens were found to be associated with severe malarial anaemia while another three antigens were associated with cerebral malaria when compared to their specific controls. While when SA and CM where compared together, a 91Kda antigen was highly associated with SA (P < 0.01), while a slightly lighter antigen of about 87 Kda was significantly associated with CM (P < 0.01). These findings have demonstrated quantitative and qualitative differences in ICs in children with SMA and CM and this underscores the potential mechanisms of the pathophysiology of the disease. Furthemore the findings of this study suggested having higher IgG4-containing ICs is a risk factor for SMA while higher IgG and also IgE-containing ICs are both associated with CM pathology. This suggest that although SMA and CM were characterized by high levels of ICs, the class and subclass make up of these ICs as well as the role that they play in each may be distinct. This study demonstrated an association between malaria antigens and severity of the disease hence there is need for full characterization of the parasite antigens. These findings may contribute to a better understanding of the role of different antibody classes and subclasses in protective or damaging mechanisms and may provide new insights into development of effective malaria control strategies and vaccine development.

CHAPTER ONE: INTRODUCTION

1.1 Background information

Plasmodium falciparum malaria is the most lethal malaria parasite of humans. Most deaths occur due to complications such as severe malaria-associated anaemia (SMA) and cerebral malaria (CM). Severe malaria is a complex syndrome which is determined by factors from both the parasite and the human host (Jakobsen *et al.*, 1995). Individuals in all continents are potentially at risk, but the greatest suffering is experienced by people in the tropical countries. Annually an estimated 300-500 million clinical cases of malaria are reported and 1.5-2.7 million deaths occur worldwide mostly among children and a further 3.2 billion people at risk. Approximately 90% of the clinical cases occur in Africa (WHO 2005, WHO, 1998). The degree of endemicity varies between countries and even between different areas in the same country (Miller *et al*., 1994). In regions of very high endemicity, the greatest suffering is borne by children less than five years of age and pregnant women, whereas in areas of low endemicity the disease affects all age groups (WHO, 2000).

The patterns of pathology also differ with changes in degree of endemicity. In areas of high endemicity, although individuals older than five years of age continue to harbour malaria parasites, the frequency of disease is greatly reduced. This acquired clinical immunity in older children is never reached in regions where there is very low or seasonal exposure to malaria parasites (WHO, 2000). The status of the naturally acquired immune responses, which mostly comprises of humoral responses, is important since in endemic areas infants and young children exhibit the highest blood parasitemias and maximal pathology including the highest incidence of cerebral malaria (Chongsuphajaisiddhi *et al.*, 1986). In sub-Saharan Africa, non-immune individuals and

children bear most of the morbidity and mortality from complicated forms of malaria. The most common complications are severe anaemia and cerebral malaria (Breman and Campbell, 1988; Miller *et al.*, 1994). Other complications such as hypoglycaemia, renal failure and non-cardiogenic pulmonary oedema can occur singly or in any combination (Miller *et al*., 1994; Sahu *et al*., 2010).

Malaria has plagued mankind with a myriad of mechanisms for avoiding host immune responses. As such strategies against both the parasite and the disease are crucial so as to come up with better approaches of preventing the severe foms of malaria. Malaria infection leads to significant elevation of blood concentrations of immunoglobulins (Igs) and this interacts with the multiple malaria antigens leading to formation of immune complexes (ICs) (Duah *et al.*, 2010; Miller *et al.*, 1994). Immunoglobulins are vital in clearing and/or neutralizing infectious agents and are crucial in presenting antigen via Fc and/or complement receptors to antigen presenting cells (APCs). IC binding to Fc-receptors (FcRs) can trigger activatory and/or inhibitory signaling pathways that culminate in a well-balanced immune response (Nimmerjahn and Ravetch 2008). However, ICs are also associated with ill health in many diseases including malaria. The elimination of malaria parasites *in vivo* relies on cellular and antibody-mediated mechanisms directed against malarial antigens (Perlmann *et al.*, 1998; Riley *et al.*, 1992), and antibody response is the prominent part in immune response against malaria (Perlman *et al.*, 1998; Cohen *et al.*, 1971; Rosenberg *et al.*, 1973; Kibukamusoke and Voller 1970; Yone *et al*., 2005). Several asexual blood stage malaria antigens may be the target of protective immunoglobulins (Igs) and some of them have been included in malaria vaccine preparations in human beings (Long, 1993). The most important biological functions of antibodies are related to their effector functions aimed at inactivation or

removal of infectious agents and their products. Antibody based protective mechanisms form the basis of exposure-based acquired immunity and passive transfers of immunoglobulin gamma (IgG), have provided protection against *P. falciparum* blood stage in humans (Bouhraun-Tayoun *et al*., 1995).

Once formed, ICs may persist in circulation and be deposited in susceptible tissues and hence become pathological. A large body of evidence suggests that immune complexes form during malaria infection (Howard and Gilladuga, 1989; Adam *et al*., 1981; Jhaveri *et al*., 1997; Stoute *et al*., 2003; Mibei *et al*., 2005; Branch *et al*., 1998; Greenwood *et al*., 1978), and a possible role for CIC in the pathogenesis of severe anaemia (SA) and cerebral malaria (CM) has been suggested (Mibei *et al*., 2005; Adam *et al;* 1981, Jhaveri *et al*., 1997). However, the contribution of these complexes to the pathogenesis of severe malaria is not well understood.

The pathologies associated with ICs differ according to class/subclass of the antibody and size of the ICs and it is becoming apparent that the functional specificity of antibodies to malaria antigens may play an important role in the protective immune responses (Duah *et al*., 2010; Graux and Gysin 1990; Bouharoun-Tayoun *et al*., 1990; Bouharoun-Tayoun and Druilhe 1992). IgG subclasses differ in their effector functions and this is related to differences in structure especially the length and flexibility of the hinge region Graux and Gysin 1990). The flexibility is related to differences in biological properties where the Fab region of the IgG molecule combine with the antigen resulting in IC which triggers the effector mechanisms mediated via the Fc part of the IgG molecule. The four human IgG subclasses show differences in their interaction with Fc

gamma receptors (FcγR's), expressed on the effector cells like monocytes, macrophages myeloid cells and dendritic cells (Bouharoun-Tayoun and Druilhe 1992).

Binding of the Fc part of IgG to FcγR is instrumental in the induction of the cell's effector functions (Van de Winkel *et al.,* 1993; 1996). In this way FcγRs play a key role in bridging IgG and antibody activity and cellular effector mechanisms which include phagocytosis, endocytosis, antibody-dependent cell cytotoxicity (ADCC), complement activation, release of a range of inflammatory mediators, antigen presentation and clearance of ICs. Interaction between FcγR and IgG antibodies is pivotal in the immune response against infectious agents (Bredius *et al.,* 1994) and since IgG differs in their affinity to FcγR, clinical manifestation and their functioning will depend upon the properties of the FcγR as well as the isotype.

IgG1 and IgG3 have been shown to predominate in anti-parasite responses thus suggesting a role of these isotypes in protection against malaria (Bouhraun-Tayoun *et al*., 1995; Caprera *et al.,* 2004; Piper *et al.,* 1999). IgG1 and IgG3 are considered to be cytophilic hence protective against *P. falciparum* malaria while IgG2 and IgG4 are thought to be non-cytophilic and non-protective and are associated with blocking of protective mechanisms offered by IgG1 and IgG3 (Bouhraun-Tayoun *et al*., 1995). IgG2 may also have a possible role by activating macrophages through FcγRIIA (Aucan *et al*., 2000).

The relationship between IgE and CM especially on sequestration of parasitized red blood cells (PRBC) has been suggested (Perlmann *et al*., 1994), and it is thought IgE-IC might be inducing local overproduction of TNF-α, which plays a major role in CM

pathogenesis (Perlmann *et al*., 1994; Perlmann *et al*., 1999). IgA-Immune complexes have been shown to bind CR1, just like IgG-IC in patients with glomerulonephritis (Coppo *et al.,* 1989). It is felt that the same could be happening in severe malarial anaemia where there is interaction of IC and CR1 and lysis of RBC. Initial infection with *P. falciparum* malaria has been shown to preferentially stimulate synthesis of IgM antibodies (Abele *et al.,* 1965). In a study by Rosenberg *et al.,* (1973), and another by Brattig et al., (2008), rising titres of IgM antibodies to red blood cells (RBCs) were found in patients with *P. falciparum* parasites and this observation was not present in non-malaria patients with high IgM concentrations or in healthy controls. These studies confirmed the association between high IgM antibody level and malarial anaemia and highlighted their concurrent development.

Although a pattern of association has been established between ICs and *P. falciparum* infection, the exact composition of these complexes is not fully understood. So far, no clear pattern of association between isotypes and protection or severity of malaria has been demonstrated. This study aimed to further delineate the composition of the immune complexes in terms of the host antibody isotype involved in their formation. The antibody isotypic composition of the CIC was compared between children with complicated and uncomplicated *P. falciparum* malaria and correlated with disease activity in an effort to establish if there is a relationship. The study was also interested in investigating any qualitative differences between these two clinical groups that could explain their distinct clinical presentations.

1.2 Problem Statement

Plasmodium falciparum malaria infection is complex and leads to generation of ICs. The role of antibodies in malaria protection is not yet fully clear and the effect of each isotype is not fully understood and largely remains a controversial subject. The role of ICs in the pathogenesis of the most severe malaria complications has not been fully evaluated. ICs can lead to cell and end organ damage by their deposition on cell surfaces and by initiating complement cascade resulting in the deposition of complement activation products on erythrocytes and other organs such as kidneys. Also cross-linking Fc receptors on effector cells like macrophages and monocytes, ICs can stimulate the production of pro-inflammatory cytokines that have been proposed to have a role in the pathogenesis of severe malaria. Since presently there is no affordable, safe and effective malaria vaccine, there is a continuous need to understand how malaria causes morbidity and mortality in the hope that this knowledge may lead to more effective ways of preventing these complications. Recent studies showed high levels of CIC in children with severe malarial anaemia and cerebral malaria and possible roles these ICs play in modifying the malaria pathogenesis in favour of severe forms of the disease have been suggested. Children with severe malaria have been shown to have elevated levels of CICs but no clear difference between children with SMA and those with CM has been shown so far. It is known that antibody classes and subclasses differ in their bilogical and physiological properties hence ICs from patients with different forms of clinical malaria may differ in the amounts of antibody class and subclass components they contain.To further investigate the relationship between the high levels of CIC and severe malaria it is worth investigating the qualitative and quantitative differences of these ICs in an effort to unravel the mystery surrounding the mechanisms of severe *P. falciparum* malaria and to give insight into potential pathogenic mechanisms of disease.

1.3 Hypotheses

This study was guided by the following null hypotheses

H_o1: There are no quantitative and qualitative differences in the composition of ICs between children with severe malarial anaemia and cerebral malaria or between children with severe malaria and uncomplicated malaria.

H_o2: There are no differences in the antigens in ICs in children with severe malarial anaemia and cerebral malaria or those with uncomplicated malaria controls.

1.4 Objectives

1.4.1 General Objectives

The following were the general objectives for the study

i) To investigate class and subclass specific immune complexes ICs) in patients with severe malarial anaemia, cerebral malaria, and compare to age and gender-matched uncomplicated malaria controls.

ii) To investigate any association between immune complexes and severity of malaria and also to characterise the malaria antigens associated with ICs in children with severe malaria and their uncomplicated malaria controls.

1.4.2. Specific objectives

i) To measure specific immunoglobulin classes and subclasses in children with severe malaria and their age and gender-matched uncomplicated malaria controls.

ii) To establish an association between specific antibody class/subclass immune complexes with severity of malaria.

iii) To establish an association, if any, between specific *P. falciparum* antigens in IC formation and associated pathophysiology in severe malaria.

1.5 Significance and Anticipated Outputs

Clinical studies have shown that 10 – 20 % of severe malaria cases die despite prompt chemotherapeutic intervention (Brewster *et al.*, 1990; WHO, 2005). Therefore, there is a need for the development of strategies to prevent the most severe forms of malaria, which in most cases are attributed to *P. falciparum* (Miller *et al.*, 1994; WHO, 1998). A large body of evidence suggests that ICs are produced during malaria infection. However, their role in the pathogenesis of the most severe malaria complications has not been fully evaluated. Quantitative and qualitative analysis of these CICs in patients with complicated and uncomplicated malaria will bring to light the exact composition and nature of the most putative ICs, which are linked to development and severity of *P. falciparum* malaria and will also enable the correlation of the host antibody class/*P. falciparum* antigen type with the severity of the disease.

Malaria is a complex disease and involves many inflammatory responses and identification of the composition of the ICs is paramount as it will guide purification of specific antigens which elicit higher levels of protectiveimmune responses. A clear understanding of factors that contribute towards pathological events associated with severe disease or those which contribute to protective responses is a vital requirement for the development of effective therapeutic and prophylactic approaches to severe *P. falciparum* malaria and will contribute to strategies to manage complicated malaria cases. The findings will contribute in identifying the possible vaccine candidate parasite antigens and will contribute to shedding more light on the nature of *P. falciparum* severe malaria pathogenesis and also in development of novel strategies to manage the severe forms of malaria like immunomodulation strategies to block the damaging pathways associted to a particular antibody class or subclass.

CHAPTER TWO: LITERATURE REVIEW

2.1 Clinical manifestation of *Plasmodium falciparum* malaria

Malaria in children differs from that in adults in terms of its varied manifestations and higher mortality especially in under-five age group (Bhave, 1996; Bruce-Chwatt, 1985; Sahu *et al*., 2010). Varied and rare manifestations pose a diagnostic challenge hence a need to keep a high index of suspicion for the diagnosis of malaria in children (Bhave, 1996; Dass *et al*., 2010). The common mode of presentation of fever, headache, body ache, anaemia and splenomegaly is seen only in about 50% of cases in which the diagnosis is easy especially when peripheral smear done in such cases is positive for malaria parasites (Bhave, 1996; Dass *et al*., 2010). The clinical presentation in a child is very much dependent on the immune status of the child. Children who have well developed immunity to malaria have fewer symptoms and less severe manifestations in spite of heavy parasitaemia Miller *et al.*, 1994). Children with poor or no immunity to malaria tend to have severe manifestations and a higher degree of pyrexia (Bhave, 1996). The symptoms also depend on the physiological diversity of the *in vivo* biology of the malaria parasite (Daily *et al.*, 2007).

Malaria-associated pathology is exclusively restricted to the asexual stages, especially asexual replication of the parasite within the erytrocyte. In the non-immune patients, *P.falciparum* infections are usually characterized by severe complications such as cerebral malaria (CM) and severe anaemia (SA) (Breman and Campell, 1998; Miller *et al*., 1994). However, other complications such as hypoglycaemia, renal failure and non-cardiogenic pulmonary oedema can occur singly or in any combination (Miller *et al*., 1994). Studies in malaria holoendemic area of western Kenya showed that children aged less than two years old experience the greatest burden of disease, with SA being the major

cause of malaria related mortality (Bloland *et al*., 1999) This supports the earlier studies which showed that SA is a predominant complication in areas of high endemicity where people are bitten by hundreds of infected mosquitoes per year (Miller *et al*. 1994; Lacritz *et al*., 1992) with peak incidence being 6.24 months (Snow *et al*., 1997). Another curious observation is that SA decreases after three years of age despite the continued infection in the population and at a time when the incidence of CM is increasing in the non-endemic regions (Miller *et al*., 1994).

Cerebral malaria is more common in areas with low endemicity with peak incidence in 3-4 year old children (Marsh, 1992). Studies by Marsh and Snow (1999) reported that the clinical manifestation of severe *P. falciparum* infection is dependent on the parasite virulence, host genetic factors and age beside the intensity of transmission and physiological changes like during pregnancy where the host defence against infection is altered (Karnad, 1996). The clinical manifestation of severe malaria such as hypoglycaemia, convulsions and SA are relatively common among children (Molyneaux *et al*., 1989; Marsh *et al*., 1995). White (1996) reported that acute renal failure, jaundice and pulmonary oedema occur more frequently in adults, while CM (coma), shock and acidosis, which often terminate in respiratory arrest, can occur at any age.

2.2 Immunity to malaria

Studies in malaria endemic areas have revealed a difference in susceptibility to *P. falciparum* malaria and also in the extent of pathology. Such differences are attributed to differences in the level of immunity either innate or acquired in the population (Duah *et al.*, 2010).

2.2.1 Innate immunity to malaria

Natural resistance to malaria based on inherited traits, which offer resistance, has been observed in some individuals living in malaria endemic areas. Several genetic polymorphisms, especially those involving red blood cells (RBCs), have been observed to be linked with resistance to *P. falciparum* infections. This could be representing a survival strategy evolved in the population due to selective pressure by the malaria parasite. Several studies have showed that the complete absence of Duffy glycoprotein on the surface of RBC confers protection against *P. vivax* (Miller *et al*., 1976, Neil, 1997, Miller, 1994). This is because this species requires Duffy glycoprotein as a receptor for merozoite invasion. Sickle cell haemoglobin (Hbs) trait has also been shown to confer resistance. Previous investigators working on transgenic mice expressing sickle cell trait showed that the mice were protected from rodent malaria and rodent cerebral malaria respectively (Shear *et al*., 1993; Kaul *et al*., 1994; and Hood *et al.,* 1996). The heterozygous sickle cell trait in humans (HbAS) had been reported to confer resistance against *P. falciparum* malaria (Allison, 1964). Studies by Hill *et al*., (1991) in Gambia and by Marsh (1992) in Kenya showed that HbAS provided more than 90% protection from both CM and SA in children.

The thalassemias, involving defects in synthesis of the α- or β-globulin chains, have also been shown to be associated with some form of protection. Willcox *et al*., (1983) reported a 50% reduction in the risk of clinical malaria among Liberian children with β-thalassaemia while α-thalassaemia was associated with a highly protective effect against the serious complication of *P. falciparum* malaria (Allen *et al*., 1997). A clear mechanism underlying these observations is not known but it has been suggested that the efficient immune clearance by phagocytosis in thalassaemias could be a contributory

factor (Brockelman *et al*., 1987). Glucose-6-phosphate dehydrogenase deficiency, which is an enzymopathy of RBC, has also been suggested to be associated with protection against *P. falciparum* malaria (Giribaldi *et al*., 1992; Ruwende *et al*., 1995). Furthermore, human ABO blood groups have been suggested to be associated with resistance to *P falciparum* malaria with persons with blood group O being relatively resistant to rossetting, a mechanism associated with disease severity (Rowe *et al*., 1995; Barragan *et al*., 2000; Rowe *et al*., 2007).

There are forms of innate immune mechanisms that aid in checking the growth of the parasite in the system prior to development of acquired immunity. Here immune cells including natural killer (NK) cells and neutrophils play some important roles in eliminating the parasite. NK cells have been reported to effect their function by lysing the *P.falciparum* schizont-infected RBCs (Orago and Facer, 1991) while neutrophils and macrophages phagocytose the parasite forms (Kumaratilake *et al*., 1994). Macrophages have also been shown to produce cytokines, mainly TNF-α and NO which have anti-parasitic effects (Dugas *et al.*, 1995).

2.2.2 Acquired immunity

The development of acquired immunity depends on repeated exposure to the parasite (Snow *et al*., 1997). Immunity to malaria only occurs after many years of recuring infections. This is believed to be due to antigenic variation and the time taken by individuals to develop immunity to invariant parts of otherwise very polymorphic antigens (Snow *et al*., 1997). Immunity to malaria manifests as milder disease symptoms and lower parasitaemia. In areas of high endemicity where individuals are continuously exposed to the parasite, although individuals after five years of age continue to harbour

parasites, the frequency of the disease is greatly reduced. This clinical immunity is never achieved in regions where there is low or seasonal exposure to malaria parasites (WHO, 2000; Ferreira *et al*., 1996; Snow *et al*., 1997). Acquired immunity to *P. falciparum* parasite involves cellular and humoral immune responses and also involves anti-parasite and anti-disease processes (Baird, 1995). It is largely dependent on host age (McGregor, 1987; Marsh, 1992), genes, level of parasite transmission, parasite stage and parasite species (Marsh, 1992).

2.2.2.1 Immunity to pre-erythrocytic stages

Early studies reported that sterile immunity against sporozoites challenge could be induced by immunization using irradiated sporozoites (Edelman *et al*., 1993; Hoffman *et al*., 1996; Nussenweig and Nussenweig, 1989). It was observed this protective immunity required persistent liver parasites for it depends on the ability of the live irradiated sporozoites to penetrate liver cells and transform into uninucleated liver trophozoites (Scheller and Azad 1995; Druilhe and Marchand, 1989). *In vitro* antigen-specific monoclonal antibodies have been shown to inhibit the invasion and development of *P. falciparum* in human hepatocytes. Another observation is that passive immunization with anti-sporozoite antibodies protects mice and monkeys against *Plasmodium* species sporozoite-induced malaria (Good and Doolan, 1999), thus indicating a role for antibodies in protection.

Studies on adoptive transfer of immune cells have shown that pre-erythrocytic immunity mainly depends on $CD8^+$ T cells (Good and Doolan, 1999; White *et al*., 1996; Doolan and Hoffman; 1999; Weiss *et al*., 1988). Adoptive transfer studies in naïve and β-2-microglobulin deficient (-/-), mice as well as depletion studies have revealed that the

parasite antigens expressed on the surface of infected host hepatocytes in context with class I MHC molecules are the major targets for protective immunity against the parasite pre-erytrocytic stage (Good and Doolan 1999; White *et al*., 1996). Studies by Doolan and Hoffman (1999) confirmed that $CD8^+$ T cells play a critical role in triggering a mechanism of adoptive immunity dependent on interferon-gamma (IFN-γ), nitric oxide (NO), interleukin-12 (IL-12) and in part on natural killer (NK) cells. IFN- γ inhibit in a dose-dependent manner the development of pre-erythrocytic forms in the liver of mice challenged with *P. berghei* sporozoites (Ferreira *et al*., 1986). Furthermore, administration of monoclonal anti-IFN- γ antibody to an immune host was shown to abrogate protective immunity (Schofield *et al*., 1997). Another study by Sedegah *et al*., (1994) was able to demonstrate that an IFN- γ-dependent recombinant IL-12 (rIL-12) induced protection against *P. yoelii* sporozoite challenge in mice and suggested that rIL-12 stimulates non-B, non-T cells to produce IFN- γ that kills intra-hepatic parasites by stimulating NO production. A role had also been pointed for $CD4^+$ T cells. Wang *et al*., (1996) reported protection against *Plasmodium yoelii* which was CD4+ T cell and IFN- γ-dependent following immunization with linear peptides from sporozoite surface protein-2 /thrombospondin related adhesive protein (SSP2/TRAP).

2.2.2.2 Immunity to erythrocytic (Blood) stages

Early studies in West Africa demonstrated that blood stage protective immunity could be achieved in children by the transfer of IgG fractions of sera from adults (Cohen *et al*., 1961). A critical role has been proposed for cytophilic antibodies (IgG1, IgG2 and IgG3) subclasses. This is supported by the observation that passively transferable protection against experimental *P. yoelii* infection in mice has been associated with cytophilic IgG2A and IgG3 subclasses (White *et al*., 1991; Lilian *et al*., 1998).

Furthermore, protected individuals have been reported to have predominantly cytophilic antibodies IgG1 and IgG3, while on the other hand, unprotected children have non-cytophilic IgG2 or IgM classes or low levels of malarial antibodies overall (Philips 1994; Sarthou *et al*., 1997). To add on this, in malaria endemic areas age-dependent increase in level of parasite specific IgG1 and IgG3 subclasses activity has been shown to be closely associated with reduced susceptibility to as well as increased chances of recovery and survival from severe *P. falciparum* malaria (Aribot *et al*., 1996; Sarthou *et al*., 1997). *In vitro* studies (Bouharoun-Tayoun *et al*., 1990; Fell and Smith, 1998) showed that cytophilic antibodies act in cooperation with monocytes and macrophages to inhibit blood stage malaria parasites. The same was also evident in parasite killing effector mechanisms like opsonization (Groux and Gysin, 1990) and phagocytosis (Urquhart, 1994) of infected erythrocytes. The mechanisms involved in antibody-mediated protection are by the cytophilic antibodies exerting anti-parasite effect through antibody-mediated cellular cytotoxicity (ADCC). There is an enhanced *in vitro* phagocytosis by neutrophils in the presence of type 1 cytokines and anti-merozoite surface antigen-2 (MSA-2) (Lakshmi and Antonio, 2000).

It has been proposed that the interaction between MSAs and IgG1 and IgG3 cytophilic antibodies bound to monocytes through the FcγRII stimulates the secretion of tumor necrosis factor-alpha (TNF-α) that inhibits *P. falciparum* intra-erythrocytic development (Bouharoun-Tayoun *et al*., 1995). Aucan *et al*., (2000) suggested a protective role for IgG3 and IgG 2, they may activate effector cells through FcγRIIA and this provides evidence for the blocking role of IgG4 in *P. falciparum* malaria. Cytokines have also been shown to be major mediators of immunity as well as pathogenesis of human malaria due to blood stage parasites. Cytokines such as IFN-γ, TNF-α, TNF-β, IL-

1 and granulocyte colony stimulating factor (GCSF) are capable of enhancing anti-parasite action of blood monocytes and neutrophils, while IL-4 inhibits monocyte/macrophage-mediated killing of *P. falciparum in vitro.* (Kumaratilake and Ferrante, 1994). Cytokines have also been reported to mediate interaction between humoral and cellular immune responses during malaria infections (Cruz Cubas *et al*., 1994).

Studies in mice have shown that susceptibility and resistance to blood-stage malaria is related to the differential activation of $CD4^+$ T-cells of T-helper type 1 (Th1 and T helper type 2 (Th2). It has been suggested that $CD4^+$ T-cells and neutrophils may account for the naturally acquired reduction of gametocytes infectivity following a blood meal (Lensen *et al*., 1997). Antibodies against some of the surface proteins like Pfs230 induce ADCC (Williamson *et al*., 1995).

2.2.3 Age and immunity to malaria

In malaria endemic areas, both the prevalence and severity of malaria infection decreases with age. However multiple infections with malaria do not confer long lasting, sterile protective immunity. This immunity to malaria is acquired more rapidly by adults than children and it requires repeated infections (Baird, 1995). Studies done in Senegal by Rogier and Trape (1993) on *P. falciparum* among children, reported similar levels of protection among children of the same age group suggesting that the acquisition of clinical protection in areas where malaria is endemic involves a progressive and homogenous decrease of the possibility of having a malarial attack.

Individuals who are repeatedly exposed to malaria develop antibodies against many sporozoite, liver-stage, blood-stage and sexual stage malaria antigens. It is thought that antibodies acting against sporozoites, liver-stage and blood-stage organisms are responsible for the decreased susceptibility to malaria infections and disease seen in adults in malaria endemic areas, and that, antibodies against the sexual stages of *Plasmodium* may reduce malaria transmission. There is an age-related decrease in the frequency and density of parasitaemia related to pubertal development (Kurtis *et al*., 2001). Studies have also shown age-related changes and the apparent difference in the pattern of IgG isotype antibodies induced predominantly by variant antigens expressed by parasite isolates with different origins hence suggesting differences could lie at the epitope level.

Age is a strong influence on parasite specific IgG subclass antibodies hence age seems to be the major factor associated with specific isotype distribution (Aribot *et al.*, 1996), especially on IgM, IgG2 and IgG3. IgG2 and IgG3 against conserved epitopes have been shown to increase with age (Aucan *et al.*, 2000). Development of protective immunity may also be due to the gradual acquisition of specific immunity to most of the parasite strains found in a given population (Marsh and Howard, 1986). Also individual factors that change with age independent of cumulative effects of repeated exposure may govern the degree of naturally acquired immunity (Baird, 1995).

2.3 Malaria infection and generation of CIC

Soluble malarial antigens as well as antibodies to various plasmodial constituents have been demonstrated in the sera of patients infected with either *P. falciparum* or *P. malariae* (Shepherd *et al*., 1982, Adam *et al.,* 1981; Blackman and Holder 1992; Camus and Hadley 1985; Jakobsen *et al.,* 1993). These antigens may be available for binding by reactive antibodies (Mohamed, 1982). This sets the stage for the formation of CICs (Jhaveri *et al.,* 1997, Tyagi and Biswas 1999) and i*n vivo* complement activation leading to hypocomplementamia which are more common in patients with malaria complications such as severe anaemia, cerebral malaria and thrombocytopenia of *P. falciparum* (Adam *et al.,* 1981). Autoantibodies like rheumatoid factor (RF), anti-ssDNA and anti-dsDNA have been observed in many patients with acute malarial infections suggesting that CICs may have in part an autoimmune nature (Jhaveri *et al.,* 1997). This autoimmune nature of malaria might be due to non-specific polyclonal stimulation of B- cells leading to emergence of different kinds of antibodies by the same infection (Jhaveri *et al*., 1997). These observations suggest that the intensity of the immune response and the associated complement activation may be important factors in the pathogenesis of malaria. In addition to complement activation, ICs can stimulate macrophages to secrete pro-inflammatory cytokines (Jarvis *et al*., 1997; Virella *et al.,* 1995) both of which can contribute to the pathogenesis of severe malaria (Abdalla *et al*., 1983; Clark and Cowden, 1992).

The fate of CICs is determined by host phagocytic system represented by circulating leukocytes and the reticuloendothelial system (RES). Overload of this system, blockade of Fc and complement receptors, or deficiency in complement receptors like CR1 present on mononuclear phagocytic cells leads to immune complexes persisting in

circulation. ICs then deposit in various organs like kidneys, vascular endothelium, choroid plexus, synovium and others (Theofilopoulus, 1980). This may be important in precipitating immune complex-mediated diseases or pathologies for they can be secondarily deposited in other susceptible tissues like blood vessels, gromeruli, liver, spleen, nerves, and synovial fluid among other tissues (Theofilopoulus, 1980; Gomez-Guerrero *et al.,* 2002). Immune complexes also activate complement system through both the classical and alternative pathways. Once the complement system has been activated, several biological activities including immune adherence, increased vascular permeability and inflammation, which are implicated in severe *P. falciparum* malaria complications, are generated that play a role in immune complex-induced pathologies (Gomez-Guerrero *et al.,* 2002). Studies in mice (Mitchell, *et al*., 1987), monkeys (Cornacoff, *et al*., 1983) and in humans (Adam *et al.,* 1981; Greenwood *et al.,* 1978; Jhaveri *et al.,* 1997; Stoute *et al.,* 2003; Mibei *et al.,* 2005) have shown a link between levels of ICs and severity of malaria infection.

2.4 Antibody generation and interaction with *Plasmodium* antigens contribute to acquisitio of immunity to malaria

Plasmodium falciparum infection leads to generation of antibodies of various classes and subclasses. These antibodies, together with other components of the immune system, comprise the first line of defence to fight the infection and confer some form of immunity. The antibodies generated will be of different specificities depending on the particular *P. falciparum* antigen that stimulated their synthesis. The generated antibodies will be of different classes/subclasses. *P. falciparum* specific IgM, IgG1 – IgG4 and IgE antibodies have been demonstrated in sera of malaria patients and inhabitants of regions where malaria is endemic (Desowitz, 1989; Wahlgren *et al.,* 1983; 1986). Antibody-

mediated protection depends on both quality (specificity and affinity), and concentration of the relevant antibodies and the balance between the different Ig classes and subclasses (Beck *et al.,* 1995; Aribot *et al.,* 1996; Ferrante and Rzepczyk, 1997), some classes being beneficial while others being damaging or unprotective.

Immunoglobulin classes and subclasses differ in their ability to interact with Fc receptors on macrophages, to activate the complement cascade, and to stimulate pro-inflammatory cytokines (Foreback *et al.,* 1997; Groux and Gysin, 1990; Lucisano Valim and Lachman 1991). The antibodies mainly comprises of IgG and IgM (Branch *et al.,* 1998; Rossenberg *et al.,* 1973; Alonso *et al.,* 1998; Kusuhara *et al.,* 2000). IgE has also been shown to be elevated in malaria infection (Maeno *et al.,* 2000; Verra *et al.,* 2004). IgE was also shown to be associated with cerebral malaria (Perlman *et al*., 1994). The anti malarial antibodies are generated against a host of *P. falciparum* antigens which are stage-specific in malaria infected individuals and some of which have been shown to correlate with clinical immunity to the disease. Such *P. falciparum* antigens includes; variant surface antigens (VSA) and anti-VSA antibodies have been shown to be protective (Kinyanjui *et al.,* 2004).

Other malaria antigens are; Circumsporozoite protein (CSP), Sporozoite surface protein 2 (SSP2), Liver stage-specific antigen-1 (LSA-1), Merozoite surface protein 1 (MSP-1) (Holder and Freeman 1984), Merozoite surface protein 2 (MSP-2) (Smythe *et al*., 1988), Erythrocyte binding antigen 175 (EBA-175) (Camus and Hadley 1985), Ring-infected erythrocyte surface antigen (RESA) and Serine repeat antigen (SERA). Others include, Apical merozoite antigen (AMA-1) (Peterson *et al*., 1989), Histidine rich protein 2 (HRP-2), Rhoptry-associated proteins (RAP-1 and RAP-2) and Erythrocyte membrane

proteins 1 (PfEMP1) (Bull *et al*., 1988; 2002). Most of these *P. falciparum* antigens are malaria vaccine candidates and others have been tried out. Examples of the leading blood stage vaccine candidates that target invasion of RBCs include: MSP-1 (Holder and Blackman, 1994; Kumar *et al*., 1995; Egan *et al*., 1996), MSP-2 (Sturchler *et al*., 1995), 175-kDa erythrocyte binding antigen (EBA-175) (Sim *et al*., 1990), apical membrane antigen-1 (AMA-1) (Collins *et al*., 1994), rhoptry associated antigen-1 (RAP-1) (Ridley *et al*., 1990), synthetic peptide including MSP-1 and CSP sequence (SPf66) (Valero *et al*., 1993; Alonso *et al*., 1994; D'Allessandro *et al*., 1995; Nosten *et al*., 1996). Those that target development of the parasite within the RBC include: *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) (Baruch *et al*., 1995; Smith *et al*., 1995), ring-infected erythrocyte surface antigen (RESA, Pf155) (Collins *et al.,* 1986) and serine-rich antigen (SERA) (Inselburg *et al*., 1991).

Individuals who are repeatedly exposed to malaria develop antibodies against many sporozoite, liver-stage, blood-stage and sexual stage malaria antigens. It is thought that antibodies acting against sporozoites, liver-stage and blood-stage organisms are responsible for the decreased susceptibility to malaria infections and disease seen in adults in malaria endemic areas, and that, antibodies against the sexual stages of *Plasmodium* may reduce malaria transmission. IgG plays a crucial role in host defence against erythrocytic stages of *P. falciparum* since passive transfer of IgG from immune African adults to children was found to kill malaria parasites (Cohen *et al.,* 1961; Bouharoun-Tayoun *et al.,* 1990). This kind of immunity is primarily by antibodies that target the erythrocytic stage of the malaria life cycle.

In a malaria vaccine trial in Tanzania, it was found out that the prevalence of naturally acquired IgG antibodies recognising malaria antigens was high and increases with age (Alonso *et al.,* 1998). The study showed IgG antibodies against soluble *P. falciparum* 66 (SPf66, which is a derivative of CSP), NANP, (a derivative of MSP, P 190) and Merozoite surface protein-1 (MSP-1 19kD) antigens were high and increases with age. This reflected the high level of natural exposure of the children to *P. falciparum*. There has been difficulty in establishing whether such antibody responses are related to protection in field trials in endemic areas. SPf66 induce IgG antibodies, which mediate protection against clinical malaria. Serum from SPf66 vaccinated individuals has been shown to inhibit parasite growth *in vitro* (Salcedo *et al.,* 1991).

Initial infection with *P. falciparum* malaria has been shown to preferentially stimulate synthesis of IgM antibodies (Abele *et al.,* 1965). In a study by Rosenberg *et al.,* (1973), rising titres of IgM antibodies to red blood cells (RBCs) were found in patients with *P. falciparum* parasites and this observation was not present in non-malaria patients with high IgM concentrations or in healthy controls. This study confirmed the association between high IgM antibody level and malarial anaemia and highlighted their concurrent development. In the most anaemic malaria patients it was also shown that serum C3 was lowest and survival time of normal RBCs was reduced (Rosenberg *et al.,* (1973). This has got a bearing on pathogenesis of *P. falciparum* malarial anaemia. In endemic areas, patients who have been repeatedly exposed to malaria since birth develop protective IgG antibodies. When passively transfused to children, these antibodies afford clinical improvement of naturally acquired malaria (Cohen *et al.,* 1961). While in the tropics, high serum levels of IgG and IgM occur frequently and are often attributable to endemic

malaria (Kibukamusoke and Voller 1970; McFerlane and Voller, 1966; McGregor *et al.*, 1970; Targett 1970).

Early studies in West Africa demonstrated that blood stage protective immunity could be achieved in children by the transfer of IgG fractions of sera from adults (Cohen *et al*., 1961). A critical role has been proposed for cytophilic antibodies (IgG1, and IgG3) and IgG2 subclasses. This is supported by the observation that passively transferable protection against experimental *P. yoelii* infection in mice has been associated with cytophilic IgG2A and IgG3 subclasses (White *et al*., 1991; Lilian *et al*., 1998). Furthermore, protected individuals have been reported to have predominantly cytophilic antibodies IgG1 and IgG3, while on the other hand, unprotected children have non-cytophilic IgG2 or IgM classes or low levels of malarial antibodies overall (Phillips 1994; Sarthou *et al*., 1997). To add to this, in malaria endemic areas age-dependent increase in level of parasite specific IgG1 and IgG3 subclass activities have been shown to be closely associated with reduced susceptibility to as well as increased chances of recovery and survival from severe *P. falciparum* malaria (Aribot *et al*., 1996; Sarthou *et al*., 1997).

Healthy adults in Gabon were shown to harbour anti-VSA IgG2 and IgG3 antibodies while healthy children had predominant IgG3 and IgG4 responses (Cabrera *et al*., 2004). *In vitro* studies (Bouharoun-Tayoun *et al*., 1990; Fell and Smith, (1998) showed that cytophilic antibodies act in cooperation with monocytes and macrophages to inhibit blood stage malaria parasites. The same was also evident in parasite killing effector mechanisms like opsonization (Groux and Gysin, 1990) and phagocytosis (Urquhart, 1994) of infected erythrocytes. The mechanisms involved in antibody-mediated protection are by the cytophilic antibodies exerting anti-parasite effect through

antibody-mediated cellular cytotoxicity (ADCC). It has been shown that there is an enhanced *in vitro* phagocytosis by neutrophils in the presence of type 1 cytokines and anti-merozoite surface antigen-2 (MSA-2) antibodies (Lakshmi and Antonio, 2000).

Although naturally acquired antibodies from clinically immune individuals belong to the IgG1 and IgG3 subclasses (Bouharoun-Tayoun *et al*., 1995), there is little correlation between levels of specific antibodies and resistance to infection or to clinical disease since the parasite itself can initiate, in an immune evasion strategy, synthesis of high-titre low affirnity antibodies leading to hypergammaglobulinaemia (Miller *et al.,* 1994, Anders *et al*., 1988). The role of antibodies in protective immunity to malaria is not completely understood but it is though to be relying on various mechanisms including inhibition of merozoite invasion of erythrocytes (Quinn and Wyler 1979), antibody-mediated phagocytosis via Fc receptors (FcRs), and complement (Pleass *et al.,* 2003) and an ab-dependent cellular inhibition (Bouharoun-Tayoun *et al.,* 1995; Badell *et al.,* 2000).

It has been proposed that the interaction between MSAs and IgG1 and IgG3 cytophilic antibodies bound to monocytes through the FcγRII stimulates the secretion of tumor necrosis factor-alpha (TNF-α) that inhibits *P. falciparum* intra-erythrocytic development (Bouharoun-Tayoun *et al*., 1995). Aucan *et al*., (2000) suggested a protective role for IgG3 and IgG2 that they may activate effector cells through FcγRIIA and this provides evidence for the blocking role of IgG4 in *P. falciparum* malaria. Cytokines have also been shown to be major mediators of immunity as well as pathogenesis of human malaria due to blood stage parasites. Cytokines such as IFN-γ, TNF-α, TNF-β, IL-1 and granulocyte colony stimulating factor (GCSF) are capable of enhancing anti-parasite action of blood monocytes and neutrophils, while IL-4 inhibits

monocyte/macrophage-mediated killing of *P. falciparum in vitro.* (Kumaratilake and Ferrante, 1994; Nyakeriga *et al.,* 2002). Cytokines have also been reported to mediate interaction between humoral and cellular immune responses during malaria infections (Cruz Cubas *et al*., 1994; Perlmann and Troye-Blomberg 2000). Antibodies against some of the surface proteins like Pfs230 induce ADCC and this contributes to desreased parasitaemia through parasite clearance (Williamson *et al.,* 1995). IgA-Immune complexes have been shown to bind CR1, just like IgG-IC in patients with glomerulonephritis (Coppo *et al.,* 1989). It is felt that the same could be happening in severe malarial anaemia where there is interaction of IC and CR1 and lysis of RBC.

2.5 CIC and severe *P. falciparum* malaria pathogenesis

A large body of evidence suggests that immune complexes form during malaria infection (Howard and Giladuga, 1989; Jhaveri *et al.,* 1997; Stoute *et al.,* 2003; Greenwood *et al.,* 1978; Mibei *et al.,* 2005), and have suggested a possible role for ICs in the pathogenesis of severe anemia (SA) and cerebral malaria (CM). However, the contribution of these complexes to the pathogenesis of severe malaria is not well understood.

ICs can complicate malaria pathology in several ways. One way is through their immunoregulatory role. ICs can interact with antigen receptor-bearing lymphocytes and sub-population of B and T cells as well as unclassified lymphocytes, macrophages and dendritic cells bearing Fc and complement (C) receptors like FcγR, CR1 and CR2 (Laborde *et al.,* 2007; Ravetch and Bolland 2001). Via such interactions immune complexes may suppress or augment immune responses. They also lead to release of

soluble suppressor factors from B and T cells (they activate regulatory T-cells) leading to effector cell blockade, blockade of antigen receptors on T-cells and inhibition of B-T cell collaboration (Theofilopoulus, 1980). Human platelets are also involved since they bear IgG (FcγR) and C1q receptors and aggregate and release nucleotides and vasoactive amines in response to immune complexes. Some of these vasoactive amines increase vascular permeability and local inflammation (Theofilopoulus, 1980), which are implicated in cerebral malaria pathology (Gupta *et al*., 1988).

The pathologies associated with ICs differ according to class/subclass of the antibody and size of the ICs and it is becoming apparent that the functional specificity of antibodies to malaria antigens may play an important role in the protective immune responses (Groux and Gysin, 1990; Bouharoun-Tayoun *et al.,* 1990; 1992). IgG subclasses differ in their effector functions and this is related to differences in structure especially the length and flexibility of the hinge region. The flexibility is related to differences in biological properties where the Fab region of the IgG molecule combine with the antigen resulting in IC which triggers the effector mechanisms mediated via the Fc part of the IgG molecule. The four human IgG subclasses show differences in their interaction with Fc gamma receptors (FcγRs), expressed on the effector cells like monocytes, macrophages myeloid cells and dendritic cells (Groux and Gysin, 1990).

Binding of the Fc part of IgG to FcγR is instrumental in the induction of the cell's effector functions (Van de Winkel and Capel, 1993). In this way FcγRs play a key role in bridging IgG and antibody activity and cellular effector mechanisms which include phagocytosis, endocytosis, antibody-dependent cell cytotoxicity (ADCC), complement activation, release of a range of inflammatory mediators, antigen presentation and

clearance of ICs. FcγR-induced phagocytosis also play a role in antigen presentation and amplification of the immune response since several FcγR-bearing cell types like macrophages and dendritic cells have the capacity to present antigens to T cells. Interaction between FcγR and IgG antibodies is pivotal in the immune response against infectious agents (Bredius *et al.,* 1994) and since IgG subclasses differ in their affirnity to FcγRs, clinical manifestation and their functioning will depend upon the properties of the FcγR as well as the isotype.

IgG1 and IgG3 have been shown to predominate in anti-parasite responses thus suggesting a role of these isotypes in protection against malaria (Bouharoun-Tayoun *et al.,* 1995; Cabrera *et al.,* 2004; Piper *et al*., 1999). IgG1 and IgG3 are considered to be cytophilic hence protective against *P. falciparum* malaria while IgG4 is thought to be non-cytophilic and non-protective and is associated with blocking of protective mechanisms offered by IgG1 and IgG3 (Bouharoun-Tayoun *et al.,* 1995). Although IgG2 has been suggested to be moreless cytophilic and hence has a possible role by activating macrophages through FcγRIIA (Aucan *et al*., 2000).

Many theories exist on the pathogenesis of cerebral malaria (Warrel *et al.,* 1990). One is that during an acute *P. falciparum* infection, the non-immune patient is unduly susceptible to rapid formation of antigen-antibody complexes which being complement fixing, tend to be deposited in the brain as well as in other tissues. Deposition of these complexes in the brain causes inflammation characterized by petechial haemorrhages in sub-cortical white matter of the brain (Howard and Gilladuga, 1989). Studies in mice have suggested that there are higher and sustained levels of immune complexes in cerebral lesions (Gupta *et al.,* 1988). Studies in human malaria have also shown elevated

levels of CIC in severe malaria (Adam *et al*., 1981; Jhaveri *et al*., 1997; Branch *et al*., 1998; Greenwood *et al.,* 1978). More recent studies in children have also found similar trend (Stoute *et al.,* 2003; Mibei *et al.,* 2005). Marked precipitation of immune complexes may be directly responsible for the lesions caused by their deposition in tissues, which leads to inflammation. These studies further suggested that immune complex deposition might block surveillance mechanism thereby contributing to development of cerebral malaria. This observation of higher CIC levels in CM supports the known fact that *P. falciparum* infection in a non-endemic area makes an individual unduly susceptible to generate higher antibody responses (Miller *et al*., 1994) thereby resulting in elevated levels of circulating immune complexes. This difference may also be explained in part by considering the relationship between complement regulatory proteins (CRP) level and CIC. Previous studies in this study area comparing CR1 levels in severe malaria, showed higher erythrocyte-CR1 in CM patients than SA patients thus suggesting that RBC CR1 may play a role in the pathogenesis of cerebral malaria perhaps by allowing rosette formation (Waitumbi *et al.,* 2000; Stoute *et al.,* 2003).

Patients with severe malarial anaemia have been shown to have low levels of CR1 (Waitumbi *et al.,* 2000). Patients with either insufficient or inefficient CR1 molecules on their RBCs may have poor circulating immune complexes clearance capacity through phagocytosis in liver and spleen, which then predisposes one to developing immune complex-mediated complications. The inability to remove immune complexes from circulation may lead to unchecked complement activation and deposition of C3b on the surfaces of erythrocytes and other cells. Consistent with this is the finding of reduced survival time of normal RBCs that has been shown in acute *P. falciparum* and *P. vivax* as well as a positive Coombs test in these patients (Rosenberg *et al*., 1973). Severe

malarial anaemia has been suggested to be mainly due to extensive phagocytosis of normal as well as parasitized erythrocytes (Brown, 1969; Waitumbi *et al.,* 2000). Also autoimmune mechanism has been suspected and studies in animal models support this idea (Zuckerman, 1969). Rising titres of IgM abs that can coat non-parasitized RBCs in vitro following infection with *P. falciparum* malaria strengthens the case for autoimmune anaemia (Rosenberg *et al*., 1973). Allonso *et al.,* (1971), suggested that malaria infection leads to thymus-dependent sensitization through IC and stimulates B-cells to produce auto antibodies to host RBCs and to gamma globulins (IgGs). There is an indication that IgM antibodies may commonly be present in patients exposed to endemic malaria (Chizzolini *et al*., 1991; Wahlgren *et al*., 1986).

Studies have suggested that circulating immune complexes cause haemolysis and/or erythrophagocytosis by immune adherence phenomenon through C3b receptors (Mohammed, 1982; Waitumbi *et al.,* 2000). Also the progressive decrease of C3 concentrations following infection with malaria (Stoute *et al.,* 2003; Waitumbi *et al*., 2002; Rosenberg *et al.,* 1973) coupled with increasing IgM-red cell ab titres and decreasing haematocrit (Rosenberg *et al*., 1973; Brattig *et al.,* 2008), suggests that complement may participate in an autoimmune haemolytic anaemia in some patients with severe *P. falciparum* malaria.

The relationship between IgE and CM especially on sequestration of PRBC has been suggested (Perlman *et al*., 1994), and it is thought IgE-IC might be inducing local overproduction of TNF-α, which plays a major role in CM pathogenesis. Studies have shown that people living in malaria-endemic areas have elevated serum levels of both total and anti-malaria specific IgE (Desowitz, 1989; Perlman *et al.,* 1994). IgE interacts

with Fc epsilon receptors (FcεRs) expressed on a variety of cell types. There are two types of IgE receptors; the low-affinity FcεRII (CD23) expressed on B cells and induced by IL-4 on macrophages (Vercelli *et al.,* 1988), some T cells, eosinophils and platelets (Delespesse *et al.,* 1992). Cross-linking of CD23 on macrophages or other CD23-bearing effector cells by IgE-containing immune complexes is thought to play a pathogenic role via TNF-mediated pathways (Perlmann *et al.,* 1994;, 1999; Dugas *et al.,* 1995). The second IgE receptor is the high affinity FcεRI which is found on mast cells and/or basophils. Cross-linking of this receptor by IgE-containing immune complexes,or in response to IL-3, basophils rapidly release cytokines such as IL-4 (Nyakeriga *et al.,* 2003),and IL-13 (Plaut *et al.,* 1989; Brunner *et al.,* 1993). Both cytokines are important in the polarization of T helper (Th), cells and results in shift to Th2 cell.

IgA-Immune complexes have been shown to bind CR1, just like IgG-IC in patients with glomerulonephritis (Coppo *et al*., 1989). It is felt that the same could be happening in severe malarial anemia where there is interaction of IC and CR1 and lysis of RBC. Initial infection with *P. falciparum* malaria has been shown to preferentially stimulate synthesis of IgM antibodies. In a study by Rosenberg *et al.,* (1973), rising titres of IgM antibodies to red blood cells (RBCs) were found in patients with *P. falciparum* parasites and this observation was not present in non-malaria patients with high IgM concentrations or in healthy controls and this study suggested an association between high IgM antibody level and malarial anaemia and highlighted their concurrent development (Rosenberg *et al.,* 1973).

2.6 Methods commonly used to purify measure ICs

The estimation of CICs in sera of patients with different diseases has been frequently used for the assessment of disease activity for the purpose of therapy and control (Krauledat *et al*., 1985). Over fifty methods have been described for the measurement of CICs in humans and animals (Jones *et al.*, 1982). Variability in sensitivity, complexity, clinical applicability and limited specificity of the available test systems complicates the choice of assay used. Thus it is well known that the application of different assay systems for the detection of CIC will lead to divergent results due to different biological or physical principles. Therefore, for the screening for serum ICs, the application of at least two or more different methods is recommended. Furthermore the test systems used should be based on different tracing principles permitting the detection of ICs of various sizes and complexity (Krauledat *et al*., 1985).

Most test systems used are based on the complement system and its breakdown products. This is because most ICs share a common property in their ability to activate complement system via classical or alternative pathway hence it seems relevant to test breakdown products. Most of these test systems are antigen non-specific and include C1q binding assay (C1qBA), PEG precipitation, conglutinin binding assay (KBA) and cryoprecipitation among others. They identify the presence of ICs by relying on the different physiochemical and biological properties of antigen-complexed antibody as opposed to free antibody (Jones *et al.*, 1982).

C1q binding of ICs is an indication for the classical method of complement activation. C1q binds weakly to monomeric IgG1, IgG2, IgG3 and IgM. Binding is enhanced when the proteins aggregate because of heating or antigen binding (Mitchell *et*

al., 1987). The C1qBA detect only those ICs that bind the first component of complement. However, C1qBA detects neither ICs with non-complement activating antibodies, nor ICs with antibodies that activate complement preferentially via the alternative pathway (IgE and IgA) (Theofilopoulus, 1980). C1q also interacts preferentially with ICs larger than 19S. It has also been shown that C1qBA is less sensitive in measuring smaller (antigen excess) than large complexes (antibody excess) and is efficient at detecting complexes of intermediate size (between 19S and 7S) (Woodroffe *et al.*, 1977). Solid phase C1qBA is very sensitive (1-10µg/ml aggregated human IgG) and have the distinct advantage over fluid phase C1q tests in that they are antibody specific and have minimum false positive results (Mitchell *et al*., 1987).

C1q binding assay was used to detect immune complexes containing IgG1, IgG2, IgG3, and IgG4 antibodies for the current study. Polyethylene glycol (3.5% w/v) has been frequently used to isolate immune complexes from serum or plasma (Digeon *et al.*, 1977; Lock and Unsworth, 2000; Golda *et al.*, 2004; Sengupta *et al.*, 2002). PEG solution at 3.5% w/v precipitates mainly immune complexes while very few free antibodies are precipitated and very little of other proteins comes down. PEG at 3.5% is thus effective in purifying immune complexes. PEG at 2.0% w/v was used to precipitate ICs for measurement of IgM, IgA and IgE containing ICs by use of sandwich ELISA for the current study. Once the ICs have been precipitated out, they can be dissociated by an acidic buffer. Ab-Ag complexes are held by weak covalent bonds, which dissociates in an acidic environment. Glycine-HCL at pH less than 3 is normally used (Kesten *et al.*, 1991; Kasuhara *et al.*, 2000). Glcine-HCL pH 2.0 was used to precipitate ICs for the current study.

Gel electrophoresis is one method which has been extensively used to separate proteins and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has been described and shown to be efficient in separating proteins and thus is useful in separation and identification of antigens (Kasuhara *et al.,* 2000; Golda *et al.,* 2004; Suarez *et al.,* 2004; Rojas and Sigel 1997; Arora *et al.,* 1991). The principle is based on the fact that proteins will migrate at different speeds depending on the molecular weight and the net charge. Two-dimensional gel electrophoresis, (2-DE) coupled with protein identification through proteomics analysis is currently the tool in protein identification technology (Görg *et al.,* 2004). 2-DE enables the separation of complex protein mixtures and delivers a map of intact proteins (Görg *et al.,* 2004). 2-DE couples isoelectric focussing, (IEF), in the first dimension with SDS-PAGE in the second dimension, and enables separation of complex mixtures of proteins according to their isoelectric points, mass, solubility and relative abundance. Depending on the gel size and pH used, 2-DE can resolve more than 5000 proteins simultaneously and can detect less than 1ng of protein per spot (Görg *et al.,* 2004). SDS-PAGE was utilised for the initial screening of IC samples for *P. falciparum* antigens followed by 2-DE for identification of the specific target *P.falciparum* proteins for proteomics analysis.

CHAPTER THREE: MATARIALS AND METHODS

3.1 The study sites

The study was carried out at Kondele KEMRI-Walter Reed Research Laboratories using participants drawn from the following hospitals:

a) Nyanza Provincial General Hospital, Kisumu. This is a major referral centre for the population living in the malaria holoendemic area of Lake Victoria basin (Figure 1).

b) Kisii District Hospital situated in Kisii town: This is a highland area with yearly malaria epidemics usually following the onset of the long rainy season (Figure 1).

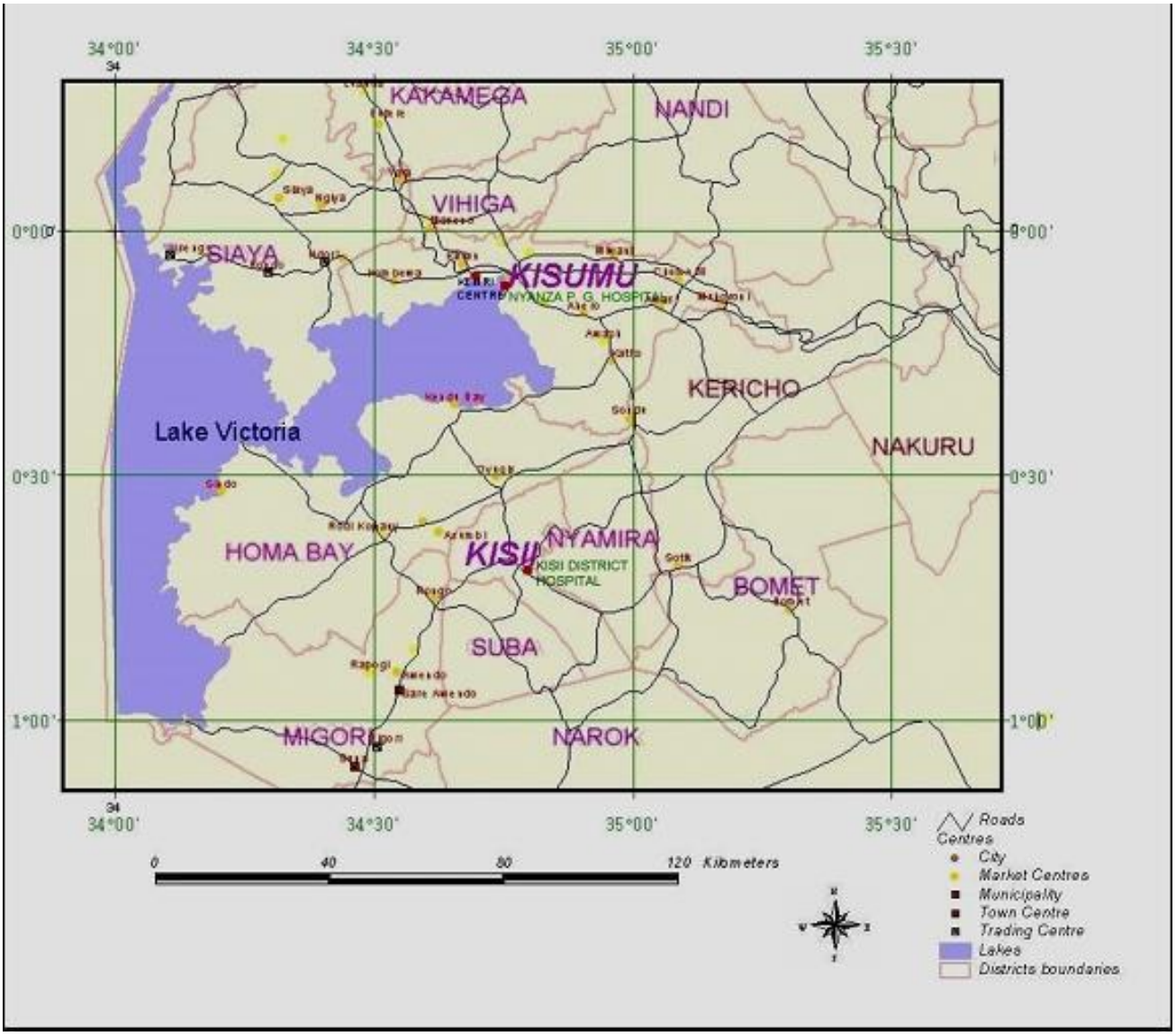


Figure 1. Map of study sites showing the location of Kisumu and Kisii towns

3.2 The study design and Patient Population

The study had a matched case-control design. Severe malaria anaemia (SMA) cases were recruited from the paediatric ward of the Nyanza Provincial General Hospital (NPGH), Kisumu, Kenya, where malaria is holoendemic. Since CM is uncommon in this area, CM cases were recruited from the paediatric ward of the Kisii District Hospital (KDH), as well as from the NPGH. KDH is located in the highlands of western Kenya where transmission is seasonal and the predominant complication is CM hence consequently receives many more CM cases than the NPGH (Hay et al., 2002).

3.2.1 Inclusion criteria.

SMA cases for this study were defined as children with asexual *P. falciparum* parasitemia by Giemsa-stained thick and thin blood smear and Hb ≤ 6 g/dL. CM was defined as asexual *P. falciparum* parasitemia by Giemsa-stained blood smear and a Blantyre coma score of ≤ 2 (Molyneux et al., 1989; Newton *et al*., 1997; Mabeza *et al*., 1995), lasting at least 30 min if there was a history of convulsions. Symptomatic uncomplicated malaria controls matched by gender and age ± 2 months were assigned to each case at a case:control ratio of 1:1 for SMA and 1:1 or 1:2 for CM, and were identified from the outpatient clinic of the same hospital where the corresponding case was recruited. Controls were defined as children with a normal mental status, a Hb > 6 g/dL, a Giemsa-stained blood smear positive for asexual *P. falciparum*, and an axillary temperature ≥ 37.5 ^{o}C. In the absence of fever, two of the following signs or symptoms were required: nausea/vomiting, irritability, poor feeding, myalgias or headache.

All children were evaluated in a standardized fashion at enrollment (visit 1) and at follow-up (visit 2) two months later. If a child failed to return for follow-up, a field

worker traveled to his/her last known domicile to determine his/her status. During follow-up, a blood sample was obtained once it was confirmed that the child was asymptomatic and free of parasitemia. If malaria persisted at the first follow-up visit, the child was re-treated and re-evaluated two weeks later. Inpatient treatment for malaria consisted of IV quinine and outpatient therapy was with artemether/lumefantrine (Kokwaro *et al.*, 2007).

3.2.2 Exclusion criteria

General exclusion criteria included evidence of concomitant serious infections (like, meningitis excluded by lumbar puncture when indicated, pneumonia, sepsis), chronic illness, or a history of blood transfusion in the three months preceding enrollment to avoid the influence of donor erythrocytes in the measurements.

Volunteers were also excluded from the study if there was an inability or unwillingness of the subject and/or the parent/guardian to give consent.

3.2.3 Ethical considerations

Participants were recruited under a human use protocol approved by the National Ethics Review Committee of the Kenya Medical Research Institute, Nairobi, Kenya and the Human Use Research Committee, the Walter Reed Army Institute of Research, Silver Spring, Maryland, USA. Informed consent was obtained from all parents or guardians.

3.3 Collection of blood samples.

Giemsa-stained thick and thin blood smears were prepared from capillary blood obtained by finger prick. A 2.5 ml sample of EDTA-anticoagulated venous blood was obtained at enrollment and 5 ml at follow-up. Following measurement of hemoglobin

levels, the EDTA-anti-coagulated blood was centrifuged and the plasma was stored at –70 ^{o}C.

3.4 Circulating immune complex analysis

Antibody classes and subclasses were determined using standard ELISA protocols. IgG and IgG subclasses ICs were determined using C1q binding assay which is a standard Elisa assay that utilises the ability of C1q to bind ICs, and aggregated immunoglobulins. IgA and IgE-containing ICs were measured usind a sandwich ELISA

3.4.1 IgG- containing immune complexes

The methodology for measurement of total IgG-containing IC was described in detail (Mibei *et al.,* 2005). Briefly, for measurement of total IgG-containing ICs a C1q-based ELISA assay was used. Wells of Immulon II HB 96-well plate (Thermo Labsystems, Helsinki, Finland) were coated overnight with 10 μg/mL of C1q (Sigma-Aldrich). Aggregated IgG, prepared from purified human IgG (Sigma-Aldrich) by heating at 63 ^{o}C for 30 min followed by size fractionation over a Sephacryl S-300 70x2.6 cm column (Amersham Pharmacia Biotech, Piscataway, NJ), served as a standard. Total IgG was detected by using goat anti-human IgG (Kirkegaard & Perry, Baltimore, MD) at dilution of 1:3000. For detection of antibody subclasses, biotin-labeled monoclonal antibodies against human IgG1 (Sigma-Aldrich) at a dilution of 1:8000, IgG2 (Becton-Dickinson, Brussels, Belgium) diluted 1:1000, IgG3 (Zymed Laboratories, South San Francisco, CA) diluted 1:2000, IgG4 (Becton-Dickinson) diluted 1:4000 were used. After incubation, the wells were emptied and washed X4 with wash buffer. Horse radish peroxidase (HRP)-labeled streptavidin (Sigma-Aldrich) was diluted 1:6000 in dilution buffer and 100 μL was added to each well followed by 30 minutes incubation at room temperature. After

washing four times, 200 μL of ABTS substrate (Kirkegaard & Perry) was added to each well and incubated for 30 min followed by measurement of the OD_{415nm}. IC level was expressed as micrograms of aggregated human IgG equivalent per ml (μg AHG Eq/ml). Calculation of subclass concentration in the standard was based on the approximate percentage concentration of each IgG subclass in the AHG standard used (Schaurer *et al.,* 2003). Positive and negative controls were used. Plate-to-plate variation was controlled by normalizing to the positive control sample by using the following formula

$ICc(\mu gAHGEq/ml) = ICuc \times Cm/Cp$ Where ICc is the corrected IC level of the sample, ICuc is the uncorrected IC level, Cm is the average concentration of the IC for the positive control and Cp is the concentration of the positive control for the plate in which the sample was tested.

3.4.2 IgM, IgE and IgA Immune complexes

To detect IgA and IgE-containing ICs, a sandwich ELISA assay was used. In order to rule out detection of free Igs, ICs were precipitated from plasma by adding 100 μL of 4% PEG (w/v) solution (PEG 6000, Fluka, St. Louis, MO), to 100 μL of plasma diluted 1:5 in borate buffer pH 8.5 (Pierce, Rockford, IL). This was mixed well and incubated overnight at 4 °C then centrifuged at 5,000 RPM for 10 minutes at 4 °C. The IC precipitate was washed twice with 200 μL 2% PEG solution. IC pellet was then re-suspended in 100 μL borate buffer pH 8.5 and stored frozen until use. Wells of an Immulon II HB 96-well plate (Thermo Labsystems) were coated with 100 μL of goat anti-Human IgM (Kirkegaard & Perry), or goat anti-human IgE antibody (Kirkegaard & Perry) or anti-human IgA (Kirkegaard & Perry) diluted 1:2000 in dilution buffer and incubated overnight at 4 °C. The plate was washed X4 with wash buffer (0.25% Tween 20 in PBS pH 7.4) then blocked for 1 hour with 200 μL of blocking buffer (PBS, 0.5% boiled casein, 1% Tween,

0.01% Thimerosal, 20 μg/ml phenol red). After four washes, 100 μL of IgM, IgE or IgA standard (Sigma-Aldrich) at various dilutions, control samples, or PEG-precipitated test samples diluted 1:50 in dilution buffer was added to duplicate wells and incubated for 1 hr at room temperature. This was followed by four washes with wash buffer and addition of 100 μL of HRP-conjugated goat anti-human IgM, IgE or IgA (Sigma-Aldrich) diluted 1:5000, 1:500 or 1:3000 respectively in dilution buffer and incubation for 1 hr at room temperature. Colour development, absorbance measurement, and normalization were as described for IgG above.

3.4.3 Immune complex dissociation and electrophoresis

The purified immune complexes were dissociated using an acidic buffer. 100μl of the purified IC sample was mixed with an equal volume of 1.5M glycine-HCL pH 2.0 and incubated for 1h at 37^{o}C. The samples were then neutralized by adding 100μl 1.5M Tris-HCL buffer pH 8.5. The dissociated immune complexes were then separated by electrophoresis on Novex 4-12% Tris-glycine precast gels (Invitrogen). 10μl of the diluted samples and standards were loaded to each well in the gel and electrophoresis done at 200 volts constant for 50 minutes.

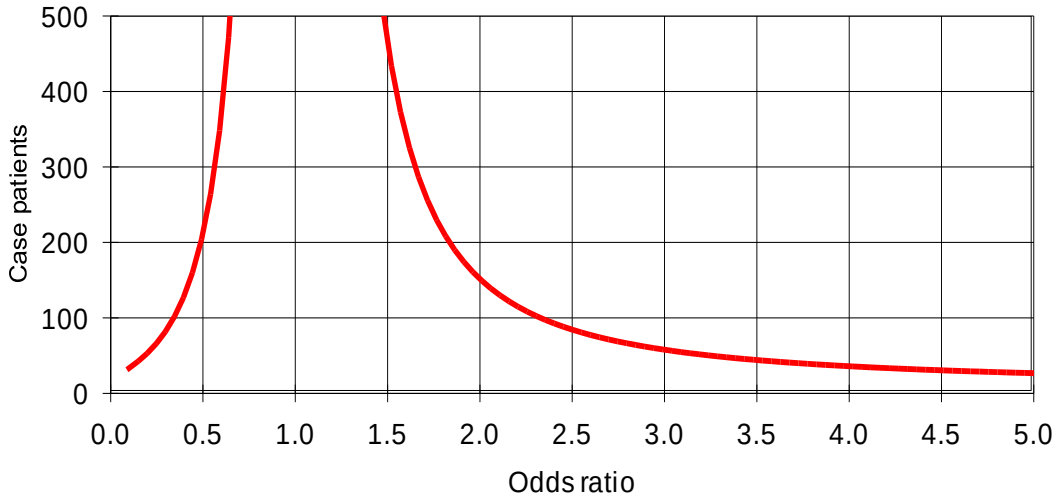
Western blotting was then done according to the procedure provided (Novex, Invitrogen). The membrane was probed with human malaria hyperimmune serum. Goat anti-human IgG HRP conjugated was used as a secondary antibody. Bands were then revealed by addition of chemiluminescense substrate (Pierce), followed by signal acquisition by exposure of photographic film, Clear Blue X-Ray film (Pierce). Preliminary parasite specific band identification and analysis was done and those samples showing specific important parasite proteins were then subjected to 2-dimensional gel

electrophoresis (2-DE) and the specific parasite spots punched out for proteomics analysis.

3.5 Sample size

There are no data on the prevalence of different classes and subclasses of CICs in children with severe malaria. Sample sizes were also limited by the rate at which cases were being enrolled. Assuming a prevalence of IgE associated CICs of 25%, it will be possible to detect an OR < 0.5 OR > 2.2 with a power of 80% and $\alpha = 0.05$. Figure 2 below depicts the relationship between sample size and odds ratio. For sample populations who are closely related, a larger sample size will be needed to be able to detect a significant difference while for distinct populations a smaller sample size will be adequate to detect significant difference.

Figure 2 A graph showing the relationship between sample size and odds ratio



3.6 Data Analyses:

Statistical analysis was performed using SPSS for windows version 11.5 (SPSS Inc., Chicago, IL) software package. Mean IgG, IgG subclass, IgE, and IgA-containing IC levels were compared between cases and controls by univariate analysis of variance with matching adjusted for home districts. Comparison of IC levels between SMA and CM cases at enrollment was done using Mann-Whitney U test due to unequal variances. Multivariate conditional logistic regression controlling for district of residence was carried out to determine the association of antibody class/subclass and severity of malaria and enrolment and follow-up. To make meaningful associations, the IC levels were converted to standard deviation units by dividing the IC units by standard deviation of antibody class or subclass for each cohort. Fishers Exact test was used to test for association of severity to specific malarial antigens. All tests were two-tailed with $\alpha \leq 0.05$.

CHAPTER FOUR: RESULTS

4.1 Demographics

A total of 75 SMA and 32 CM cases were enrolled and matched to 74 and 52 symptomatic uncomplicated malaria controls respectively. The symptomatic uncomplicated malaria controls were identified from the outpatient clinic of the same hospital where the corresponding case was recruited. The demographics and clinical characteristics of the study participants were recently reported (Owuor *et al.,* 2008). Table 1 summarizes the demographic characteristics of study participants at enrolment. The mean age (standard deviation) for SMA cases was 16.9 (13.7) months and that of their controls was 16.8 (13.3) months. The same parameters for CM cases and their controls were 33.1(19.2) and 33.6 (16.4) months respectively. There were no significant differences between cases and controls in the gender proportions, the district of origin, ethnic origin, or parasite density (Owuor *et al.,* 2008). There were also no significant differences in the parasite densities between CM and SMA cases during visit 1 (Owuor *et al.,* 2008). 65 SMA cases and 63 uncomplicated malaria controls presented for follow-up. There were five deaths among patients with SMA (6.7% mortality) all of which occurred during the initial hospitalization. There was one death among the SMA controls (1.3% mortality) which was due to an episode of severe diarrhoea. Among CM cases and their controls 27 and 44 presented for follow-up, respectively. One in-hospital death occurred among CM cases recruited at KDH (4.2% mortality) and no deaths occurred among CM cases at NPGH.

The age range for CM cases was 3 months to 7 years and for SMA cases was 5 months to 7 years. Despite the overlap in ages, the mean difference in age for the two clinical groups was significant ($P < 0.001$ by an independent samples t test). There were

no significant differences in the mean parasite densities although CM cases had comparatively higher parasitaemia.

Table 1. Demographic profiles of the study groups

Variable	Severe Anaemia		Cerebral Malaria	
	Cases (N = 75)	Controls (N = 74)	Cases (N = 32)	Controls (N = 52)
Mean age (SD) in months	16.9 (13.7)	16.8 (13.3)	33.1 (19.2)	33.6 (16.4)
No. Females (%)	33 (44)	32 (43)	16 (50)	29 (56)
Mean Hemoglobin (SD) in g/dl	4.7 (0.9)	8.8 (1.7)	9.0 (1.7)	9.3 (1.6)
Mean Parasite density (SD)	4468 (6742)	4427 (4687)	6493 (6274)	5180 (5887)

NOTE. Figures in parentheses represent the Standard Deviation (SD), N - Sample Size

4.2 Antibody class and sub-class specific IC levels in cases and controls

Immune complex levels were measured to identify differences between cases and controls and between children with SA and CM. Figures 3 through Fig. 10 show the IC levels for each group and visit. CM cases had elevated IC levels as compared to SA cases at visit 1. The difference was significant for IgG total-ICs ($P < 0.0001$), IgG1 ICs ($P < 0.001$), IgG2 ICs ($P = 0.001$), IgG4 ICs ($P < 0.0001$) and IgA-containing ICs ($P < 0.01$) (Mann Whitney U test). These observations support earlier suggestions that CM patients are unduly susceptible to generating higher levels of ICs (Howard and Gilladuga, 1989). CM cases had significantly higher IgE-containing IC levels than their symptomatic controls ($P = 0.01$) (Fig 10), and elevated IgG total-containing ICs ($P = 0.03$) (Fig 3). Following malaria treatment, the levels of IC in both SMA and CM cases

declined suggesting that the higher IC levels and severe malaria were causally related to the malaria infection.

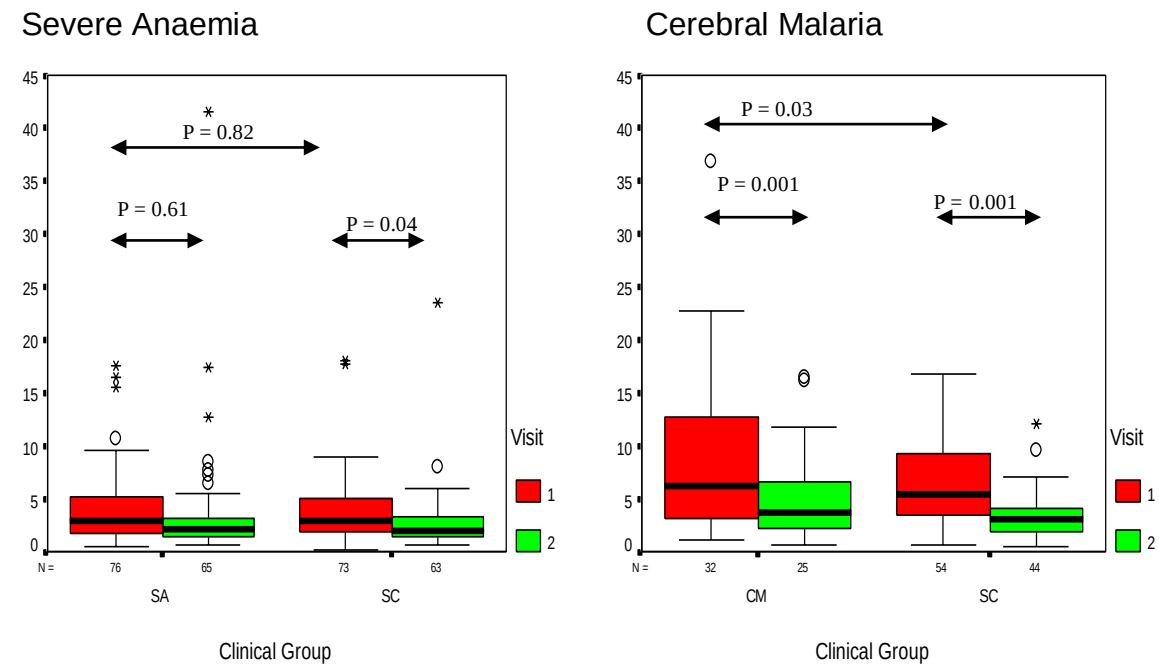


Figure 3. Box and whisker blots showing total IgG-IC levels in severe malarial anaemia and cerebral malaria and the symptomatic controls during enrolment and at follow-up.

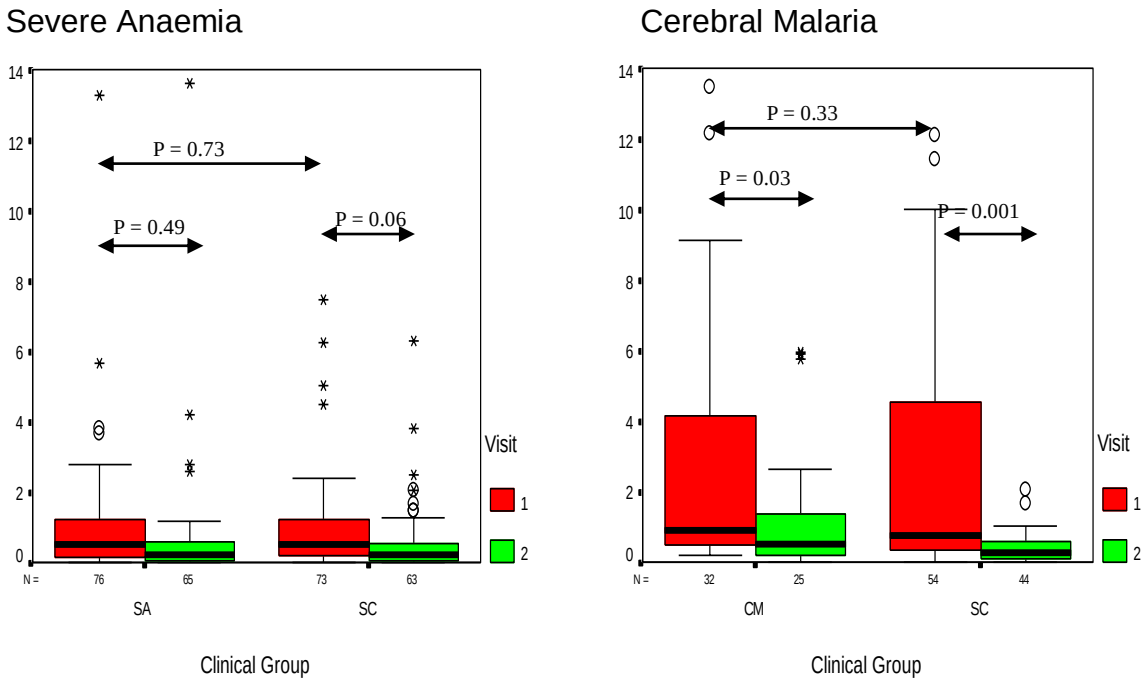


Figure 4. Box and whisker blots showing IgG1-IC levels in severe malarial anaemia and cerebral malaria and the symptomatic controls during enrolment and at follow-up

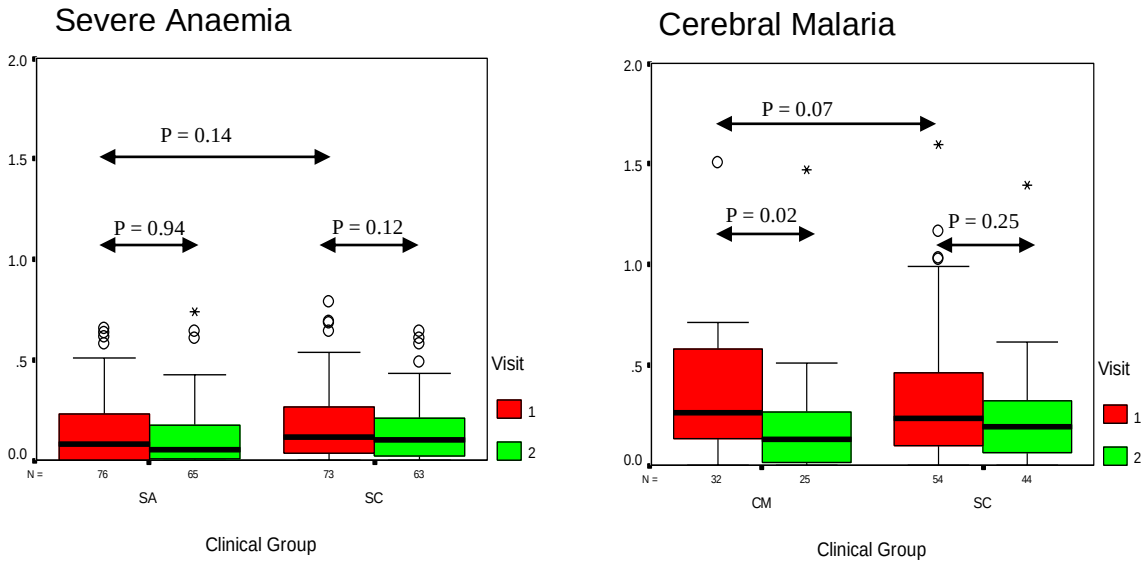


Figure 5. Box and whisker blots showing IgG2-IC levels in severe malarial anaemia and cerebral malaria and the symptomatic controls during enrolment and at follow-up

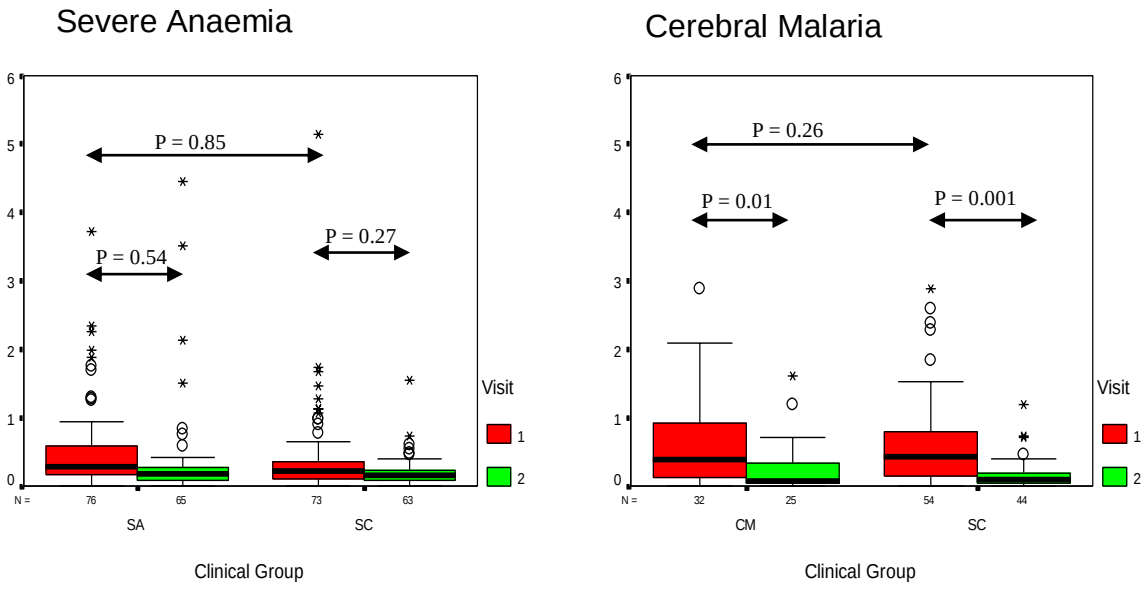


Figure 6. Box and whisker blots showing IgG3-IC levels in severe malarial anaemia and cerebral malaria and the symptomatic controls during enrolment and at follow-up.

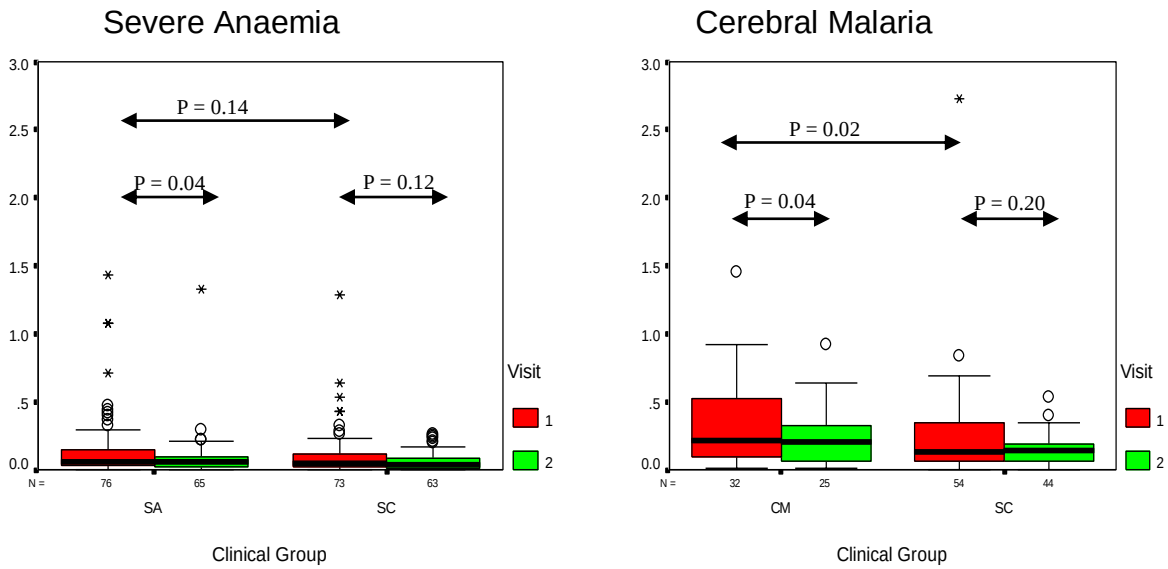


Figure 7. Box and whisker blots showing IgG4-IC levels in severe malarial anaemia and cerebral malaria and the symptomatic controls during enrolment and at follow-up.

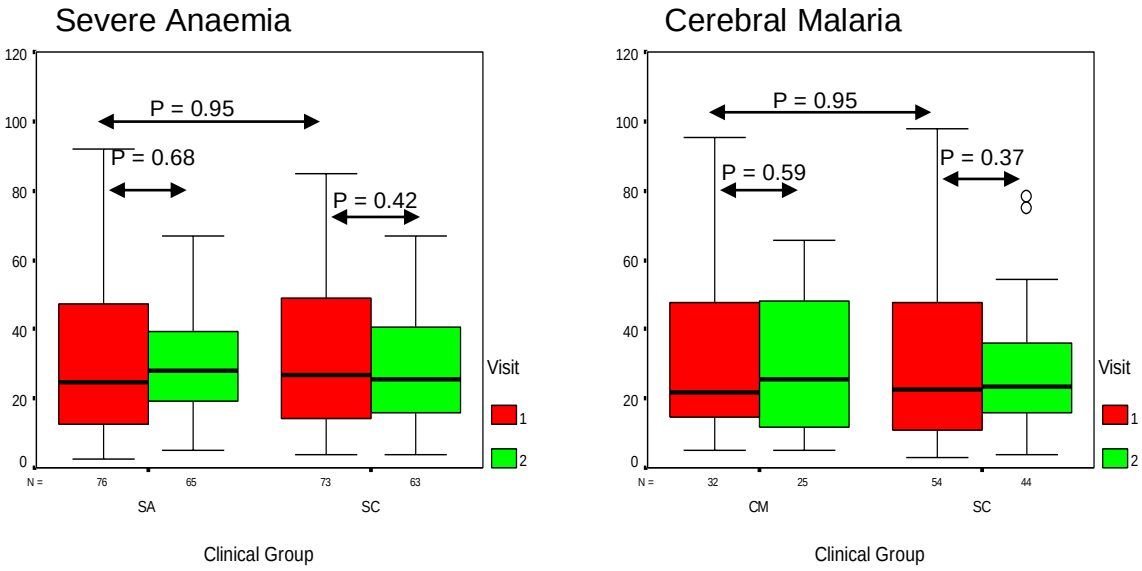


Figure 8. Box and whisker blots showing IgM-IC levels in severe malarial anaemia and cerebral malaria and the symptomatic controls during enrolment and at follow-up.

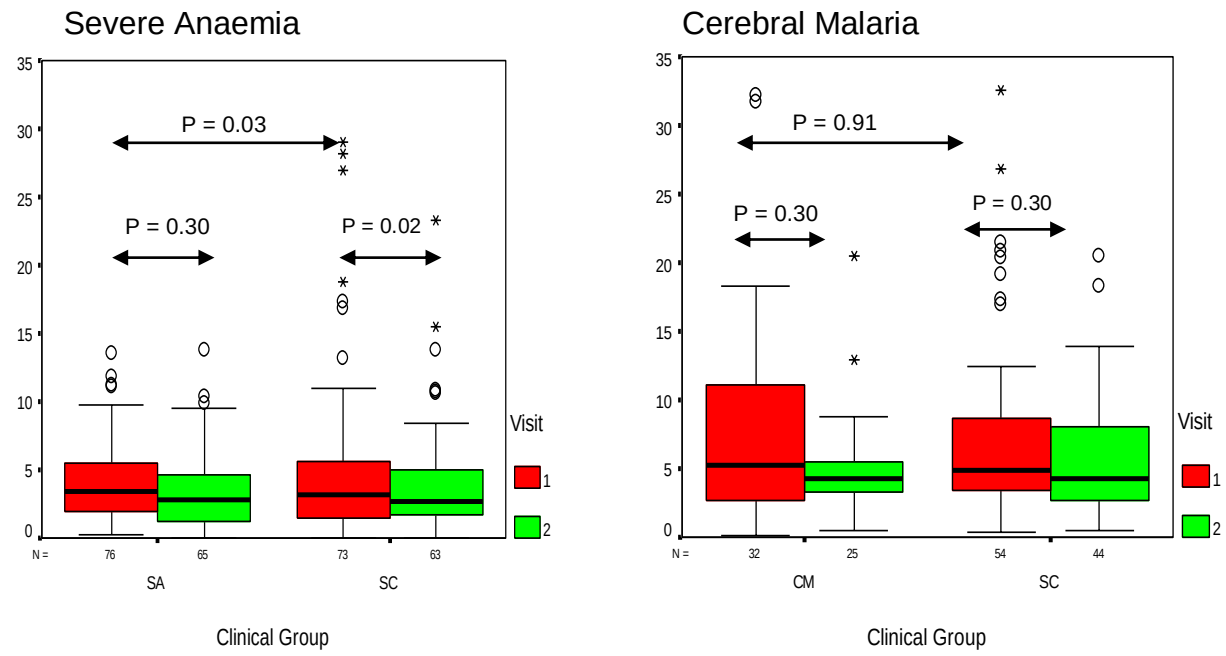


Figure 9. Box and whisker blots showing IgA-IC levels in severe malarial anaemia and cerebral malaria and the symptomatic controls during enrolment and at follow-up

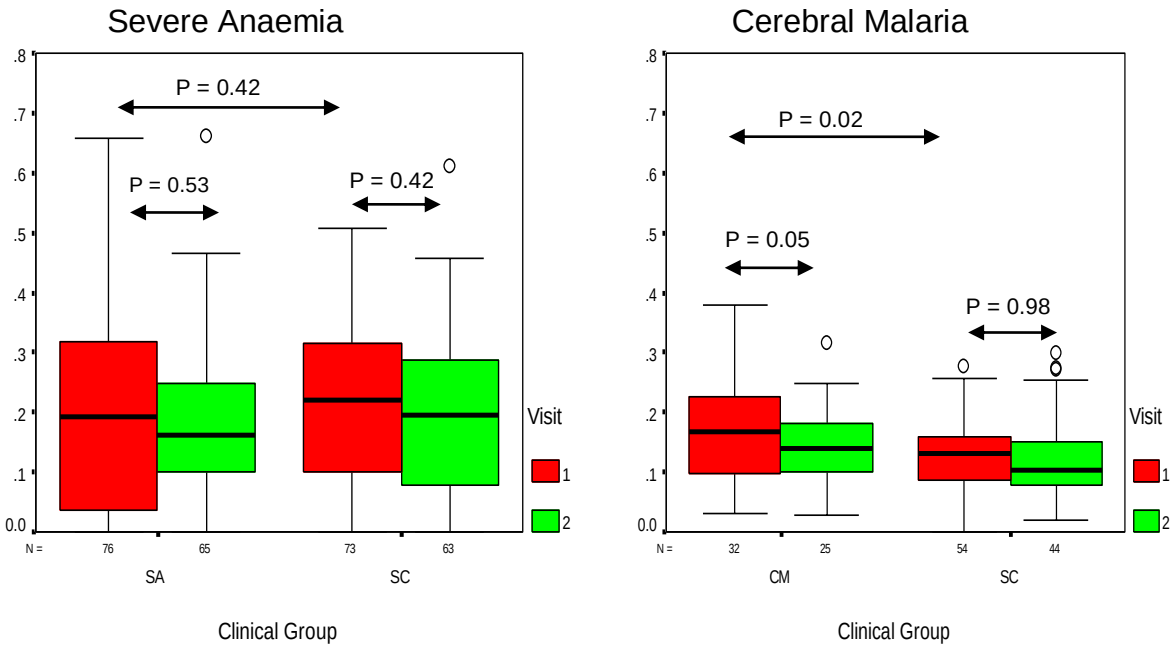


Figure 10. Box and whisker blots showing IgE-IC levels in severe malarial anaemia and cerebral malaria and the symptomatic controls during enrolment and at follow-up.

4.3 Logistic regression analysis of specific antibody class/subclass-containing immune complexes

Logistic regression test was carried out to check the association of antibody class/subclass and severity of malaria and to make meaningful associations; the IC levels were converted to standard deviation units by dividing by standard deviation of antibody class or subclass for each cohort. The resulting values were then used to check the association between each antibody isotype-containing IC and severity of malaria. Table 2 and 3 summarizes the findings. IgG4 antibody-containing immune complexes were associated with the risk of developing severe malarial anaemia (OR = 3.11, 95% CI = 1.01 to 9.56, P = 0.05**)** while IgG total antibody-containing ICs were associated with the risk of developing cerebral malaria (OR = 2.58, 95% CI = 1.20 to 5.53, P = 0.02**)**. IgE-containing immune complexes also were associated with an increased risk of developing cerebral malaria (OR = 3.27, 95% CI = 1.38 to 7.78, P = 0.01) (Table 2).

During follow-up, IgG-containing ICs were associated with an increased risk of severe malarial anaemia whereas IgE-containing ICs were protective against severe malarial anaemia. For CM cases and controls, IgG1-containing ICs were associated with increased risk of CM whereas IgG2- and IgA-containing ICs were associated with a decreased risk of CM (Table 3).

Table 2. Conditional logistic regression for immune complex levels at enrolment.

	Severe Anaemia			Cerebral Malaria		
Class/subclass IC	OR	95% CI for OR	*P* value	OR	95% CI for OR	*P* value
Total IgG	0.46	0.14 to 1.49	0.20	2.58	1.20 to 5.53	0.02
IgG1 IC	1.28	0.62 to 2.65	0.51	0.90	0.79 to 1.04	0.14
IgG2 IC	0.56	0.27 to 1.15	0.12	0.85	0.44 to 1.64	0.64
IgG3 IC	1.71	0.74 to 3.94	0.21	0.81	0.54 to 1.20	0.29
IgG4 IC	3.11	1.01 to 9.56	0.05	1.03	0.77 to 1.38	0.82
IgA IC	0.74	0.50 to 1.08	0.12	0.65	0.38 to 1.11	0.12
IgE IC	0.88	0.43 to 1.81	0.73	3.27	1.38 to 7.78	0.01

NOTE. OR, Odds Ratio, CI, Confidence intervals

Table 3 Conditional logistic regression for immune complex levels at follow-up

	Severe Anaemia			Cerebral Malaria		
Class/subclass IC	OR	95% CI for OR	*P* value	OR	95% CI for OR	*P* value
Total IgG	21.02	2.12 to 208	0.01	1.58	0.44 to 5.61	0.48
IgG1 IC	4.11	0.22 to 77.56	0.35	2.07	1.18 to 3.62	0.01
IgG2 IC	0.24	0.06 to 1.03	0.06	0.13	0.02 to 0.95	0.05
IgG3 IC	2.52	0.04 to 169	0.67	0.45	0.08 to 2.54	0.36
IgG4 IC	34.83	0.31 to 3914	0.14	1.37	0.35 to 5.36	0.65
IgA IC	0.53	0.27 to 1.04	0.07	0.12	0.02 to 0.86	0.04
IgE IC	0.23	0.06 to 0.85	0.03	0.53	0.16 to 1.71	0.29

NOTE. OR, Odds Ratio, CI, Confidence intervals

4.4 *P. falciparum* parasite specific antigens which participate in IC formation

Immune complexes were purified from patient's sera using 2% polyethylene glycol (PEG), and 1–dimensional polyacrilamide gel electrophoresis (PAGE) and Western blotting done. There were several different *P. falciparum* antigens detected from the blots probed with human hyperimmune serum. *P. falciparum* antigen analysis was done with Fisher's exact test and six specific *P. falciparum* antigens were found to be significantly associated with severe malarial anaemia while one (Ag #5) was found to be associated with both SMA and CM. Another three different antigens were found to

be significantly associated with cerebral malaria. Figure 11 shows the positive control antigen profile when probed with human hyperimmune sera.

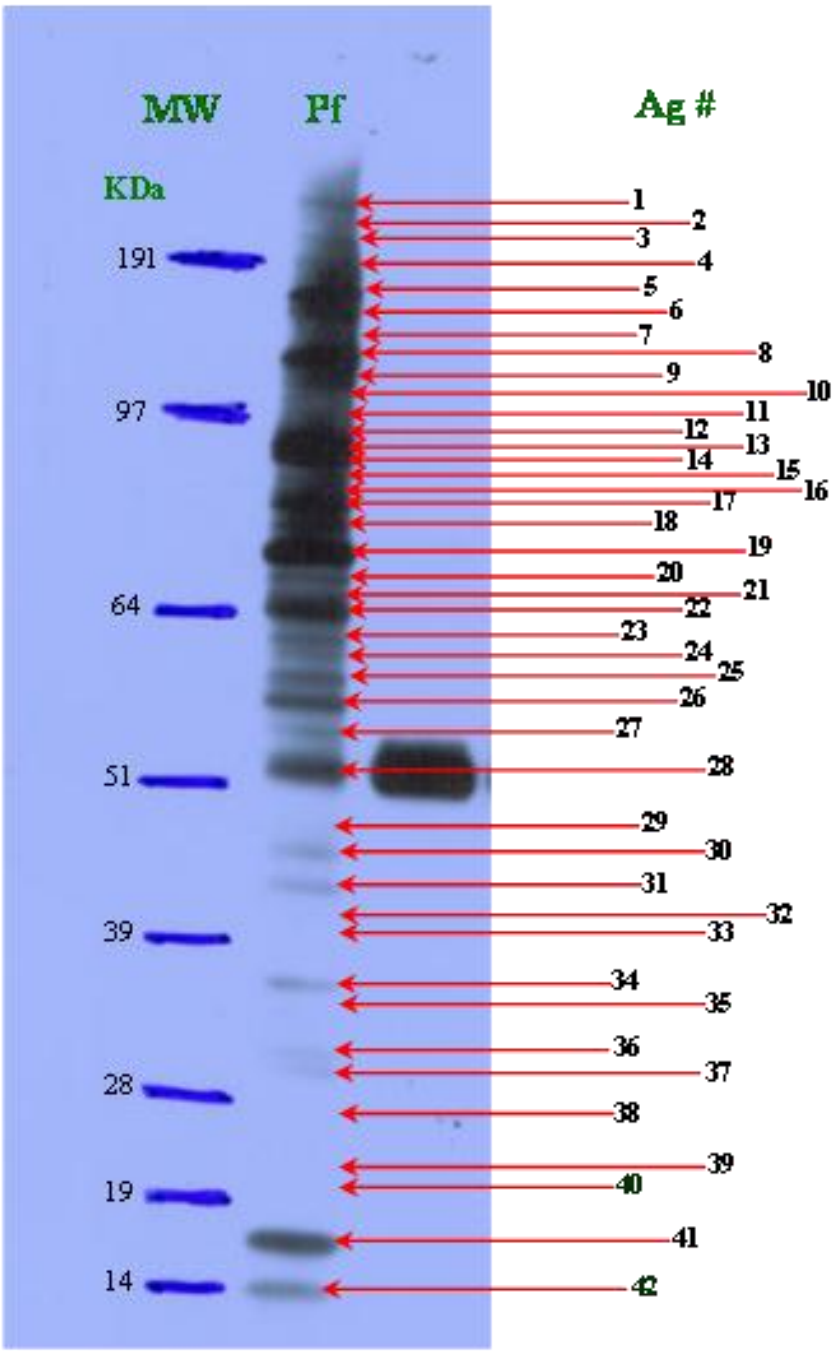


Figure 11. *P. falciparum* crude extract antigen profile. The various antigens were assigned hypothetical numbers for identification and matching.
Note: **MW**- molecular weight marker, **Pf** – *P. falciparum* crude extract lane, **Ag #** - the different parasite specific bands which could be detected with human hyperimmune serum.

Severe malarial anaemia when compared with symptomatic controls, was significantly associated with the following *P. falciparum* antigens: Ag #5, (P = 0.004), Ag #13 (P = 0.03), Ag #15 (0.0009), Ag #18 (P = 0.01), Ag #22 (P = 0.002), Ag #25 (P = 0.001) and Ag #26 (P = 0.03) (Table 5). While the following antigens were significantly associated with cerebral malaria: Ag #6 (P = 0.01), Ag #17 (P = 0.005) and Ag #30 (P = 0.02) (Table 6).

When SA and CM were compared together, it was found that Ag #15 was highly associated with SA (P = 0.002), while Ag #17 was significantly associated with CM (P = 0.003) (Table 7). Figures 12-14 are representative blots showing the significant parasite bands.

Table 5. *Plasmodium falciparum* specific antigens significantly associated with severe malarial anaemia. Analysis done by 2X2 contingency tables Fisher's Exact Test

		SA		Controls		
Pf Antigen	MW	Positive	Negative	Positive	Negative	P value
Ag # 5	113	21	50	6	57	0.004
Ag # 13	109	20	51	8	55	0.03
Ag # 15	91	33	38	12	51	0.001
Ag # 18	85	35	36	17	46	0.01
Ag # 22	73	12	59	1	62	0.002
Ag # 25	65	15	56	2	61	0.002
Ag # 26	62	40	31	24	39	0.03

Note: **SA** - Severe anaemia cases, **Pf** - *P. falciparum* , **Ag** - antigen
MW - approximate molecular weight (kDa)

Table 6. *Plasmodium falciparum* specific antigens significantly associated with cerebral malaria. Analysis done by 2X2 contingency tables Fisher's Exact Test

		CM		Controls		
Pf Antigen	MW	Positive	Negative	Positive	Negative	P value
Ag # 5	113	5	23	0	26	0.05
Ag # 6	109	9	19	1	25	0.01
Ag # 17	87	16	12	5	21	0.005
Ag # 30	40	14	14	5	21	0.02

Note: CM - Cerebral malaria cases, Pf - *P. falciparum* , Ag - antigen
MW - approximate molecular weight (kDa)

Table 7. *Plasmodium falciparum* specific antigens significantly associated with severe malaria. Analysis done by 2X2 contingency tables Fisher's Exact Test

		SA		CM		
Pf Antigen	MW	Positive	Negative	Positive	Negative	P value
Ag # 15	91	33	38	4	24	0.002
Ag # 17	87	17	54	16	12	0.003

Note: SA - Severe anaemia CM - Cerebral malaria cases, Pf - *P. falciparum* , Ag - antigen, MW - approximate molecular weight (kDa)

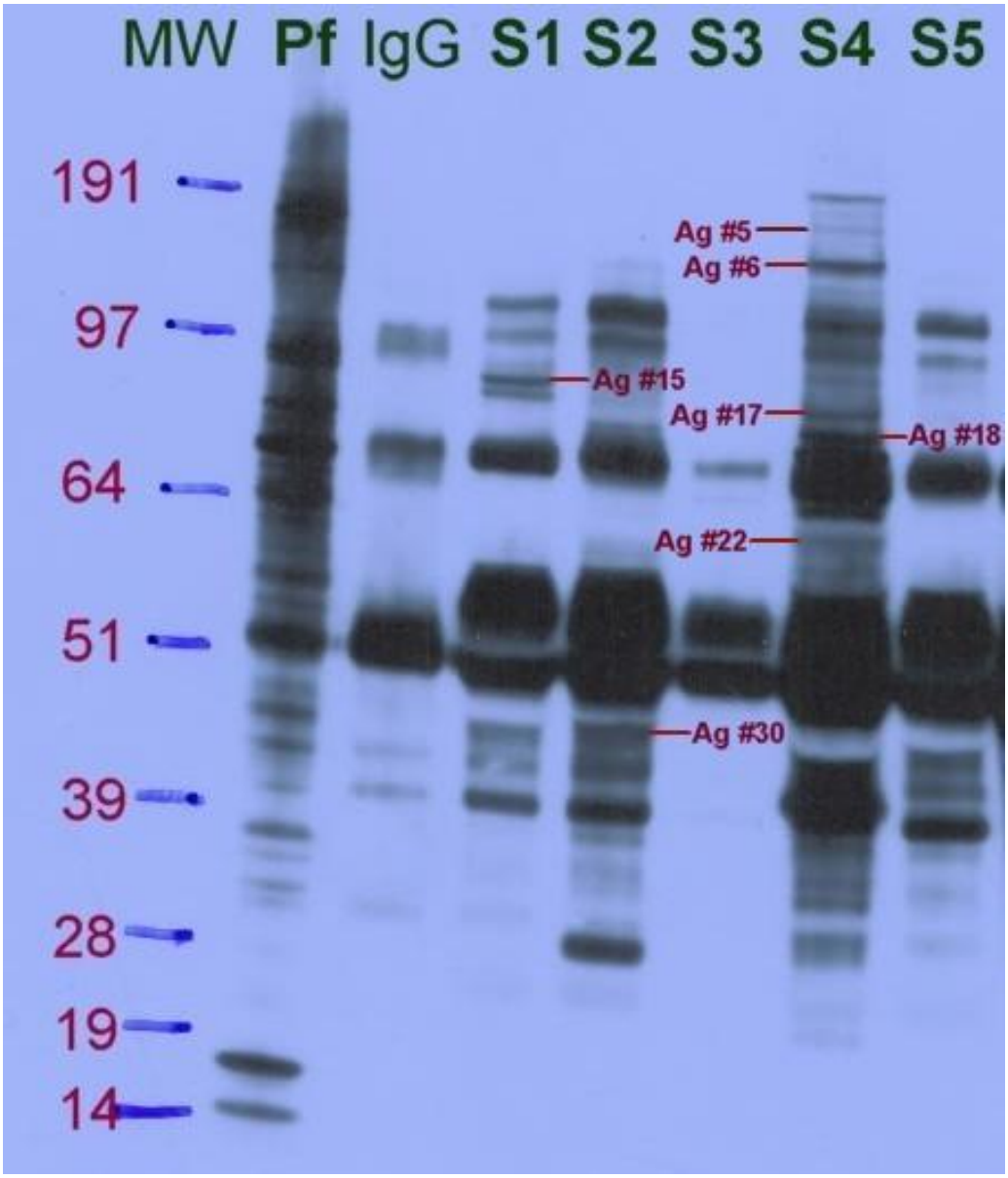


Figure 12. *P. falciparum* antigen profiles of representative test samples showing some of the significant *P. falciparum* parasite bands.
ICs were dissociated, electrophoresed on 1D gel blotted by Western transfer and probed with human hyperimmune serum.
Note lanes: MW- Molecular weight marker, Pf – Crude extract positive control, IgG – human IgG negative control, S1-S5 – test IC samples.

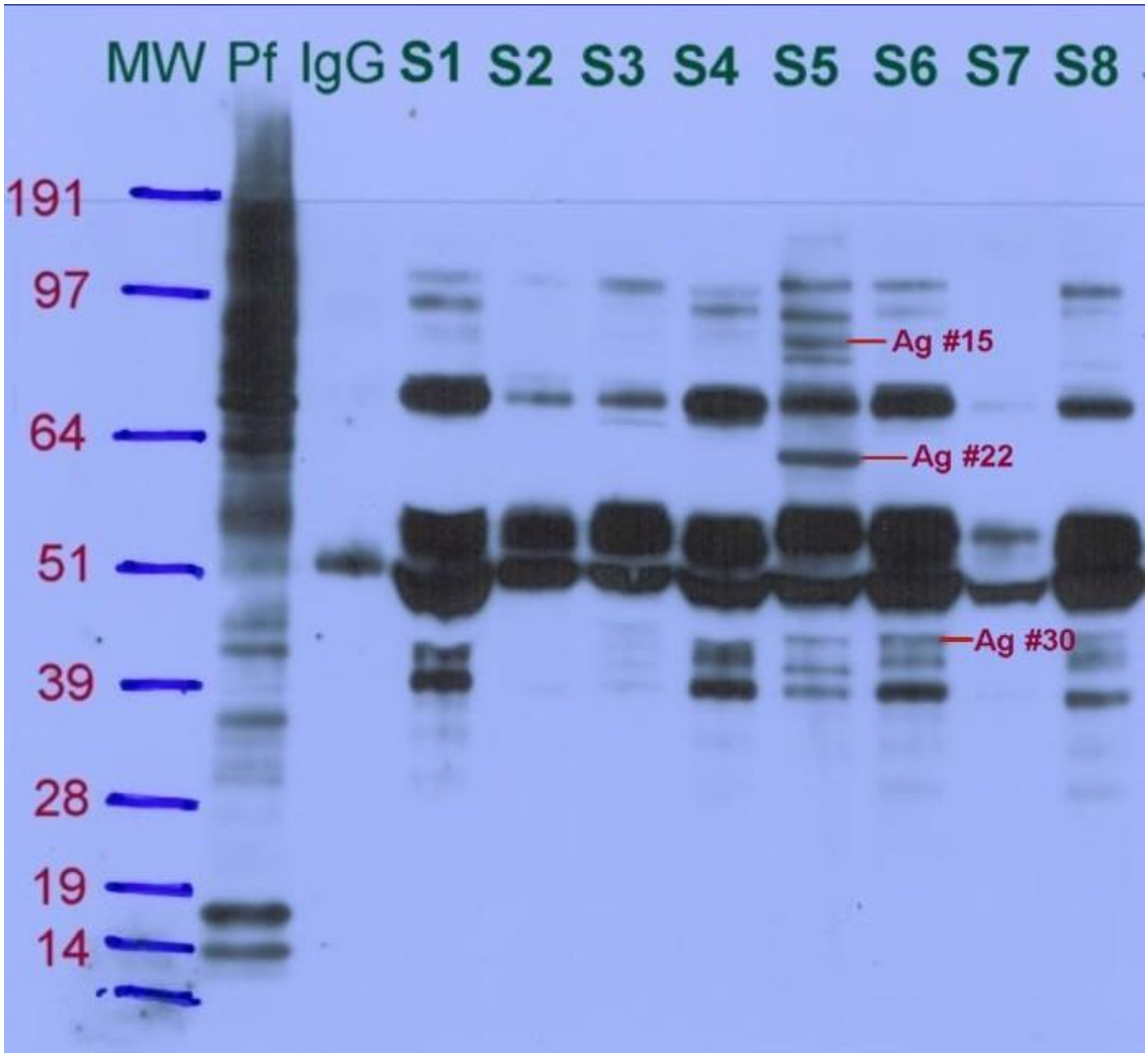


Figure 13. *P. falciparum* antigen profiles of representative test samples showing some of the significant *P. falciparum* parasite bands.
ICs were dissociated, electrophoresed on 1D gel blotted by Western transfer and probed with human hyperimmune serum.
Note lanes: **MW**- Molecular weight marker, **Pf** – Crude extract positive control, **IgG** – human IgG negative control, **S1-S8** – test IC samples.

Having identified specific *P. falciparum* antigen bands which are significantly associated with the severe forms of malaria, some samples which had shown such bands in 1D-gels were further analysed on 2D-gels. This was to isolate the specific spots from other co-migrating proteins and would enable identification through proteomics. *P. falciparum* crude extract was run to get a whole picture of the parasite antigens (Fig. 14). A few selected samples were also processed for 2-DE and analysed and specific

spots were punched out for proteomics. Figure 15 is a representative 2-DE blot while Figure 16 is a representative silver-stained 2-DE gel to reveal *P. falciparum* antigens.

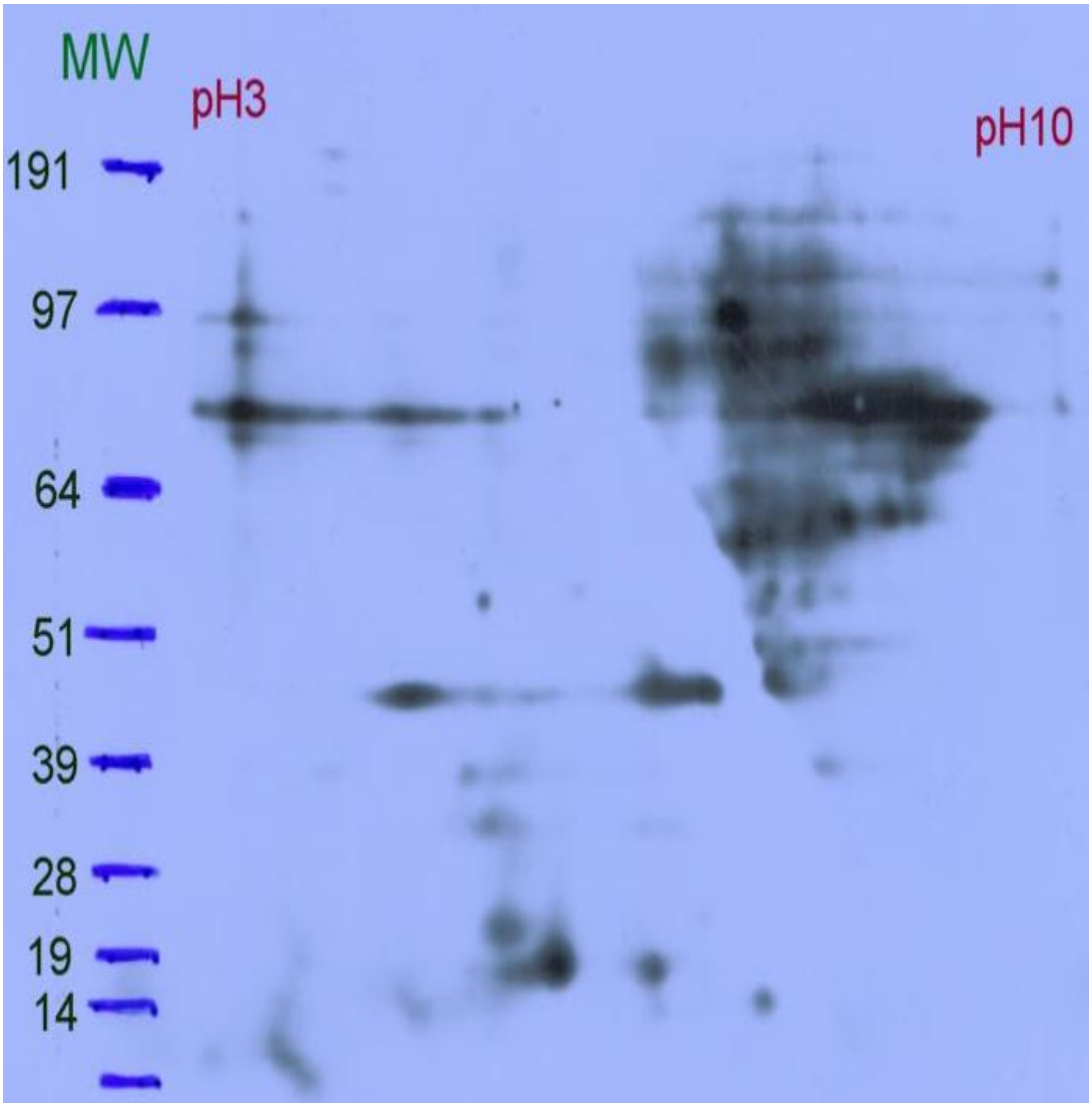


Figure 14. *P. falciparum* crude extract 2-DE antigen profile.
Crude extract was focussed and 2-D PAGE done, blotted by Western transfer and probed with anti-*P. falciparum* antiserum.
Note: MW- molecular weight marker (kDa).

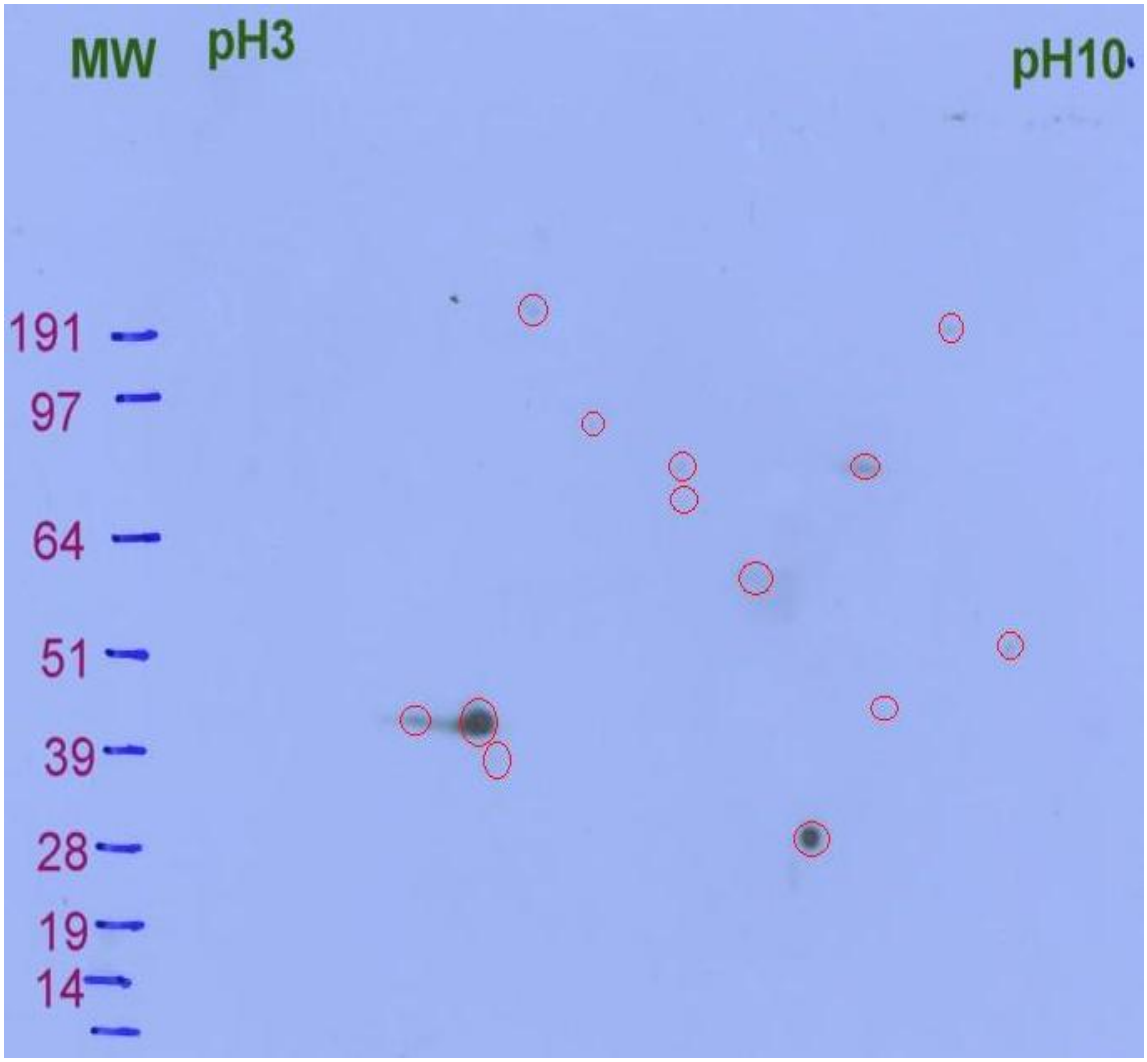


Figure 15. *P. falciparum* antigen profiles of representative SA sample showing some of the significant *P. falciparum* parasite spots.
ICs were dissociated, electro-focussed and 2-DE done, blotted by Western transfer and probed with human hyperimmune serum.
Note: **MW**- Molecular weight marker (kDa).

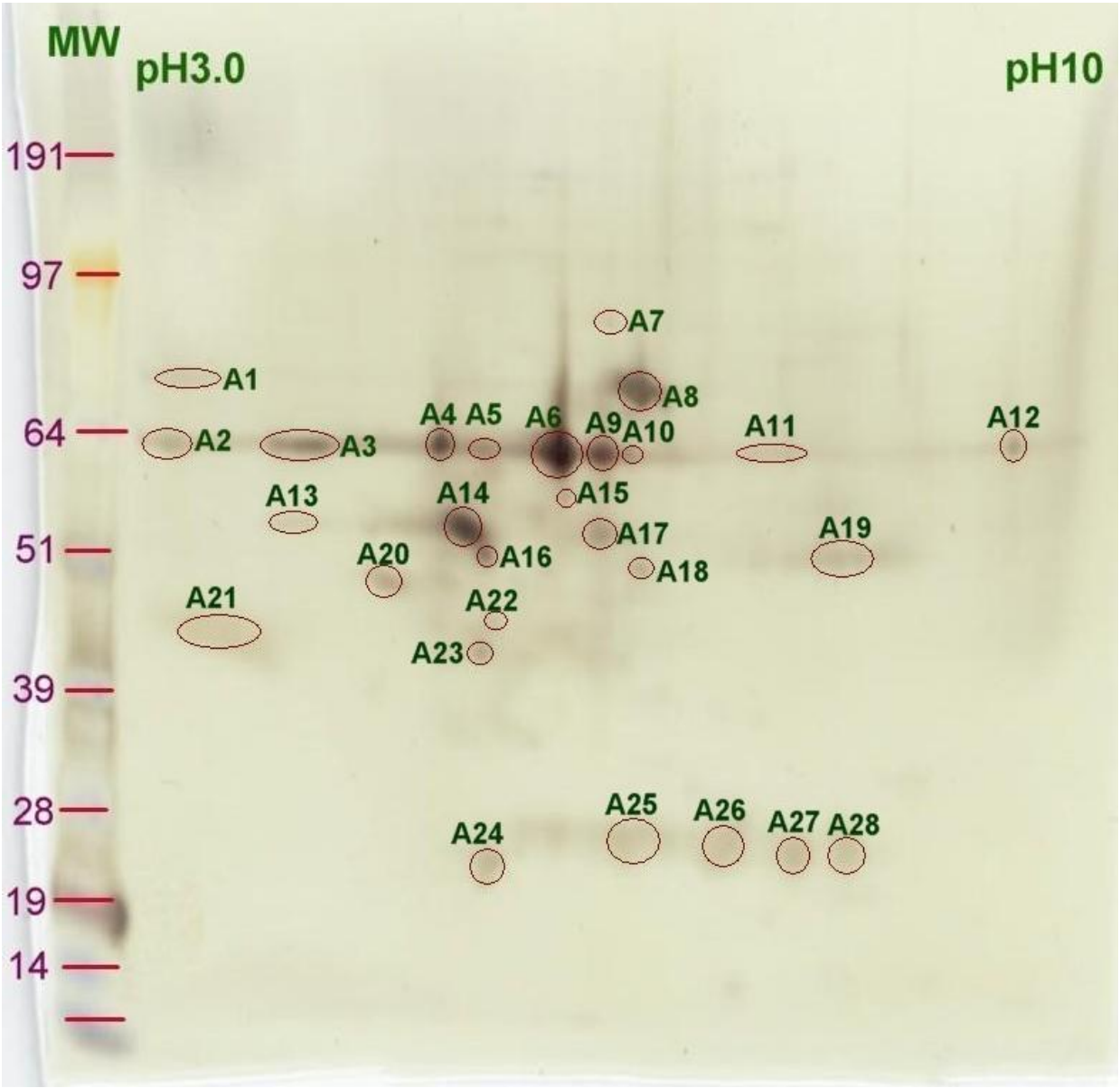


Figure 16. *P. falciparum* antigen profiles of representative SA sample showing some of the significant *P. falciparum* parasite antigens.

ICs were dissociated in glycine-HCl pH 2.0, solubilized in NaOH, IgG/human albumin depleted then electro-focussed and 2-DE done.The gel was then silver stained to reveal the protein spots.

Note: **MW**- Molecular weight marker (kDa).

CHAPTER FIVE: DISCUSSION

5.1 Antibody class/subclass specific IC levels in *P. falciparum* malaria cases and controls

Plasmodium falciparum malaria infection leads to generation of ICs. The role of antibodies in malaria protection is not yet fully clear and the effect of each isotype is not fully understood and largely remains a controversial subject. ICs can lead to cell and end organ damage by their deposition on cell surfaces and by initiating complement cascade resulting in the deposition of complement activation products on erythrocytes and other organs such as kidneys (el Shoura, 1994). Also cross-linking Fc receptors on effector cells like macrophages and monocytes, ICs can stimulate the production of pro-inflammatory cytokines (Virella *et al*., 1995; Nyakeriga *et al.*, 2003) that have been proposed to have a role in the pathogenesis of severe malaria (Clark and Cowden 1992). Despite the accumulating evidence linking antibodies and/or immune complexes to malaria, no clear pattern of association between antibody class or subclass and clinical form of severe malaria has emerged. Furthermore most of the study findings have been based on total or anti-malarial antibody levels and not on complexed antibodies. Therefore the main objective of this study was to determine the composition of IC both quantitatively and qualitatively in terms of antibody class/subclass and and to relate this to severity of *P. falciparum* malaria. It was aimed at analysing the relationship between IC antibody class/subclass and the severity of *P. falciparum* malaria.

In general, IC levels were elevated at visit 1 during enrollment and diminished in all groups in response to malaria treatment (Table 1) which is consistent with previous observations from the same study area (Mibei *et al*., 2005) and suggests that malaria infection leads to IC formation. However, unlike previous observations (Mibei *et al*.,

2005), the decline in IC levels with treatment was not statistically significant for SMA cases. The reasons for this are unclear. One possible explanation is the expansion of the SMA case definition to children with Hgb ≤ 6 g/dL. However, a subgroup analysis of children with Hgb ≤5 g/dL revealed similar results. Another possible explanation is the decreased ability of children with SMA to clear ICs from circulation. Interestingly, it has been reported from the same study area, that red cells of children with SMA have acquired deficiencies in CR1/CD35 (Waitumbi *et al.,* 2000; 2004), which is a key complement receptor that, among other functions, binds opsonized ICs from circulation. Red cells carry ICs bound via CR1 to the liver and spleen where they are removed (Davies *et al*., 1990). Therefore, the decreased ability of children with SMA to clear ICs may be due to the deficiency of red cell CR1. A third possible explanation is that in the previous study the follow-up was four months as opposed to two months in this study. On the other hand, IC levels of children with CM and their controls were lower at follow-up than at enrolment.

Children with SMA, except for high levels of IgG4-containing ICs, did not differ significantly from their controls at enrollment (Figure 3 – Figure 10). This observation was confirmed by the logistic regression analysis which showed that IgG4-containing ICs was the only independent predictor of SMA although the statistical significance was borderline (P = 0.05) (Table 2). The mechanism by which IgG4-containing ICs may increase the risk of SMA is not immediately clear. Antibody-based protective mechanisms like preventing adherent interactions, forms the basis of acquired immunity and is dependent on generation of cytophilic IgG antibody isotypes in the anti-parasite antibody repertoire. Although IgG4 is common on the surface of red cells from patients with malaria and in patients with autoimmune hemolytic anaemia (Dubarry *et al.,* 1993;

Facer, 1980), it is known to be noncytophilic, meaning that it is a poor inducer of phagocytosis (Aase, 1994, Groux, 1990). In addition, IgG4 is a poor activator of the complement cascade and a poor inducer of pro-inflammatory cytokines upon binding to FcγRI (CD64) receptors on macrophages (Foreback *et al.,* 1997; Lucisano and Lachmann 1991). A number of studies have also found an association between high levels of total IgG4 or IgG4 anti-malarial antibodies and increased susceptibility to clinical malaria attacks or severe malaria (Schreiber *et al.,* 2006; Aucan *et al.,* 2000). Conversely, an association between IgG1 and/or IgG3 and protection against uncomplicated and complicated malaria has been observed in several studies (Yone *et al.,* 2005; Tangteerawatana *et al.,* 2007; Sarthou *et al.,* 1997; Aribot *et al.,* 1996; Ndungu *et al.,* 2002). IgG1 and IgG3 subclasses have also been shown to predominate in anti-parasite responses in semi-immune individuals (Cabrera *et al.,* 2004; Piper *et al.,* 1999).

The basis for the protection of IgG1 and IgG3 subclass antibody has been attributed to their efficient cooperation with effector cells hence greater opsonophagocytic activity and their complement activation capacity (Groux and Gysin 1990; Aase, 1994). Unlike non-cytophilic isotypes, IgG3 has been shown to be most efficient for complement activation while IgG1 was more efficient for cell lysis with human complement (Bindon *et al.,* 1988). The role of IgG1 and IgG3 antibodies in binding and activating monocytes, promoting phagocytosis in protected adults and leading to the inhibition of parasite growth has been shown (Bouharoun-Tayoun *et al*., 1990; Bouharoun-Tayoun and Druilhe, 1992). In contrast non-cytophilic IgG4 and IgG2 were found to predominate in non-protected children and primary attack adults (Bouharoun-Tayoun and Druilhe 1992; Wahlgren *et al*., 1983). Protection or

susceptibility to *P. falciparum* malaria in relation to IgG subclasses could be said to be related to interaction with effector and/or accessory cells. IgG molecules interact with the effector and/or accessory cells via FcγRs expressed on the cell surfaces. Interaction between FcγR and IgG antibodies is pivotal in the immune response against infectious agents (Bredius *et al.,* 1994) and since IgG subclasses differ in their affirnity to FcγRs, clinical manifestation of their function will depend upon the properties of the FcγRs as well. Once an IC has been formed its fate is determined by the nature of interaction between it the IC and these cells. Although IgG4 is a weak complement activator, unlike IgG1and 3, binds only FcγRI (CD64) which was shown not to be effective in parasite growth inhibition and clearance (Bouharoun-Tayoun *et al.,* 1990).

5.2 Logistic regression analyses – ICs and association with severe *P. falciparum* malaria

Children with SMA had higher levels of IgG4-containing immune complexes and in a conditional regression analysis, IgG4-containing ICs was the only predictor of SMA at borderline significance (P = 0.05) (Table 2). The mechanism by which IgG4-containing immune complexes may contribute to SMA is not clear. IgG4 has been shown to inhibit the IgG1and IgG3-mediated opsonization of infected erythrocytes (Groux and Gysin, 1990). It is thought to be antagonistic for protective IgG1 and IgG3 antibodies (Bouharoun-Tayoun *et al.,* 1990; Bouharoun-Tayoun and Druilhe, 1992, Groux and Gysin, 1990). It competes with the cytophilic antibodies for the same epitopes and may therefore block cytotoxicity mediated by antibody-activated effector cells (Aucan *et al.,* 2000). In *in vitro* studies, IgG4 was shown to inhibit the IgG1- IgG3-mediated opsonization of infected erythrocytes (Groux and Gysin, 1990). It also blocks antibody-dependent protective mechanisms against *Schistosoma mansoni* and especially

inhibits eosinophil-mediated killing of schistosomula (Khalife *et al.,* 1989). In malaria IgG4 may block cytophilic antibody-dependent cellular cytotoxicity mediated by effector cells like monocytes and this would lead to persistence of the parasite thereby worsening or altering the disease progression.

It is worth to note that IgG4 weakly binds complement and also weakly binds only one FcγR, FcγR1 hence could be said to be poor in initiating the important effector functions of IgG, opsonophagocytosis and cellular cooperation. Therefore, it is possible that the basis of the association between IgG4-containing ICs and increased susceptibility to SMA may be simply the fact that the antibody response is biased against a non-protective subclass or a subclass that blocks protective antibody responses leading to an increase in the risk of repeated infections. The incidence of SMA is highest in areas of the world where there is intense transmission and presumably the greatest likelihood of repeated infections.

Children with CM had higher levels of IgG-containing immune complexes when compared to their controls and also when compared to children with SMA, (Figures 3-10). This is consistent with previous findings from the same study area and the findings of others (Mibei *et al.,* 2005; Adam *et al.,* 1981). When compared to their controls, the most significant difference in subclass antibodies was higher IgG4 in CM cases. Further more children with CM had eleveted IC levels at visit 1 compared to SA and this observation support earlier suggestions that CM patients are unduly susceptible to generating higher levels of ICs (Howard and Gilladuga, 1989). In addition, levels of IgE-containing ICs were higher in CM cases than in their controls. In the conditional logistic regression model, which controlled for the effects of all IC classes and subclasses, total IgG and IgE-

containing ICs were the only independent predictors of CM, (Table 2). Other investigations have revealed an association between total IgE levels and CM and other forms of severe malaria (Calissano *et al.,* 2003; Perlmann *et al.,* 1994). The finding of IgG and IgE deposits in brain capillaries of CM fatalities containing sequestered parasitised red cells is in support of the role of ICs in the pathogenesis of CM (Maeno *et al.,* 2000). It has been proposed that these ICs can lead to local overproduction of TNF-α, a cytokine that has been implicated in the pathogensis of CM (Clark and Cowden, 1992), from monocytes by crosslinking their Fc receptors (Perlmann *et al.,* 1999). This could be achieved by the interaction of ICs carried on red cells sequestered in brain capillaries with Fc receptors on monocytes/macrophages and endothelial cells (Virella *et al.,* 1995; Beynon *et al.,* 1997; Chou *et al.,* 1985). The higher the levels of IgG ICs, the higher the load of ICs on red cells and the greater likelihood of an interaction between red cells and macrophages.

IgE in association with FcεR-bearing effector cells may give rise to reactions which are pathogenic or protective. Studies have shown that cross linking of FcεRII (CD23), by IgE-containing ICs leads to cellular activation resulting in production of nitric oxide (NO) and Tumor necrosis factor-α (TNF-α) (Dugas *et al.,* 1995), the two important effector molecules in malaria. TNF- α has been shown to be an important pathogenic factor in CM causing malaria fever, tissue lesions and haemorrhages (Clark *et al.,* 1991). TNF- α is primarily released from effector cells in presence of IgE-ICs and its concentration was shown to correlate well with mortality from CM (Kwiatkowski *et al.,* 1990). The finding of significantly higher IgE-containing ICs in CM cases compared to controls is consistent with the findings of Perlman *et al.,* (1994). Cross linking of CD23 on monocytes or other cells by IgE-ICs is thought to play a pathogenic role mediated via

pro-inflammatory cytokine pathways (Perlman *et al.,* 1994; Dugas *et al.,* 1995; Perlman *et al.,* 1999). Comparison of IgE and TNF concentration in blood of patients with uncomplicated and severe malaria indicated a positive association with the severity of the disease (Perlmann *et al.,* 1997). This was confirmed by the current study where IgE-ICs were associated with CM severity. Furthermore IgE-ICs have been shown to be potent inducers of Interleukin-4 (IL-4), (Nyakeriga *et al.,* 2003), and serum IgE levels correlate well with IL-4-producing cells in blood (Elghzali *et al.,* 1997), thus plays a vital regulatory role in the switch from TH1 to Th2 cells. This T-helper cell polarization is important as it results in shift from IgM/IgG to IgE secretion by B-cells (Desowitz *et al.,* 1997), secretion of pro-inflammatory cytokines including Il-4 and more importantly such responses may antagonize Th1 responses and cell mediated immunity (Seder and Paul, 1994; Kumaratilake and Ferrante, 1992; Kwiatkowski 1995; Kwiatkowski *et al.,* 1993). This may alter the course of infection significantly in favour of CM.

The finding of an association between IgE-ICs and CM and not SA despite the two groups having similar IgE-IC profile raises a question as to why the difference in manifestation. It seems the nature of immune response in an individual does not only depend on the qualitative composition of ICs nor the specific concentration of the antibodies but may be modified based on parasite virulence (Gupta *et al.,* 1988), the host genetic factors and most importantly level of immunity related to variation in malaria exposure (Ferreira *et al.,* 1996; Marsh *et al.,* 1989; McGuire *et al.,* 1994). CM patients utilized for this study came from an area with seasonal malaria transmission hence their exposure is low compared to SA patients. IgE has also been shown to be associated with decreased parasitaemia (Desowitz, 1989) and was linked with acquisition of immunity with increased parity (Desowitz *et al.,* 1993) thus reflecting a progressive enhancement of

immunity mediated by IgE with parity or with exposure. Therefore, IgE and other antibody classes in SA individuals or exposed individuals may have greater anti-parasite and anti-disease activity while the same antibodies in non-exposed individuals may mediate damage. Further elucidation of such pathogenic and/or protective mechanisms will be of considerable interest for vaccine development and better disease management.

The relationships between ICs and severe malaria were quite different during follow-up compared to the acute malaria period, (Table 3). Although participants during follow-up were negative for malaria by microscopy and did not exhibit any clinical signs of disease, it is reasonable to assume that the ICs detected were residual from the previous malaria infection, and/or derived from continuous antigenic malaria challenge and other infections. These intervening events may be equally important in determining who ultimately develops malaria complications. Whereas during acute malaria IgG-containing ICs were somewhat protective against SMA, (Table 2), total IgG IC seemed to be deleterious at follow-up, (Table 3). This observation is consistent with the argument that continuous antigenic stimulation and IC formation can lead to complement activation and erythrophagocytosis that are key for the development of SMA (Stoute, 2005). On the other hand, the presence of IgE-containing ICs seemed to be protective against SMA. Anti-malarial IgE has been observed to be protective from subsequent infections in a longitudinal study (Bereczky *et al.,* 2004) and again suggests that the avoidance of recurrent malaria infections is important in avoiding SMA. Furthermore, since the parasite-induced switch from IgM/IgG to IgE production appears to occur late during an immune response (Helmby *et al.,* 1996; Desowitz *et al.,* 1997), it may be said to be associated with an increase in avidity. In any event the binding of IgE antibodies to many parasite antigens is easily detectable *in vitro* despite the large excess of IgG (Perlmann *et*

al., 1994). The association between IgG or IgE-containing ICs and CM disappeared during convalescence. Instead, a surprisingly protective effect from non-cytophilic and non-complement activating ICs (IgG2 and IgA) was seen whereas the presence of IgG1-containing ICs was found to be a risk factor.

The role of IgG2 in protection cannot be fully explained. Studies have suggested a role for IgG2 mediated by FcγRIIa (Deleron *et al.,* 1997; Bouharoun-Tayoun *et al.,* 1995). This low affirnity FcγR was shown to be involved in growth inhibition of *P. falciparum* but not the high affirnity FcγRI (Bouharoun-Tayoun *et al.,* 1995). It seems binding of low affirnity FcγRIIa-H131 by IgG2 complexed to malaria antigens initiates a more efficient immune response by monocytes or other effector cells. The cumulative effect of this binding may contribute to more efficient parasite clearance mechanisms and overall better disease management which could account for the current observation. This is supported by studies by Deloron *et al.,* (1997) which showed higher IgG2 levels were associated with low risk of acquiring *P. falciparum* malaria from birth to 6 months. IgG2 and IgG3 against conserved *P. falciparum* epitopes have been shown to increase with age in malaria endemic regions (Aribot *et al.,* 1997). Furthermore FcγRIIa-131H/H was associated with low *P. falciparum* density in Kenyan infants (Shi *et al.,* 2001), thus suggesting IgG2 is crucial in acquired immunity to clinical *P. falciparum* malaria. As to why the same was not observed during visit 1 cannot be easily explained.

In summary, these findings suggest that although both SMA and CM are characterized by high levels of ICs, the class and subclass make-up of these ICs as well as the role they play in each may be distinct. While CM is characterized by high levels of IgG and IgE-containing ICs that may serve to stimulate local production of pro-

inflammatory cytokines in sequestered brain capillaries, SMA is characterized by persistent lower level IgG-containing ICs in the intervening period between malaria attacks, the sub-class make up of which may interfere with adequate immune responses and may allow repeated infections.

5.3 *P. falciparum* specific antigens in immune complexes associated with severe malaria

Soluble malarial antigens as well as antibodies to various plasmodial constituents have been demonstrated in the sera of patients infected with either *P. falciparum* or *P. malariae* (Shepherd *et al*., 1982; Adam *et al.,* 1981; Blackman and Holder 1992; Camus and Hadley 1985; Jakobsen *et al.,* 1993). These antigens may be available for binding by reactive antibodies (Mohamed, 1982). This sets the stage for the formation of CICs (Jhaveri *et al.,* 1997; Tyagi and Biswas, 1999) and i*n vivo* complement activation leading to hypocomplementamia which are more common in patients with malaria complications such as severe anaemia, cerebral malaria and thrombocytopenia of *P. falciparum* (Adam *et al.,* 1981). Furthermore, repeated exposure to parasite antigens contribute to development of acquired immunity to malaria (Snow *et al*., 1997). Immunity to malaria only occurs after many years of recuring infections. This is believed to be due to antigenic variation and the time taken by individuals to develop immunity to invariant parts of otherwise very polymorphic antigens. Immunity to malaria manifests as lessened disease symptoms and lower parasitaemia. A large body of evidence shows that antibody responses against *P. falciparum* variable antigens on the surface of RBC contribute a lot to acquired immunity against malaria. (Bull *et al.,* 1998; Dodoo *et al.,* 2001; Marsh *et al.,* 1989; Tebo *et al.,* 2002). Furthermore, adhesion of infected RBC to vascular endothelium

via these variable antigens is thought to contribute to the pathogenesis of malaria (Groux and Gysin, 1990; Tebo *et al.,* 2002; Schreiber *et al.,* 2006).

Several protective malaria antigens were isolated from immune complexes and the significant *P. falciparum* antigens were different in CM and SMA cases (Table 5 – Table 7). This finding may point to different immunological responses in the two categories. Although several antigens detected were shared across the clinical categories, there were those which were found to be characteristic to a particular group. Severe malarial anaemia when compared with symptomatic controls, was significantly associated with the following *P. falciparum* antigens; Ag #5, ($P = 0.004$), Ag #13 ($P = 0.03$), Ag #15 ($P < 0.001$), Ag #18 ($P = 0.01$), Ag #22 ($P = 0.002$), Ag #25 ($P < 0.001$) and Ag #26 ($P = 0.03$) (Table 5). While the following antigens were significantly associated with cerebral malaria; Ag #6 ($P = 0.01$), Ag #17 ($P = 0.005$) and Ag #30 ($P = 0.02$) (Table 6).

When SA and CM were compared together, it was found that a 91Kda antigen (Ag #15), was highly associated with SA ($P = 0.002$), while a slightly lighter antigen of about 87Kda (Ag #17) was significantly associated with CM ($P = 0.003$) (Table 7). As to whether the two antigens are related or different remains to be seen. Identification of these various antigens will be of paramount importance as it will enable purification of such antigens which elicit higher levels of protective immune responses thus being considered as vaccine candidates.

Malaria antigen analysis is vital in helping to understand and profile the various antigens in relation to their contribution to protection and/or pathogenesis of the disease.

Malaria infection is complex and involves many inflammatory responses which may enhance cell to cell interractions (cytoadhearance) and cell stimulation involving both malaria-derived antigens or toxins and host derived factors such as antibodies and cytokines (Wahlgren 1986; Cubas *et al*., 1994; Hommel *et al*., 1996; Jakobsen *et al*., 1995). Malaria antigens and toxins are mostly stage-specific. It has been observed that during malaria infection, the peaks of parasitaemia fluctuate over time (Miller *et al.*, 1994). This is due to presentation of different antigenic determinants and this is as survival strategy of the parasite. Merozoites and late stage trophozoites or schizonts undergo antigenic variation thus rendering the host immune responses ineffective. This varietion originates from genetic recombination in the mosquitoes and also through existance of several variable antigens gene families in the genome of the parasite. The large resorvoir of variant antigens give the parasite the ability to avoid specific and non-specific immune clearance by the host (Chen *et al.*, 2000).

Several blood stage antigens may be the target of protective antibodies and some of which have been correleted with clinical immunity. Such malaria antigens have been included in malaria vaccine preparations in humans (Matuschewski and Muller, 2007). RESA, MSP-1 and MSP-2 were shown to be targets of protective immunity in experimental models (Collins *et al.*, 1986; Ling *et al.*, 1994; Saul *et al.*, 1992; Goshnick *et al.*, 2004,) and also in humans (Taylor *et al.*, 1998). These antigens are also recognised by naturally acquired antibody (Perlmann *et al.*, 1989; Riley *et al.*, 1992; Taylor *et al.*, 1995).

Malaria toxins are parasite derived molecules which induce the human host to over produce serum-bound factors and include parasite-derived molecules secreted or

released from parasite at late stages (Trophozoites and schizont stages) and are contained among the glycosylphosphatidylinositol (GPI)-anchored proteins. Parasite products either directly damage host tissues or more importanly stimulate the overproduction of host cytokines (Jakobsen *et al.*, 1995; Schofield and Hackett, 1993). Studies have shown that *P. falciparum* cultures contain antigens which stimulate the secretion of TNF-α and IL-1 and other cytokines from various host cells (Bate *et al.*, 1989; Wahlgren *et al.*, 1995). Moderate amounts of cytokines such as TNF-α, IFN-γ and IL-1 are necessary for the human host in fighting invading microorganisms but over induction of host cytokines can vary the disease pathogenesis. High circulating levels of TNF- α and high fever occur at the rapture of schizonts and this suggests antigens from schizonts might have potential toxic effects (Karunaweera *et al.*, 1992). Also *P. falciparum* metabolite hemozoin released after schizont rapture is toxic and has been suggested to induce IL-2 production (Ockenhouse *et al.*, 1992).

Previous studies on human antibody responses to malaria have focused on the antigenic specificity of the response but it is increasingly becoming apparent that it is important to consider the quality of the immune response. This involves both the affirnity of the antibody for the antigen and the nature of Fc which determines the effector functions of the antibody including antibody mediated cellular cytotoxicity which has been described as a potentially important mechanism of anti-malarial immunity. It has also been proposed that a predominance of IgG1 and IgG3 is required for an effective anti-malarial immunity (Groux and Gysin, 1990). Thus an ideal vaccine candidate should be one which elicits the appropriate antibody class/subclass and also an effective cell mediated immunity.

These results together with complete antigen analysis and identification will guide future studies and enable picking those specific antigens which stimulate the appropriate immune response in the human host and will guide on malaria vaccine development as has been shown by the ability of different antigens to stimulate different arms of the immune system with varrying levels of protection.

CHAPTER SIX: SUMMARY OF CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

a) This study has revealed an association between immune complexes and severe malaria especially IgG4-ICs and SMA and also IGE-ICS and CM. This underscores the potential mechanisms underlying the pathogenesis of the severe forms of malaria disease. Furthermore, these mechanisms may be distinct in the two clinical groups and this suggests IgG4-ICs and IgE-ICs could be used as markers for SMA and CM respectively.

b) The study demonstrated association between the antigens and the severity of malaria but the specific antigens involved remains unknown. This finding underscores the need for comprehensive characterization of these antigens to assist in identifying potential vaccine candidates.

c) Based on the findings of this study, the pathophysiology of malaria is very complex and may involve the contribution of both the host antibody as well as the parasite antigen. These findings have highlighted the role ICs play in malaria hence agents that could block the interraction of ICs with cell surface FcRs may represent novel approaches for managing CM and SMA.

6.2 Suggestions for future research work

a). This study has demonstrated differences in antibody class/subclass that participates in formation of immune complexes which contribute in severe malaria pathologies. The specific mechanisms responsible for malaria pathophysiology is unclear, thus there is need to further investigate these immune complexes mechanistically in order to shed more light on the exact mechanisms anti-malarial antibodies complexed with malaria antigens contribute to modifying the course of infection.

b). This study has clearly demonstrated that there are both qualitative and quantitative differences in immune complexes from children with severe *P. falciparum* malaria compared to those without. It would be interesting to execute a similar study but using cross-sectional samples so as to include the element of age. This will enable pinpointing the most important qualitative differences at the age most severely affected by *P. falciparum* malaria.

c). Based on the findings of this study and other studies done earlier, the differences in manifestation, it is not yet conclusively clear what is responsible for the difference in clinical manifestation of severe *P. falciparum* malaria that is, cerebral malaria and severe malarial anaemia. Other studies have pointed to age difference, while others have implicated parasitaemia level, endemicity in relation to exposure and level of immunity, has also been implicated as well as cytokine and complement functioning. It would be necessary to come up with other studies to shed further light on this issue.

d). IgA and IgG2 were found to be protective against celebral malaria yet the two are are non-cytophilic and non-complement activating. Their role in protection deserves further investigation.

e). This study identified specific *P.falciparum* antigens that are associated with severe malaria. Their full characterization is important so as to enable selection of parasite antigens as candidate antigens in malaria vaccine development.

6.3 Limitations of the study

The study was intended to include proteomics analysis of the various malaria antigens isolated from the immune complexes. This however could not be achieved due to funding issues which made it difficult to carry out the analysis abroad for this could not be done locally.

The other limitation of the study was lack of additional functional assays to support the identification of immune complex isotypes and antigen characterization. The additional functional assays would have elucidated the effector functions induced and associated pathology. This would have enriched the findings and give a mechanistic explanations of the observed differences between the different malaria clinical groups.

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APPENDICES

Appendix 1 Reagents

Phosphate buffered saline

Dissolve

0.46g $NaH2PO_4$, 2.3g Na_2HPO_4, 18.0g NaCL in 1 litre Distilled de-ionized water, adjust pH to 7.4, filter sterilized and store at 4^oC.

Can also be prepared from pre-prepared packs also

Wash buffer

PBS/ 0.5% Tween- Take 2.5 ml of Tween and mix with 47.5ml of PBS

Blocking buffer

PBS, 0.5% boiled casein, 1% Tween, 0.01% Thimerosal, 20 μg/ml phenol red-Dissolve 5g of Casein in 300ml of deionized water, add 2ml of 5N NaOH

-Boil, allow to cool

-add 100ml 10X PBs

-add 0.1g thimerosal

-add 0.02g phenol red

-adjust pH to 7.4 and top up volume to 1 litre with deionized water

Appendix 2 Published Paper

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Distinct pattern of class and subclass antibodies in immune complexes of children with cerebral malaria and severe malarial anaemia

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SUMMARY

Plasmodium falciparum *infection can lead to deadly complications such as severe malaria-associated anaemia (SMA) and cerebral malaria (CM). Children with severe malaria have elevated levels of circulating immune complexes (ICs). To further investigate the quantitative differences in antibody class/subclass components of ICs in SMA and CM, we enrolled 75 children with SMA and 32 children with CM from hospitals in western Kenya and matched them to 74 and 52 control children, respectively, with uncomplicated symptomatic malaria. Total IgG IC levels were always elevated in children with malaria upon enrolment, but children with CM had the highest levels of any group. Conditional logistic regression showed a borderline association between IgG4-containing IC levels and increased risk of SMA (OR = 3·11, 95% CI 1·01–9·56, P = 0·05). Total IgG ICs (OR = 2·84, 95% CI 1·08–7·46, P = 0·03) and IgE-containing ICs (OR = 6·82, OR 1·88–24·73, P ≤ 0·01) were associated with increased risk of CM. These results point to differences in the contribution of the different antibody class and subclass components of ICs to the pathogenesis of SMA and CM and give insight into potential mechanisms of disease.*

Keywords *cerebral malaria, circulating immune complexes, malaria, anaemia*

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INTRODUCTION

Plasmodium falciparum is the most lethal malaria parasite of humans. Most deaths occur due to complications such as severe malaria-associated anaemia (SMA) and cerebral malaria (CM). Malaria infection leads to development of malaria-specific antibodies and to antigenemia (1). Consequently, immune complexes (ICs) form during malaria infection (2,3). ICs can activate complement and deposit in tissues stimulating an inflammatory response (4). Therefore, some investigators have suggested a possible role for ICs in the pathogenesis of SMA and CM (5,6).

In a previous study, we showed increased levels of ICs in children with severe malaria but no clear differences between children with SMA and CM (6). Because immunoglobulin classes and subclasses differ in their ability to interact with Fc receptors on macrophages, activate the complement cascade and stimulate pro-inflammatory cytokines (7–9), we reasoned that ICs from patients with different forms of clinical malaria may differ in the amounts of antibody class and subclass components they contain. Therefore, in the present study, we investigated the association between the amount of antibody class and subclass components of ICs and the risk of SMA and CM.

MATERIALS AND METHODS

Study design and patient population

Participants were recruited under a human use protocol approved by the Human Use Research Committee, the Walter Reed Army Institute of Research, Silver Spring, MD, USA, and the National Ethics Review Committee of the Kenya Medical Research Institute, Nairobi, Kenya. Informed consent was obtained from all parents or guardians. The study had a matched case-control design. In comparison to our previous studies, we modified the case definition for

SMA cases from Hb ≤ 5 g/dL to Hb ≤ 6 g/dL to increase the probability of finding red cells with complement (C3) deposition in support of other studies (10). Thus, SMA cases, defined as children with asexual *P. falciparum* parasitemia by Giemsa-stained thick and thin blood smear and Hb ≤ 6 g/dL, were recruited from the paediatric ward of the Nyanza Provincial General Hospital (NPGH), Kisumu, Kenya, where malaria is holoendemic. Because CM is uncommon in this area, CM cases were recruited from the paediatric ward of the Kisii District Hospital (KDH), as well as from the NPGH. KDH is located in the highlands of western Kenya where transmission is seasonal and consequently receives many more CM cases than the NPGH (11). CM was defined as asexual *P. falciparum* parasitemia by Giemsa-stained blood smear and a Blantyre coma score of ≤ 2 (12), lasting at least 30 min if there was a history of convulsions. Symptomatic uncomplicated malaria controls matched by gender and age ± 2 months were assigned to each case at a case:control ratio of 1 : 1 for SMA and 1 : 1–2 for CM, and were identified from the outpatient clinic of the same hospital where the corresponding case was recruited. Controls were defined as children with a normal mental status, a Hb > 6 g/dL, a Giemsa-stained blood smear positive for asexual *P. falciparum* and an axillary temperature = 37·5°C. In the absence of fever, we required two of the following signs or symptoms: nausea/vomiting, irritability, poor feeding, myalgias or headache. General exclusion criteria also included evidence of concomitant serious infections (i.e. meningitis excluded by lumbar puncture when indicated, pneumonia, sepsis), chronic illness or a history of blood transfusion in the 3 months preceding enrolment to avoid the influence of donor erythrocytes in our measurements.

All study participants were evaluated in a standardized fashion at enrolment (visit 1) and at follow-up (visit 2) 2 months later. If a child failed to return for follow-up, a field worker travelled to his/her last known domicile to determine his/her status. During follow-up, a blood sample was obtained once it was confirmed that the child was asymptomatic and free of parasitemia. If malaria persisted at the first follow-up visit, the child was re-treated and re-evaluated 2 weeks later. Inpatient treatment for malaria consisted of IV quinine and outpatient therapy was with artemether/lumefantrine (13).

Collection and processing of blood samples

Giemsa-stained thick and thin blood smears were prepared from capillary blood obtained by finger prick. A 2·5-mL sample of EDTA-anticoagulated venous blood was obtained at enrolment and 5 mL at follow-up. Following measurement of haemoglobin levels, the EDTA-anticoagulated blood was centrifuged and the plasma was stored at −70°C until later use.

Measurement of circulating ICs

The methodology for measurement of total IgG ICs was described in detail before (6). Briefly, we used a C1q-based ELISA assay. Wells of Immulon II HB 96-well plates (Thermo Labsystems, Helsinki, Finland) were coated overnight with 10 μg/mL of C1q (Sigma-Aldrich, St Louis, MO). Aggregated IgG, prepared from purified human IgG (Sigma-Aldrich) by heating at 63°C for 30 min followed by size fractionation over a Sephacryl S-300 70 × 2·6 cm column (Amersham Pharmacia Biotech, Piscataway, NJ), served as a standard. Control and test plasma were diluted 1 : 50 in dilution buffer (PBS/0·5% boiled casein, 0·025% Tween, 0·01% Thimerosal, 20 μg/mL phenol red) and 100 μL added to duplicate wells followed by incubation for 1 h at room temperature. The wells were emptied and washed four times with wash buffer (PBS, 0·025% Tween). Total IgG was detected by using horse radish peroxidase (HRP)-conjugated goat antihuman IgG (Kirkegaard & Perry, Baltimore, MD) at dilution of 1 : 3000. For detection of antibody subclasses we used biotin-labelled monoclonal antibodies against human IgG1 (Clone 8c/6-39, Sigma-Aldrich) at a dilution of 1 : 8000, IgG2 (Clone G18-21, Becton-Dickinson, Brussels, Belgium) at a dilution of 1 : 1000, IgG3 (Clone HP6047, Zymed Laboratories, South San Francisco, CA) at a dilution of 1 : 2000 and IgG4 (Clone JDC-14, Becton-Dickinson) at a dilution of 1 : 4000. After incubation, the wells were emptied and washed four times with wash buffer. HRP-conjugated streptavidin (Sigma-Aldrich) was diluted 1 : 6000 in dilution buffer and 100 μL was added to each well followed by 30 min incubation at room temperature. After washing four times, 200 μL of ABTS substrate (Kirkegaard & Perry) was added to each well and incubated for 30 min followed by measurement of the $OD_{415\,nm}$. Total IgG and subclass IC levels were expressed as micrograms of aggregated human IgG equivalent per ml (μg AHG /mL). Calculation of each subclass concentration in the standard was based on the approximate percentage concentration of each IgG subclass (14). Positive and negative control samples were used in every plate. Plate-to-plate variation was controlled by normalizing to the positive control sample by using the following formula

$$ICc(\mu gAHGEq/mL) = ICuc \times Cm/Cp$$

where ICc is the corrected IC level of the sample, ICuc is the uncorrected IC level, Cm is the average concentration of the IC for the positive control for the study and Cp is the

concentration of the positive control for the plate in which the sample was tested.

To detect IgA and IgE-containing ICs, a sandwich ELISA assay was used. In order to rule out detection of free Igs, ICs were precipitated from plasma by adding 100 µL of 4% PEG (w/v) solution (PEG 6000, Fluka, St Louis, MO) to 100 µL of plasma diluted 1 : 5 in borate buffer pH 8·5 (Pierce, Rockford, IL). This was mixed well and incubated overnight at 4°C followed by centrifugation at 5000 r.p.m. for 10 min at 4°C. The IC precipitate was washed twice with 200 µL 2% PEG solution. IC was then re-suspended in 100 µL borate buffer pH 8·5 and stored frozen until used. Wells of Immulon II HB 96-well plates (Thermo Labsystems) were coated with 100 µL of goat antihuman IgE antibody or antihuman IgA (Kirkegaard & Perry) diluted 1 : 2000 in dilution buffer and incubate overnight at 4°C. The plate was washed four times with wash buffer (0·25% Tween 20 in PBS pH 7·4) and then blocked for 1 h with 200 µL of blocking buffer (PBS, 0·5% boiled casein, 1% Tween, 0·01% Thimerosal, 20 µg/mL phenol red). After four washes, 100 µL of IgE or IgA standard (Sigma-Aldrich) at various dilutions, control samples or PEG-precipitated test samples diluted 1 : 50 in dilution buffer was added to duplicate wells and incubated for 1 h at room temperature. This was followed by four washes with wash buffer and addition of 100 µL of HRP-conjugated goat antihuman IgE or IgA (Sigma-Aldrich) diluted 1 : 500 or 1 : 3000, respectively, in dilution buffer and incubation for 1 h at room temperature. Colour development, absorbance measurement and normalization were as described for IgG above. The quantities were expressed as µg of human immunoglobulin Ig/mL.

Statistical analysis

Statistical analysis was performed using SPSS for windows version 11·5 (SPSS Inc., Chicago, IL) software package. Mean IgG, IgG subclass, IgE and IgA-containing IC levels were compared between cases and controls by multivariate ANOVA with matching and adjustment for home districts. Because most multiple comparison procedures include more comparisons than was of interest *a priori*, increasing the probability of a Type II error, we used a primary analysis that consisted of pairwise comparisons using least squares. A secondary analysis consisted of correction for multiple comparisons using Tukey's honestly significant difference test. Comparison of IC levels between SMA and CM cases at enrolment and follow-up was done using Mann–Whitney *U*-test due to unequal variances. Multivariate conditional logistic regression controlling for district of residence, and haemoglobin level where appropriate, was carried out to determine the association of antibody class/subclass and severity of malaria at enrolment and follow-up. To make meaningful associations, the IC levels were converted to standard deviation (SD) units by dividing by SD of antibody class or subclass for each cohort. All tests were two-tailed with $\alpha \leq 0{\cdot}05$.

RESULTS

Demographics

Seventy-five SMA and 32 CM cases were enrolled and matched to 74 and 52 symptomatic uncomplicated malaria controls, respectively. The demographics and clinical characteristics of the study participants were recently reported (10). The mean age (SD) for SMA cases was 16·9 (13·7) months and that of their controls was 16·8 (13·3) months. The same parameters for CM cases and their controls were 33·1 (19·2) and 33·6 (16·4) months, respectively. There were no significant differences between cases and controls in the districts of origin, ethnic origin or parasite density (10). There were also no significant differences in the parasite densities between CM and SMA cases at enrolment (10). Sixty-five SMA cases and 61 of their uncomplicated malaria controls presented for follow-up. There were five deaths among patients with SMA (6·7% mortality), all of which occurred during the initial hospitalization. There was one death among the SMA controls (1·3% mortality), which was due to an episode of severe diarrhoea. Twenty-seven CM cases and 44 of their controls presented for follow-up. One in-hospital death occurred among CM cases recruited at KDH (4·2% mortality) and no deaths occurred among CM cases at NPGH. Most of the missed follow-up visits were due to the parents declining further participation in the study or moves out of the study area.

IC levels

Comparison of IC levels between enrolment and follow-up

Table 1 summarizes the IC levels among cases and controls at the time of enrolment (visit 1) and follow-up (visit 2). The number of samples actually tested was slightly smaller than the total number of cases and controls due to insufficient volume of some samples. ICs were nearly always higher at enrolment than at follow-up. This is consistent with previous observations of increased level of ICs during malaria infection (6). However, unlike our previous observations (6), the decline in IC levels with treatment was not statistically significant for SMA cases. The reasons for this are unclear. One possible explanation is the expansion of the SMA case definition from the traditional Hb ≤ 5 g/dL to Hb ≤ 6 g/dL. However, a subgroup analysis of children with Hb ≤ 5 g/dL revealed similar results (data not shown).

Table 1 Mean (SD) IC levels among cases and controls at enrolment and follow-up

	SMA cases				SMA controls				SMA cases vs. controls			
			Visit 1 vs. visit 2				Visit 1 vs. visit 2		Visit 1		Visit 2	
IC type[a]	Visit 1 $N = 74$	Visit 2 $N = 63$	Mean difference 95% CI	P	Visit 1 $N = 70$	Visit 2 $N = 59$	Mean difference 95% CI	P	Mean difference 95% CI	P	Mean difference 95% CI	P
IgG	4·1 (3·5)	3·5 (5·6)	−0·9 to 1·5	0·61	3·9 (1·3)	2·7 (3·1)	0·0 to 2·5	0·04	−1·1 to 1·4	0·82	−0·2 to 2·5	0·11
IgG1	1·0 (1·8)	0·6 (1·8)	−0·3 to 0·6	0·49	1·0 (1·4)	0·6 (1·0)	−0·01 to 0·85	0·06	−0·5 to 0·3	0·73	−0·3 to 0·7	0·41
IgG2	0·1 (0·2)	0·1 (0·2)	−0·04 to 0·04	0·94	0·2 (0·2)	0·1 (0·2)	−0·01 to 0·07	0·12	−0·07 to 0·01	0·14	−0·04 to 0·04	0·99
IgG3	0·5 (0·6)	0·4 (0·7)	−0·1 to 0·3	0·54	0·4 (0·7)	0·3 (1·1)	−0·1 to 0·4	0·27	−0·2 to 0·2	0·85	−0·2 to 0·3	0·52
IgG4	0·2 (0·3)	0·1 (0·2)	0·0 to 0·1	0·04	0·1 (0·2)	0·1 (0·1)	−0·01 to 0·11	0·12	−0·02 to 0·11	0·14	−0·04 to 0·10	0·36
IgA	4·0 (3·0)	3·4 (2·8)	−0·6 to 2·1	0·30	5·3 (6·3)	4·0 (4·1)	0·2 to 3·0	0·02	−3·0 to −0·2	0·03	−2·2 to 0·9	0·38
IgE	0·2 (0·2)	0·2 (0·1)	−0·03 to 0·05	0·53	0·2 (0·2)	0·2 (0·1)	−0·02 to 0·06	0·42	−0·06 to 0·02	0·42	−0·06 to 0·03	0·58
	CM cases				CM controls				CM cases vs. controls			
			Visit 1 vs. visit 2				Visit 1 vs. visit 2		Visit 1		Visit 2	
	Visit 1 $N = 32$	Visit 2 $N = 25$	Mean difference 95% CI	P	Visit 1 $N = 49$	Visit 2 $N = 40$	Mean difference 95% CI	P	Mean difference 95% CI	P	Mean difference 95% CI	P
IgG	8·5 (4·7)	4·9 (3·4)	1·2 to 6·2	< 0·01[b]	6·6 (4·0)	3·3 (2·3)	1·3 to 5·2	< 0·01[b]	0·2 to 5·0	0·03	−0·3 to 4·8	0·09
IgG1	3·2 (2·4)	1·3 (1·8)	0·2 to 3·2	0·03[b]	2·8 (3·4)	0·4 (0·5)	1·2 to 3·6	< 0·01	−0·7 to 2·1	0·33	−0·1 to 3·0	0·07
IgG2	0·6 (1·0)	0·2 (0·3)	0·0 to 0·5	0·02[b]	0·3 (0·3)	0·2 (0·3)	−0·1 to 0·3	0·25	−0·01 to 0·46	0·07	−0·2 to 0·3	0·72
IgG3	0·8 (1·3)	0·3 (0·4)	0·1 to 1·0	0·01[b]	0·6 (0·7)	0·2 (0·3)	0·2 to 0·8	< 0·01[b]	−0·2 to 0·6	0·26	−0·2 to 0·6	0·42
IgG4	0·5 (0·8)	0·2 (0·2)	0·0 to 0·5	0·04	0·3 (0·4)	0·1 (0·1)	−0·1 to 0·3	0·20	0·1 to 0·5	0·02	−0·1 to 0·3	0·25
IgA	7·8 (7·9)	5·3 (4·0)	−1·6 to 5·2	0·30	7·6 (7·0)	6·1 (4·8)	−1·3 to 4·1	0·30	−3·4 to 3·0	0·91	−1·3 to 4·1	0·74
IgE	0·2 (0·2)	0·1 (0·1)	0·0 to 0·1	0·05	0·1 (0·1)	0·1 (0·1)	−0·05 to 0·04	0·98	0·01 to 0·11	0·02[b]	−0·1 to 0·1	0·98

[a]Units are in μg of aggregated human IgG equivalent (μg AHG)/mL for total IgG and subclasses. For IgA and IgE, units are in μg of human Ig/mL.
P-values were obtained by using multivariate analysis of variance with matching with adjustment for home districts.
[b]*P*-values were significant after adjustment for multiple comparisons using Tukey's honestly significant difference test.

Only IgG4-containing ICs were higher among SMA cases than controls in the primary analysis but not after correcting for multiple comparisons. CM cases, on the other hand, had relatively high IC levels at enrolment for most IgG subclasses and IgE which declined significantly upon follow-up. The only significant difference between CM cases in KDH and their counterparts enrolled in NPGH was that at follow-up CM cases enrolled in KDH had lower IgA-containing ICs than in NPGH, mean (SD) for KDH = 4·4 (5·2) and for NPGH = 10·1 (7·1), $P = 0·01$. CM controls enrolled in KDH differed from those enrolled in NPGH in that the latter had higher levels of IgG2 and IgE-containing ICs during follow-up, IgG2 KDH mean (SD) = 0·26 (0·27) and NPGH mean (SD) = 0·09 (0·12) $P = 0·03$, and for IgE KDH mean (SD) = 0·13 (0·07) and NPGH mean (SD) = 0·09 (0·03) $P = 0·04$.

Comparison of IC levels between cases and controls

There was no statistically significant difference in IC levels between SMA cases and their controls except that IgA-containing ICs were higher in controls than in cases at enrolment, but this difference disappeared after adjustment for multiple comparisons (Table 1). On the other hand, CM cases had higher total IgG, IgG4 and IgE-containing IC levels than their controls at enrolment. The strongest difference was with IgE-containing ICs which held up to adjustment for multiple comparisons. All these differences disappeared at follow-up.

Comparison of IC levels between SMA and CM cases

Table 2 summarizes the comparison between SMA and CM cases at enrolment and follow-up. CM cases had higher levels of all ICs except for IgG3 and IgE than SMA cases at

Table 2 Comparison of IC levels between SMA and CM cases at enrolment and follow-up

IC type[a]	Visit 1[b]			Visit 2[b]		
	SMA ($N = 74$)	CM ($N = 32$)	P[c]	SMA ($N = 63$)	CM ($N = 25$)	P[c]
IgG	2·9 (0·4–17·5)	6·1 (1·2–36·8)	< 0·01	2·1 (0·5–41·6)	3·6 (0·5–16·4)	0·02
IgG1	0·5 (0·0–13·3)	0·9 (0·2–16·2)	< 0·01	0·2 (0·0–13·6)	0·5 (0·0–5·9)	0·01
IgG2	0·1 (0·0–0·6)	0·3 (0·0–4·6)	< 0·01	0·0 (0·0–0·7)	0·1 (0·0–1·5)	0·27
IgG3	0·3 (0·0–3·7)	0·4 (0·0–7·0)	0·55	0·2 (0·0–4·4)	0·1 (0·0–1·6)	0·17
IgG4	0·1 (0·0–1·4)	0·2 (0·0–4·5)	< 0·01	0·0 (0·0–1·3)	0·2 (0·0–0·9)	< 0·01
IgA	3·3 (0·3–13·5)	5·2 (0·1–32·2)	0·01	2·8 (0·3–13·8)	4·3 (0·5–20·4)	0·01
IgE	0·2 (0·0–0·6)	0·2 (0·0–1·2)	0·80	0·1 (0·0–0·6)	0·1 (0·0–0·3)	0·49

[a]Units are in μg of aggregated human IgG equivalent (μg AHG)/mL for IgG and subclasses. For IgA and IgE units are in μg of human Ig/mL. [b]Values represent medians and ranges in parenthesis. [c]P-values obtained using Mann–Whitney U-test.

Table 3 Conditional logistic regression for immune complex levels at enrolment

IC type	Severe anaemia[a]			Cerebral malaria[b]		
	OR	95% CI OR	P	OR	95% CI OR	P
IgG	0·46	0·14–1·49	0·20	2·84	1·08–7·46	0·03
IgG1	1·28	0·62–2·65	0·51	0·86	0·71–1·03	0·10
IgG2	0·56	0·27–1·15	0·12	1·66	0·65–4·23	0·29
IgG3	1·71	0·74–3·94	0·21	0·85	0·46–1·55	0·59
IgG4	3·11	1·01–9·56	0·05	0·81	0·54–1·21	0·30
IgA	0·74	0·50–1·08	0·12	0·30	0·11–0·84	0·02
IgE	0·88	0·43–1·81	0·732	6·82	1·88–24·73	< 0·01

[a]Adjusted for home districts. [b]Adjusted for home districts and haemoglobin levels.

Table 4 Conditional logistic regression for immune complex levels at follow-up

IC type	Severe anaemia[a]			Cerebral Malaria[a]		
	OR	95% CI OR	P	OR	95% CI OR	P
IgG	28·69	2·75–299·75	< 0·01	1·26	0·24–6·64	0·78
IgG1	3·38	0·16–72·94	0·44	2·44	1·25–4·75	0·01
IgG2	0·21	0·05–0·96	0·04	0·11	0·01–1·17	0·07
IgG3	4·65	0·04–538·69	0·53	0·59	0·05–6·76	0·67
IgG4	71·80	0·50–10349·65	0·09	0·77	0·17–3·56	0·74
IgA	0·64	0·64–1·26	0·19	0·07	< 0·01–1·04	0·05
IgE	0·23	0·06–0·89	0·03	0·47	0·09–2·43	0·37

[a]Adjusted for home districts and haemoglobin levels.

enrolment. These differences persisted during follow-up except for IgG3.

Conditional logistic regression

We carried out multivariate conditional logistic regression to identify the subclass and class of antibody-containing ICs that independently were most strongly associated with severe malaria. Table 3 summarizes the findings upon enrolment. IgG4-containing ICs were the only independent predictors of SMA. Although the effect was large, the level of significance was borderline ($P = 0·05$). On the other hand, total IgG and IgE-containing ICs were most strongly associated with the development of CM whereas IgA-containing ICs seemed to be protective.

Assuming that the condition of the children at follow-up was similar to their baseline state, we tested for the association between class and subclass IC levels and severe malaria during follow-up (Table 4). Total IgG IC levels were associated with an increased risk of SMA whereas IgE-containing ICs were protective against SMA during follow-up (Table 4). IgG1-containing ICs were associated with increased risk of CM, and there was a trend of association between IgG2 and IgA-containing ICs and decreased risk of CM.

DISCUSSION

Plasmodium falciparum malaria infection leads to generation of ICs which can deposit on endothelial surfaces and activate the complement cascade, resulting in the production of a number of pro-inflammatory mediators (15). Also, by cross-linking Fc receptors on effector cells such as macrophages and monocytes, ICs can stimulate the production of pro-inflammatory cytokines (16) that have been proposed to have a role in the pathogenesis of severe malaria (17). Despite the accumulating evidence linking ICs to the pathogenesis of malaria (2,5,6,18–20), no clear pattern of association between antibody class or subclass and the clinical form of severe malaria has emerged. Therefore, the main objective of this study was to determine the composition

of ICs in terms of antibody class/subclass and to relate this to the severity of *P. falciparum* malaria.

In general, IC levels were high at enrolment and diminished in all groups in response to malaria treatment (Table 1). This is consistent with our previous observations (6) and suggests that the initial malaria infection was responsible for the elevated IC levels. However, unlike our previous observations (6), the decline in IC levels with treatment was not statistically significant for SMA cases. We believe the explanation lies in the differences in the length of follow-up between the two studies. The former study had a follow-up period of 4 months as opposed to 2 months in the present study. Therefore, children with SMA may have a lower capacity to clear ICs from circulation since they require a longer period of time to do so. Interestingly, we have reported that red cells of children with SMA have acquired deficiencies in CR1/CD35 (10,21,22), a key complement receptor that, among other functions, binds opsonized ICs from circulation. Red cells carry ICs bound via CR1 to the liver and spleen where they are removed (23). Therefore, the decreased ability of children with SMA to clear ICs may be due to the deficiency of red cell CR1.

Children with CM had higher levels of total IgG ICs than their controls and children with SMA (Tables 1 and 2). This is consistent with our previous findings and the findings of others (5,6). When compared to their controls, the most significant difference in IC subclass antibody composition was higher level of IgG4-containing ICs in CM cases at enrolment. In addition, levels of IgE-containing ICs were higher in CM cases than in their controls. In the conditional logistic regression model, which controlled for the effects of all IC classes and subclasses, total IgG and IgE-containing IC levels were the only independent predictors of CM at enrolment (Table 3), while IgA, normally considered to have a role in mucosal immunity, was protective. Other investigators have reported an association between total IgE levels and CM and other forms of severe malaria (24,25). The finding of IgG and IgE deposits in brain capillaries of CM fatalities containing sequestered parasitized red cells is in support of the role of ICs in the pathogenesis of CM (19). It has been proposed that these ICs can lead to local overproduction of TNF-α, a cytokine that has been implicated in the pathogensis of CM (17), from monocytes by cross-linking their Fc receptors (26). This could be achieved by the interaction of ICs carried on red cells sequestered in brain capillaries with Fc receptors on monocytes/macrophages and endothelial cells (27–29). The higher the levels of IgG ICs, the higher the load of ICs on red cells and the greater likelihood of an interaction between red cells and macrophages.

On the other hand, except for high levels of IgG4-containing ICs, children with SMA did not differ significantly from their controls at enrolment (Table 1). This observation was confirmed by the logistic regression analysis which showed that the presence of IgG4-containing ICs was the only independent predictor of SMA at enrolment although the statistical significance was borderline ($P = 0{\cdot}05$) (Table 3). The mechanism by which IgG4-containing ICs may increase the risk of SMA is not immediately clear. Although IgG4 is common on the surface of red cells from patients with malaria and in patients with autoimmune haemolytic anaemia (30,31), it is known to be noncytophilic, meaning that it is a poor inducer of phagocytosis (8,32). In addition, IgG4 is a poor activator of the complement cascade and a poor inducer of pro-inflammatory cytokines upon binding to FcγRI (CD64) receptors on macrophages (7,9). A number of studies have also found an association between high levels of total IgG4 or IgG4 antimalarial antibodies and increased susceptibility to clinical malaria attacks or severe malaria (33,34). Conversely, an association between IgG1 and/or IgG3 and protection against uncomplicated and complicated malaria has been observed in several studies (35–39). The basis for the protection of IgG1 and IgG3 subclass antibody has been attributed to their greater opsonophagocytic activity and their complement activation capacity (8,32). IgG4 has been shown to inhibit the IgG1and IgG3-mediated opsonization of infected erythrocytes (8). Therefore, it is possible that the basis of the association between IgG4-containing ICs and increased susceptibility to SMA may be simply the fact that the antibody response is biased towards a nonprotective subclass or a subclass that blocks protective antibody responses leading to an increase in the risk of repeated infections. The incidence of SMA is highest in areas of the world where there is intense transmission and presumably the greatest likelihood of repeated infections.

Assuming that the condition of the participants at follow-up is a good reflection of their baseline between malaria attacks, we felt that studying the relationships between ICs and severe malaria during this visit could be enlightening. Although participants during follow-up were negative for malaria by microscopy and did not exhibit any clinical signs of disease, it seems reasonable to assume that the ICs detected at this time were residual from the previous malaria infection, and/or derived from continuous antigenic malaria challenge and other infections during the intervening follow-up period. These intervening events may be equally important in determining who ultimately develops malaria complications. Whereas during acute malaria total IgG IC levels were associated with protection against SMA (Table 3), these seemed to be deleterious at follow-up (Table 4). This observation is consistent with our argument that continuous antigenic stimulation and IC formation can lead to complement activation and are key for the development of SMA (40). On the other hand, the presence

of IgG2 and IgE-containing ICs during follow-up seemed to be protective against SMA. Antimalarial IgE has been observed to be protective from subsequent infections in a longitudinal study (41). The mechanism for the association between IgG2-containing ICs and decreased risk of SMA is not clear but in some studies antimalarial IgG2 has been associated with resistance against malaria (34). These data again suggest that the avoidance of recurrent malaria infections is important in preventing SMA. The association between IgG or IgE-containing ICs and CM disappeared during follow-up. Instead, we were surprised to see borderline protective effect from noncytophilic and noncomplement activating ICs (IgG2 and IgA) while the presence of IgG1-containing ICs was found to be a risk factor. The role of IgA in protection from CM deserves further investigation.

In summary, our findings suggest that although both SMA and CM are characterized by high levels of ICs, the class and subclass make-up of these ICs as well as the role that they play in each may be distinct. While CM is characterized by high levels of IgG and IgE-containing ICs that may serve to stimulate local production of pro-inflammatory cytokines in sequestered brain capillaries, SMA is characterized by persistent lower level IgG ICs in the intervening period between malaria attacks, the subclass make-up of which may interfere with adequate immune response and allow repeated infections.

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Appendix 3. Abstracts for conferences/workshops/seminars

CLASS AND SUBCLASS ANTIBODY ANALYSIS OF CIRCULATING IMMUNE COMPLEXES IN CHILDREN WITH SEVERE *PLASMODIUM FALCIPARUM* MALARIA

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Abstract
The exact mechanisms underlying pathogenesis of severe forms of *Plasmodium falciparum* malaria are not fully understood yet they are associated with a lot of morbidity and mortality. Studies have shown a link between severe *P. falciparum* malaria and levels of circulating immune complexes (CIC) but the exact role that these CICs play in the pathogenesis of severe *P. falciparum* malaria is still unclear. This study aimed to investigate the qualitative differences in antibody classes and subclasses in serum immune complexes (IC) between children with severe *Plasmodium falciparum* malaria and those with uncomplicated malaria. The study showed a general increase in levels of IgG-ICs as a result of *P. falciparum* infection in severe malaria cases and their symptomatic controls. Although IgG IC levels were elevated in children with severe malaria upon enrolment, children with CM had the highest levels of any group. Conditional logistic regression showed a borderline association between IgG4-containing ICs and increased risk of SMA (OR = 3.11, 95% CI 1.01 to 9.56, P = 0.05). Total IgG-containing ICs (OR = 2.58, 95% CI 1.20 to 5.53, P = 0.02) and IgE-containing ICs (OR = 3.27, OR 1.38 to 7.78, P = 0.01) were associated with increased risk of CM.

The 54th American Society of Tropical Med and Hyg Annual conference, Washington DC 11th-15th Dec 2005

Appendix 4. Research authorization ducument

Appendix 5. Antibody class/subclass immune complex data

				Standardized IC ug/ml AHG Equivalent							
ID	**SAMPLE_D**	**Visit**	**Match Code**	**Total IgG**	**IgG1 IC**	**IgG2 IC**	**IgG3 IC**	**IgG4 IC**	**IgM IC**	**IgA IC**	**IgE IC**
CM1001	5-Aug-2003	1	67067	3.069	0.478	0.349	0.086	0.26	11.152	3.671	1.198
CM1001	17-Oct-2003	2	67067	2.476	0.286	0.229	0.095	0.235	8.68	4.491	0.168
CM1002	13-Aug-2003	1	67134	12.983	5.77	0.154	1.321	0.244	28.945	3.446	0.095
CM1004	24-Oct-2003	1	67067	5.215	0.583	0.109	0.787	0.284	18.498	2.983	0.065
CM1004	4-Feb-2004	2	67067	3.279	0	0	0.141	0.103	29.536	6.329	0.085
CM1005	6-Nov-2003	1	67134	3.247	0.404	0	0.067	0.063	59.696	4.281	0.148
CM1005	25-Feb-2004	2	67134	1.925	0	0	0.007	0.042	13.075	3.095	0.065
CM1006	24-Nov-2003	1	67402	6.785	0.465	0	0.239	0.097	15.484	31.679	0.217
CM1006	16-Feb-2004	2	67402	2.786	0	0	0.014	0.079	11.96	6.592	0.029
CM1007	12-Feb-2004	1	67469	2.741	0.383	0	0	0.007	16.025	1.939	0.065
CM1008	3-Mar-2004	1	67067	16.844	12.074	0.784	1.527	0.578	11.573	6.281	0.075
CM1008	5-May-2004	2	67067	3.839	0.304	0.038	0.041	0.17	9.086	5.773	0.115
CM1009	18-May-2004	2	67469	2.353	0.052	0	0.054	0.142	19.079	2.935	0.045
CM1010	28-Apr-2004	1	67469	3.29	0.052	0	0.08	0.102	37.409	8.068	0.109
CM1010	19-Jul-2004	2	67469	2.757	0	0	0.029	0.172	49.802	12.644	0.151
CM1011	13-May-2004	1	67737	13.325	9.137	0.018	1.337	0.917	13.582	5.17	0.038
CM1011	15-Jul-2004	2	67737	7.243	5.908	0.058	0.139	0.172	26.611	8.721	0.139
CM1012	31-May-2004	1	67402	1.88	0	0	0.013	0.01	9.5	19.118	0.135
CM1012	30-Aug-2004	2	67402	1.723	0	0.097	0.001	0	3.931	1.796	0.069
CM1013	2-Jun-2004	1	67402	5.196	0	0	0.104	0.078	11.242	3.415	0.158
CM1013	26-Aug-2004	2	67402	3.433	0.333	0.322	0.062	0.238	9.432	3.542	0.112
CM1014	15-Jul-2004	1	67737	4.8	0.716	0.209	0.148	0.406	6.631	3.928	0.158
CM1014	16-Sep-2004	2	67737	3.812	0.201	0.25	0.121	0.247	15.311	8.722	0.078
CM1015	26-Oct-2004	2	68005	6.091	1.2	0.262	0.058	0.122	22.022	6.691	0.055
CM1017	11-Jan-2005	1	68139	4.583	0.655	0.256	0.255	0.142	29.351	15.063	0.154
CM1018	12-Jan-2005	1	67737	8.909	4.619	0.461	0.328	0.069	23.981	7.734	0.256
CM1019	12-Jan-2005	1	68005	6.164	0.274	0.229	0.185	0.135	33.262	8.603	0.271
CM1020	13-Jan-2005	1	68139	11.058	9.922	0.255	0.464	0.131	57.357	12.468	0.128
CM1021	19-Jan-2005	1	68407	4.057	0.192	0.269	0.226	0.201	95.313	32.246	0.17
CM1100	6-Aug-2003	1	73700	13.939	2.437	0.243	0.47	0.786	16.646	10.638	0.131
CM1100	16-Oct-2003	2	73700	2.211	0.112	0.012	0.029	0.277	5.126	5.544	0.131
CM1101	11-Aug-2003	1	73767	14.508	16.18	0.415	0.851	0.17	27.251	4.749	0.134
CM1101	13-Oct-2003	2	73767	6.622	5.947	0.157	0.066	0.205	65.438	12.904	0.314
CM1102	19-Aug-2003	1	73700	6.284	0.303	0.17	0.27	0.518	41.056	12.225	0.069
CM1102	21-Oct-2003	2	73700	6.993	0.294	0.249	0.19	0.4	14.909	4.248	0.151
CM1103	20-Aug-2003	1	73700	2.038	0.413	0.349	0.013	0.162	4.288	4.392	0.145
CM1104	26-Aug-2003	1	73968	1.95	0.879	0.274	0.105	0.111	4.95	2.14	0.092
CM1104	29-Oct-2003	2	73968	3.992	0.192	0.249	0.017	0.145	11.834	2.611	0.152
CM1105	27-Aug-2003	1	74035	6.302	0.61	0.584	0.127	0.06	21.523	4.325	0.1
CM1106	2-Sep-2003	1	73767	5.654	0.432	0.074	0.142	0.331	10.72	9.345	0.092
CM1106	3-Nov-2003	2	73767	4.162	0.283	0.062	0.0089	0.278	8.445	1.765	0.253
CM1107	10-Sep-2003	1	73968	8.121	4.273	0.427	0.035	0.078	4.161	20.912	0.119
CM1107	11-Nov-2003	2	73968	2.669	0.088	1.395	0	0.1	21.806	2.281	0.082
CM1108	17-Sep-2003	1	74035	15.701	5.77	1.022	2.882	2.73	3.119	3.252	0.135
CM1109	24-Sep-2003	1	74303	8.626	3.587	0.468	0.407	0.568	12.094	8.101	0.273
CM1109	27-Nov-2003	2	74303	3.786	2.615	0.314	0.447	0.5	6.648	2.518	0.07
CM1110	3-Oct-2003	1	74370	5.494	0.525	0.279	1.251	0.232	32.454	13.59	0.07
CM1110	2-Dec-2003	2	74370	1.633	0	0.02	0.041	0.21	12.503	3.8	0.07
CM1111	8-Oct-2003	1	74370	4.628	0.385	0.1	0.191	0.274	38.412	9.158	0.066
CM1111	8-Dec-2003	2	74370	2.008	0	0.528	0.174	0.227	17.536	13.745	0.096
CM1112	31-Oct-2003	1	74035	3.004	0.245	0.239	0.001	0.345	57.23	26.78	0.275
CM1112	5-Feb-2004	2	74035	4.49	1.654	0.224	0.144	0.341	18.957	3.062	0.096
CM1113	4-Nov-2003	1	74370	1.135	0.467	0.463	0.765	0.002	49.556	7.66	0.13
CM1113	6-Feb-2004	2	74370	1.63	0	0.219	0.001	0.02	16.228	7.148	0.074
CM1114	24-Nov-2003	1	74638	5.922	0.333	0.114	0.122	0.11	20.913	4.683	0.055
CM1114	5-Feb-2004	2	74638	2.237	0.141	0.185	0.044	0.141	10.756	2	0.1
CM1115	25-Feb-2004	1	74705	36.804	13.464	4.611	6.975	4.547	4.95	8.224	0.082
CM1115	27-Apr-2004	2	74705	7.048	0.595	0.139	0.705	0.221	23.349	7.391	0.122
CM1116	1-Mar-2004	1	73767	3.835	0.781	0.075	0.745	0.254	22.585	1.812	0.144
CM1116	3-May-2004	2	73767	3.25	0.199	0.145	0.159	0.184	26.343	2.681	0.129
CM1117	3-Mar-2004	1	74839	7.473	2.935	0.189	0.301	0.449	56.722	5.38	0.123
CM1117	5-May-2004	2	74839	5.092	0.979	0	0.332	0.372	8.442	3.276	0.103
CM1118	9-Mar-2004	1	74839	5.075	0.635	0.069	0.428	0.081	3.168	3.727	0.055

CM1119	18-Mar-2004	1	74303	9.592	4.635	0.648	0.483	0.372	16.544	3.49	0.093
CM1119	18-May-2004	2	74303	0.486	0	0.105	0.053	0.122	18.615	6.346	0.1
CM1120	18-Mar-2004	1	75040	2.087	0.537	0.149	0.384	0.023	7.639	2.375	0.171
CM1121	19-Mar-2004	1	74705	11.266	11.424	1.595	2.285	0.56	10.163	4.588	0.092
CM1121	24-May-2004	2	74705	4.84	1.002	0.613	0.0264	0.344	28.985	10.15	0.135
CM1122	22-Mar-2004	1	74705	4.878	0.519	0.249	0.254	0.211	43.144	5.318	0.055
CM1122	24-May-2004	2	74705	4.794	0.793	0.274	0.198	0.188	23.308	20.47	0.107
CM1123	7-Apr-2004	1	73968	8.883	3.26	0.558	0.581	0.113	14.811	2.99	0.085
CM1123	8-Jun-2004	2	73968	3.059	0.601	0.309	0.153	0.096	11.494	2.021	0.217
CM1125	29-Apr-2004	1	74839	6.63	1.357	0.189	0.776	0.083	40.722	21.461	0.092
CM1125	14-Jul-2004	2	74839	5.608	0.455	0.12	0.468	0.145	10.985	4.877	0.07
CM1126	21-May-2004	1	75442	2.195	0.973	0.219	0.141	0.048	19.258	0.0648	0.119
CM1127	25-May-2004	1	75509	15.813	12.143	4.123	2.089	0.4	51.088	13.282	0.314
CM1128	26-May-2004	1	75576	11.857	4.681	0.708	0.598	0.468	5.181	0.57	0.166
CM1128	29-Jul-2004	2	75576	1.288	0.324	0.054	0.068	0.065	42.27	3.962	0.151
CM1129	27-May-2004	1	75643	5.942	1.327	0.152	0.464	0.3	19.429	0.607	0.169
CM1129	30-Jul-2004	2	75643	16.106	5.738	1.472	1.615	0.917	9.338	4.373	0.18
CM1130	31-May-2004	1	75710	9.759	6.307	0.682	0.704	0.756	50.955	11.992	0.133
CM1131	3-Jun-2004	1	75576	9.205	2.556	0.545	0.352	0.58	50.095	32.61	0.161
CM1132	3-Jun-2004	1	75442	13.354	3.744	0.339	0.639	0.29	3.843	1.618	0.154
CM1133	4-Jun-2004	1	75911	3.233	0.85	0.151	0.047	0.196	58.979	0.452	0.213
CM1133	9-Aug-2004	2	75911	2.651	1.388	0.029	0.082	0.32	43.747	0.491	0.195
CM1134	27-Aug-2004	2	75911	2.237	0.058	0.345	0	0.032	30.447	2.7	0.136
CM1135	8-Jun-2004	1	75576	16.696	9.066	1.031	0.881	0.691	46.484	4.836	0.131
CM1135	12-Aug-2004	2	75576	12.079	0.966	0.455	1.196	0.531	23.283	2.969	0.142
CM1136	16-Jun-2004	1	75442	9.999	1.731	0.987	0.14	0.075	97.974	20.426	0.166
CM1136	1-Sep-2004	2	75442	3.499	0.637	0.39	0.066	0.078	9.65	1.813	0.151
CM1137	17-Jun-2004	1	76179	2.637	0.573	0.086	0.101	0.09	48.337	3.765	0.186
CM1137	20-Aug-2004	2	76179	1.107	0.339	0.119	0.064	0.51	29.444	3.418	0.247
CM1138	19-Aug-2004	2	74638	1.469	0.154	0.307	0.041	0.152	39.772	2.069	0.103
CM1139	21-Jun-2004	1	75710	10.64	5.052	1.161	0.46	0.63	22.969	4.381	0.172
CM1139	23-Aug-2004	2	75710	5.942	0.555	0.559	0.129	0.33	51.418	11.307	0.139
CM1140	23-Jun-2004	1	76380	6.021	0.82	0.481	0.246	0.22	84.72	11.453	0.186
CM1140	10-Sep-2004	2	76380	7.663	1.41	0.43	0.492	0.38	41.836	2.927	0.166
CM1141	30-Aug-2004	2	74638	1.26	0.339	0.274	0.052	0.122	19.477	3.205	0.092
CM1142	1-Jul-2004	1	75040	6.647	3.844	0.482	0.232	0.357	25.985	4.972	0.111
CM1142	2-Sep-2004	2	75040	3.145	0.712	0.39	0.094	0.245	12.362	2.635	0.1
CM1143	21-Sep-2004	2	76581	2.669	0.603	0.326	0.036	0.247	50.356	6.005	0.133
CM1144	14-Jul-2004	1	75040	4.219	0.716	0.288	0.041	0.054	16.655	16.895	0.085
CM1144	14-Sep-2004	2	75040	3.19	0.913	0.372	0.105	0.068	17.253	2.267	0.103
CM1145	15-Jul-2004	1	76715	16.44	2.77	0.617	2.883	1.449	41.665	18.304	0.315
CM1145	5-Oct-2004	2	76715	11.743	0.872	0.131	1.189	0.64	13.54	4.061	0.048
CM1146	16-Jul-2004	1	76782	12.335	8.335	0.578	0.988	0.9	29.647	5.282	0.124
CM1146	23-Sep-2004	2	76782	3.72	0.526	0	0.35	0.049	25.381	5.089	0.04
CM1147	21-Jul-2004	1	76849	3.99	2.412	0	0.46	0.053	18.241	2.911	0.087
CM1147	23-Sep-2004	2	76849	1.331	0.2	0	0	0.031	50.042	4.285	0.058
CM1148	30-Jul-2004	1	76581	0.628	0	0	0	0.031	49.501	0.335	0.193
CM1148	30-Sep-2004	2	76581	1.569	0.237	0	0.068	0.142	29.7	18.232	0.273
CM1149	11-Aug-2004	1	76715	8.487	6.489	0.022	1.838	0.046	5.951	4.382	0.04
CM1149	14-Oct-2004	2	76715	1.424	0.503	0	0.228	0.017	29.321	10.154	0.096
CM1150	14-Sep-2004	1	76581	6.372	4.553	0.138	2.383	0.03	56.844	7.457	0.193
CM1150	16-Nov-2004	2	76581	1.804	0	0	0.043	0.022	78.221	13.854	0.299
CM1151	16-Sep-2004	1	75911	6.534	1.051	0.022	1.023	0.343	78.665	4.571	0.166
CM1151	18-Nov-2004	2	75911	9.552	0.218	0	0.016	0.108	74.858	4.646	0.139
CM1152	23-Sep-2004	1	77184	1.415	0.397	0	0	0.032	46.735	9.926	0.189
CM1152	25-Nov-2004	2	77184	1.898	0.195	0	0.261	0.046	48.128	4.992	0.242
CM1153	5-Oct-2004	1	76849	9.913	7.684	0.792	2.591	0.461	67.873	11.041	0.158
CM1153	8-Dec-2004	2	76849	1.908	0.104	0.174	0.123	0.061	27.668	4.221	0.048
CM1154	7-Oct-2004	1	76782	5.56	4.732	0.081	0.654	0.08	5.7	1.506	0.086
CM1154	9-Dec-2004	2	76782	1.926	2.063	0.076	0.251	0.078	18.879	2.43	0.269
CM1155	21-Oct-2004	1	77385	1.174	0.195	0	0	0.03	63.005	6.41	0.224
CM1155	16-Dec-2004	2	77385	0.553	0.1	0	0	0.015	17.406	2.653	0.161
CM1156	16-Nov-2004	1	76179	5.472	1.842	0.08	0.978	0.111	79.171	17.332	0.142
CM1156	4-Feb-2005	2	76179	4.512	0.287	0.217	0.403	0.182	47.093	5.025	0.172
CM1157	7-Dec-2004	1	76715	3.477	2.082	0.246	0.734	0.067	17.326	6.378	0.152
CM1157	24-Feb-2005	2	76715	3.409	0.175	0.148	0.713	0.028	25.139	7.35	0.058
CM1162	10-Feb-2005	1	75710	9.277	0.633	0.197	1.436	0	20.997	4.569	0.151
CM1162	27-Apr-2005	2	75710	1.891	0.087	0.176	0.177	0.142	36.462	2.199	0.185
CM1163	12-Ap2005	1	76179	2.45	0.118	0.137	0.271	0.041	28.667	0.725	0.195
CM1164	3-Feb-2005	1	76380	4.624	0.407	0.167	0.45	0.006	21.626	0.881	0.235

CM1164	18-Apr-2005	2	76380	1.528	0.063	0.058	0.05	0.006	16.41	0.53	0.196
CM1166	7-Apr-2005	2	76782	3.061	0.094	0	0.3	0.016	27.31	6.055	0.02
CM1167	31-Jan-2005	1	76849	0.63	0.013	0.362	0.978	0.189	41.012	5.668	0.086
CM1167	13-May-2005	2	76849	3.234	0.168	0.317	0.266	0.153	38.092	4.382	0.225
CM1168	11-Feb-2005	1	77184	3.999	0.163	0.137	0.317	0.062	8.251	3.901	0
CM1168	14-Apr-2005	2	77184	4.175	0.671	0.133	0.356	0.07	35.105	13.121	0.105
SA1001	14-Jul-2003	1	83083	2.507	0.796	0.004	0.305	0.095	12.301	2.336	0.179
SA1001	30-Oct-2003	2	83083	1.225	0.272	0.165	0.041	0.064	9.397	1.443	0.143
SA1002	16-Jul-2003	1	83083	0.104	0.374	0.353	0.102	0.065	5.448	3.196	0.234
SA1002	17-Sep-2003	2	83083	0.678	0.237	0.165	0.065	0.057	20.066	5.282	0.207
SA1003	16-Jul-2003	1	83249	1.856	0.347	0.094	0.168	0.055	11.654	2.429	0.17
SA1003	2-Oct-2003	2	83249	1.522	0.233	0.084	0.185	0.067	17.465	2.016	0.225
SA1004	17-Jul-2003	1	83332	2.358	0.465	0.129	0.352	0.472	56.649	5.701	0.141
SA1004	24-Oct-2003	2	83332	3.15	0.481	0.004	0.033	0.108	48.704	7.48	0.225
SA1005	18-Jul-2003	1	83415	3.98	2.233	0.281	0.932	0.444	29.321	9.043	0.36
SA1005	23-Sep-2003	2	83415	1.248	0.145	0.049	0	0.078	28.116	3.42	0.231
SA1006	18-Jul-2003	1	83498	1.123	0.244	0	0.096	0.055	51.867	11.142	0.296
SA1007	24-Jul-2003	1	83581	1.433	0.218	0	0.135	0.032	23.606	0.852	0.087
SA1007	25-Sep-2003	2	83581	0.756	0.126	0	0	0.03	35.342	1.089	0.1
SA1008	25-Jul-2003	1	83664	5.594	1.237	0.121	3.713	0.07	43.98	2.336	0.111
SA1008	13-Oct-2003	2	83664	2.694	0.632	0	0.323	0.029	40.1	0.764	0.118
SA1009	28-Jul-2003	1	83747	1.661	0.325	0	0.02	0.061	6.422	0.852	0.095
SA1009	19-Nov-2003	2	83747	1.549	0.218	0	0.033	0.1	39.2	3.141	0.155
SA1010	31-Jul-2003	1	83581	1.396	0.233	0.013	0	0.048	52.918	2.485	0.102
SA1010	2-Oct-2003	2	83581	0.877	0.142	0.022	0	0.048	15.765	0.115	0.126
SA1011	1-Aug-2003	1	83913	2.896	0	0.001	0.3	0.029	60.492	11.213	0.187
SA1011	7-Oct-2003	2	83913	1.311	0	0	0.08	0.024	37.618	1.415	0.106
SA1012	2-Aug-2003	1	83996	6.416	0.924	0.037	0.448	0.031	85.118	7.711	0.148
SA1012	10-Oct-2003	2	83996	6.463	0	0.019	1.502	0.023	66.933	5.125	0.18
SA1013	4-Aug-2003	1	83332	7.356	5.025	0.221	1.132	0.429	17.345	3.707	0.179
SA1013	8-Oct-2003	2	83332	2.01	0	0	0.055	0.021	19	3.806	0.152
SA1014	8-Aug-2003	1	84162	16.477	2.245	0.207	2.255	1.08	28.521	0.823	0.078
SA1014	14-Oct-2003	2	84162	2.034	0	0	0.183	0.031	5.569	0.381	0.101
SA1015	10-Aug-2003	1	83498	6.038	1.755	0.162	0.193	0.048	52.396	5.153	0.508
SA1016	11-Aug-2003	1	83913	8.995	1.629	0.006	1.741	0.016	15.943	1.09	0.1
SA1016	13-Oct-2003	2	83913	1.385	0	0	0.105	0.009	29.436	1.652	0.118
SA1017	12-Aug-2003	1	83664	4.157	0	0.06	0.206	0.052	5.7	0.882	0.171
SA1017	15-Oct-2003	2	83664	2.604	0	0.074	0.114	0.037	15.977	1.919	0.072
SA1018	12-Aug-2003	1	83249	3.783	0	0	0.55	0.017	49.165	3.356	0.455
SA1018	16-Oct-2003	2	83249	2.153	0	0	0.126	0.016	10.559	1.952	0.412
SA1019	12-Aug-2003	1	84577	1.828	0	0	0.158	0.045	71.385	1.741	0.114
SA1019	21-Oct-2003	2	84577	2.027	0	0	0.196	0.063	25.841	1.177	0.135
SA1020	14-Aug-2003	1	84162	4.209	0	0	0.227	0.079	65.564	28.2	0.208
SA1020	17-Oct-2003	2	84162	2.457	0	0.01	0.055	0.034	29.057	2.307	0.216
SA1021	20-Aug-2003	1	84743	5.204	1.12	0.266	0.652	0.395	27.195	2.756	0.156
SA1021	27-Oct-2003	2	84743	1.677	0	0.1	0.218	0.115	42.854	1.978	0.146
SA1022	21-Aug-2003	1	84826	2.54	0	0.028	0.1	0.031	34.278	0.616	0.088
SA1022	6-Nov-2003	2	84826	2.4	0	0	0.174	0.037	25.602	0.617	0.102
SA1023	22-Aug-2003	1	84826	5.525	0.033	0.129	0.898	0.042	39.788	3.231	0.171
SA1023	22-Oct-2003	2	84826	3.94	0	0.193	0.153	0.039	25.286	2.549	0.206
SA1024	28-Aug-2003	1	84992	7.715	1.243	0.206	1.303	0.06	16.595	2.279	0.203
SA1024	3-Nov-2003	2	84992	1.735	0	0.12	0.121	0.039	46.493	2.633	0.127
SA1025	3-Sep-2003	1	85075	2.979	0.077	0.266	0.366	0.053	20.009	1.661	0.178
SA1025	4-Nov-2003	2	85075	2.159	0.015	0.124	0.245	0.047	51.888	4.49	0.66
SA1026	11-Sep-2003	1	83996	8.786	0.374	0.101	0.991	0.165	75.123	0.705	0.145
SA1026	10-Nov-2003	2	83996	2.68	0	0.056	0.227	0.062	29.483	1.059	0.197
SA1027	15-Sep-2003	1	85241	1.69	0.346	0	0.16	0.049	12.189	0.713	0.219
SA1027	3-Dec-2003	2	85241	1.596	0	0.156	0.234	0.054	45.702	3.654	0
SA1028	17-Sep-2003	1	85324	2.079	0	0.156	0.239	0.04	48.837	3.393	0.02
SA1029	17-Sep-2003	1	85407	2.368	0.531	0.043	0.126	0.008	12.767	2.117	0.037
SA1029	4-Dec-2003	2	85407	1.419	0.35	0.02	0.11	0	33.746	3.471	0.119
SA1030	18-Sep-2003	1	84992	1.907	0.655	0.02	0.076	0	17.071	3.321	0.114
SA1030	19-Nov-2003	2	84992	1.06	0.279	0.025	0.11	0.001	62.286	10.584	0.141
SA1031	19-Sep-2003	1	85573	3.691	0.412	0.017	0.359	0.001	26.719	2.66	0.037
SA1031	25-Nov-2003	2	85573	3.919	1.18	0.028	0.149	0	48.318	8.927	0.054
SA1032	19-Sep-2003	1	84743	1.217	0.283	0.094	0.11	0.009	14.372	1	0.278
SA1033	22-Sep-2003	1	84577	3.446	0.604	0.032	0.431	0.012	19.673	2.305	0.122
SA1033	24-Nov-2003	2	84577	2.115	0.432	0.046	0.291	0.011	50.882	1.682	0.143
SA1034	22-Sep-2003	1	85822	5.571	1.957	0.123	0.853	0.032	44.855	3.366	0.657
SA1034	24-Nov-2003	2	85822	2.096	0.351	0.069	0.151	0.012	32.024	1.214	0.103

SA1035	22-Sep-2003	1	85822	2.713	0.238	0.028	0.204	0.001	10.946	1.344	0.246
SA1035	21-Nov-2003	2	85822	2.461	0.228	0.038	0.481	0.004	22.396	1.424	0.086
SA1036	23-Sep-2003	1	85988	3.802	0.594	0.075	0.367	0.108	22.243	6.806	0.457
SA1036	1-Dec-2003	2	85988	1.355	0.868	0.028	0.116	0.007	21.334	5.272	0.246
SA1037	25-Sep-2003	1	86071	5.122	0.94	0.061	0.539	0.007	47.184	3.967	0.277
SA1037	3-Dec-2003	2	86071	3.028	0.578	0.107	0.167	0.004	27.716	1.189	0.167
SA1038	26-Sep-2003	1	86154	1.963	0.294	0	0.443	0.056	7.644	2.448	0.627
SA1038	28-Nov-2003	2	86154	1.494	0.304	0.043	0.253	0.016	5.371	1.002	0.43
SA1039	26-Sep-2003	1	85075	5.056	0.273	0.046	1.469	0.029	8.591	1.002	0.23
SA1040	30-Sep-2003	1	86071	1.813	0.268	0.102	0.257	0.042	8.433	0.713	0.222
SA1040	2-Dec-2003	2	86071	2.011	0.294	0.064	0.319	0.026	22.7	5.325	0.24
SA1041	30-Sep-2003	1	86403	10.735	1.37	0.268	0.623	0.716	46.561	6.858	0.281
SA1041	28-Nov-2003	2	86403	8.493	1.17	0.213	0.236	0.122	28.712	4.386	0.455
SA1042	1-Oct-2003	1	83415	1.429	0.361	0.025	0.143	0.004	12.8	2.462	0.197
SA1042	3-Dec-2003	2	83415	1.411	0.309	0.015	0.089	0.01	15.6	3.1	0.189
SA1043	2-Oct-2003	1	86569	1.841	0.5	0.061	0.188	0.026	2.603	1.871	0.277
SA1043	9-Dec-2003	2	86569	1.936	0.557	0.043	0.099	0.013	12.195	2.081	0.261
SA1044	6-Oct-2003	1	85573	2.889	0.79	0.051	0.262	0.008	3.937	0.66	0.046
SA1044	5-Dec-2003	2	85573	3.587	1.255	0.069	0.161	0.016	56.702	10.68	0.457
SA1045	7-Oct-2003	1	85324	17.757	0.738	0.095	5.144	0.014	39.584	13.187	0.037
SA1045	2-Feb-2004	2	85324	23.524	0.733	0.2	8.252	0.058	23.757	3.077	0
SA1046	7-Oct-2003	1	85407	5.104	0.655	0.3	0.344	0.174	26.569	2.055	0.133
SA1046	8-Dec-2003	2	85407	3.557	0.505	0.1195	0.182	0.104	26.169	2.658	0.33
SA1047	18-Dec-2003	2	85988	1.042	0.346	0.03	0.131	0.011	14.22	2.134	0.251
SA1048	8-Oct-2003	1	86984	4.856	0.353	0.129	0.41	0.152	78.262	6.025	0.295
SA1049	13-Oct-2003	1	85241	1.746	0.5	0.015	0.099	0.03	16.743	1.003	0.469
SA1049	15-Dec-2003	2	85241	1.208	0.376	0.023	0.068	0.017	12.923	0.791	0.135
SA1050	21-Oct-2003	1	86154	2.725	0.164	0.125	0.143	0.089	22.648	1.081	0.171
SA1050	18-Dec-2003	2	86154	2.948	0.204	0.129	0.15	0.053	22.398	1.608	0.079
SA1051	21-Oct-2003	1	83747	1.204	0	0.167	0.112	0.074	11.408	4.283	0.115
SA1052	1-Nov-2003	1	87316	6.096	0.041	0.461	0.275	0.136	56.463	4.075	0.316
SA1052	9-Feb-2004	2	87316	5.423	0	0.378	0.265	0.182	57.157	3.621	0.466
SA1053	10-Nov-2003	1	87399	1.489	0.145	0.231	0.216	0.141	52.542	11.846	0.151
SA1053	12-Feb-2004	2	87399	2.876	0	0.091	0.428	0.127	51.508	7.856	0.163
SA1054	10-Nov-2003	1	87482	3.635	0.171	0.508	0.274	0.081	12.65	1.107	0.326
SA1054	23-Feb-2004	2	87482	3.131	0	0.606	0.203	0.073	19.738	0.4	0.233
SA1055	15-Nov-2003	1	86569	6.952	1.3	0.201	0.306	0.042	27.916	2.633	0.291
SA1055	13-Feb-2004	2	86569	5.593	0.601	0.239	0.178	0.035	31.185	2.029	0.411
SA1056	8-Dec-2003	1	87648	15.501	3.665	0.658	1.883	1.078	15.148	1.042	0.316
SA1056	20-Feb-2004	2	87648	2.528	0	0.249	0.049	0.079	21.452	1.172	0.316
SA1057	15-Dec-2003	1	87399	5.182	0.086	0.323	0.232	0.263	41.927	2.748	0.199
SA1057	20-Feb-2004	2	87399	4.276	0	0.335	0.176	0.17	30.806	3.784	0.279
SA1058	15-Dec-2003	1	87814	4.009	0	0.273	0.189	0.028	27.648	1.621	0.446
SA1058	20-Feb-2004	2	87814	1.837	0	0.249	0.23	0.022	16.397	0.432	0.342
SA1059	9-Feb-2004	1	87897	7.15	0.164	0.187	1.76	0.038	83.864	9.697	0.509
SA1059	19-Apr-2004	2	87897	4.017	0.275	0.283	0.359	0.118	53.047	1.364	0.168
SA1060	9-Feb-2004	1	87980	2.903	0.803	0.289	0.23	0.048	57.927	2.911	0.322
SA1060	4-May-2004	2	87980	5.301	0.588	0.351	0.269	0.074	18.806	2.105	0.451
SA1061	10-Feb-2004	1	88063	17.557	2.582	0.61	1.982	1.427	2.966	1.172	0.279
SA1062	10-Feb-2004	1	87648	5.157	0.985	0.432	0.477	0.093	55.244	0.914	0.86
SA1063	13-Feb-2004	1	88229	7.87	1.358	0.58	0.528	0.294	25.372	1.813	0.404
SA1063	21-Apr-2004	2	88229	7.167	0.627	0.644	0.181	0.216	13.196	2.104	0.326
SA1064	17-Feb-2004	1	88312	8.999	2.771	0.634	0.656	0.327	40.76	2.943	0.227
SA1064	3-May-2004	2	88312	41.577	0.601	0.403	4.452	0.102	30.379	0.593	0.248
SA1065	25-Feb-2004	1	88395	5.85	0.985	0.39	0.332	0.361	53.533	4.789	0.322
SA1066	25-Feb-2004	1	87980	5.84	0.666	0.327	0.336	0.078	17.722	3.363	0.237
SA1066	25-May-2004	2	87980	7.986	0.558	0.638	0.61	0.084	61.844	23.329	0.404
SA1067	11-Mar-2004	1	88561	3.219	0	0.091	0.231	0.063	6.487	2.161	0.328
SA1067	19-May-2004	2	88561	3.48	0.047	0.259	0.265	0.096	23.829	3.097	0.291
SA1068	2-Mar-2004	1	88644	4.46	0.607	0.273	0.216	0.28	4.166	2.572	0.324
SA1068	15-Jun-2004	2	88644	3.64	0.294	0.253	0.159	0.21	26.37	3.4	0.299
SA1069	2-Mar-2004	1	86984	2.229	0	0.181	0.165	0.048	43.844	0.657	0.264
SA1069	21-May-2004	2	86984	2.364	0.015	0.2	0.124	0.094	8.308	0.753	0.332
SA1070	5-Mar-2004	1	88561	6.734	1.371	0.533	0.331	0.641	8.137	1.748	0.251
SA1070	14-May-2004	2	88561	4.516	0.217	0.576	0.111	0.21	8.897	2.297	0.389
SA1071	8-Mar-2004	1	86403	17.99	6.238	0.504	1.278	1.288	50.291	9.023	0.476
SA1071	2-Jun-2004	2	86403	1.511	0	0.187	0.124	0.253	40.546	2.422	0.25
SA1072	8-Mar-2004	1	88063	4.263	1.702	0.691	1.125	0	64.836	5.243	0.368
SA1072	22-Jul-2004	2	88063	2.562	1.676	0.491	0.365	0	43.973	2.426	0.42
SA1073	15-Mar-2004	1	88229	2.194	1	0.161	0.327	0.171	19.124	2.813	0.337

SA1073	17-May-2004	2	88229	0.616	0.39	0.172	0.168	0.091	15.459	1.267	0.14
SA1074	16-Mar-2004	1	88644	2.31	2.167	0.36	0.645	0.23	53.323	5.422	0.285
SA1074	31-May-2004	2	88644	3.663	2.492	0.324	0.729	0.259	50.395	5.089	0.181
SA1075	16-Mar-2004	1	89225	0.951	0.962	0.06	0.087	0.028	14.484	4.146	0.198
SA1075	20-May-2004	2	89225	0.878	0.818	0.115	0.185	0.026	21.223	3.731	0.203
SA1076	16-Mar-2004	1	89308	1.693	0.569	0.033	0.267	0	47.319	9.31	0.349
SA1076	4-Jun-2004	2	89308	1.734	0.685	0.091	0.386	0.087	29.864	6.118	0.282
SA1077	17-Mar-2004	1	87316	2.289	2.382	0.324	0.03	0.125	4.557	1.589	0.497
SA1077	20-May-2004	2	87316	0.783	1.445	0.297	0.204	0.166	33.29	4.821	0.248
SA1078	17-Mar-2004	1	89474	1.616	3.794	0.232	0.415	0.178	14.178	3.814	0.364
SA1078	19-May-2004	2	89474	2.815	4.205	0.417	0.584	0.149	31.466	5.533	0.417
SA1079	18-Mar-2004	1	87897	1.485	0.979	0.069	0.309	0.005	15.596	1.589	0.205
SA1079	27-May-2004	2	87897	1.807	2.069	0.186	0.537	0.003	52.766	5.927	0.259
SA1080	22-Mar-2004	1	88312	0.896	0.818	0.055	0.129	0	79.78	3.137	0.404
SA1080	31-May-2004	2	88312	1.311	1.221	0.104	0.401	0	66.995	2.329	0.284
SA1081	22-Mar-2004	1	89723	5.426	5.643	0.125	0.601	0.42	12.668	4.617	0.203
SA1081	25-May-2004	2	89723	1.198	2.57	0.26	0.392	0.294	13.738	3.62	0.229
SA1082	24-Mar-2004	1	89474	4.224	2.311	0.253	0.779	0.281	43.561	4.783	0.315
SA1082	26-May-2004	2	89474	1.903	2.025	0.239	0.454	0.16	30.518	3.62	0.251
SA1083	25-Mar-2004	1	89723	1.828	4.509	0.435	0.268	0.322	10.032	3.234	0.229
SA1083	8-Jun-2004	2	89723	1.483	1.462	0.3	0.232	0.194	3.624	3.289	0.337
SA1084	30-Mar-2004	1	89225	1.653	1.761	0.68	0.343	0.147	19.892	4.312	0.273
SA1085	5-Apr-2004	1	88395	2.601	2.373	0.268	0.254	0.169	22.794	1.395	0.263
SA1085	8-Jun-2004	2	88395	3.563	3.811	0.431	0.325	0.234	12.476	1.975	0.388
SA1086	7-Apr-2004	1	90138	3.944	2.15	0.271	0.565	0.147	16.433	4.312	0.414
SA1086	12-Jul-2004	2	90138	3.226	1.069	0.133	0.388	0.143	33.309	2.848	0.315
SA1087	8-Apr-2004	1	90221	4.793	0.997	0.246	0.398	0.055	49.295	4.091	0.371
SA1088	15-Apr-2004	1	90304	1.407	1.89	0.413	0.618	0.13	43.319	4.728	0.289
SA1088	1-Jul-2004	2	90304	1.028	0.774	0.424	0.36	0.12	22.805	3.207	0.19
SA1089	22-Apr-2004	1	90387	3.194	2.088	0.165	0.554	0.106	75.243	5.117	0.349
SA1089	3-Jul-2004	2	90387	2.04	2.794	0.182	0.379	0.06	39.224	4.618	0.32
SA1090	30-Apr-2004	1	89308	1.388	1.051	0.147	0.145	0.03	14.741	2.683	0.278
SA1090	29-Jun-2004	2	89308	1.501	1.131	0.165	0.154	0.018	30.398	5.617	0.264
SA1091	10-May-2004	1	90221	7.002	7.484	0.534	1.677	0.033	64.52	26.975	0.32
SA1091	12-Jul-2004	2	90221	3.447	6.304	0.218	1.551	0.028	55.157	13.819	0.311
SA1092	11-May-2004	1	90138	5.343	1.935	0.64	0.512	0.043	31.938	5.033	0.337
SA1092	29-Jul-2004	2	90138	1.047	1.186	0.169	0.227	0.005	40.943	4.784	0.307
SA1093	14-May-2004	1	90719	1.769	2.132	0.24	0.255	0.016	27.712	3.786	0.355
SA1094	17-May-2004	1	90304	5.772	1.311	0.068	1.069	0.115	20.629	2.655	0.324
SA1094	3-Aug-2004	2	90304	0.777	0.158	0	0.087	0	15.265	2.986	0.264
SA1095	17-May-2004	1	90885	1.198	0.067	0	0.163	0.001	21.902	0.523	0
SA1095	26-Jul-2004	2	90885	2.071	0.237	0	0.271	0.005	5.61	0.747	0
SA1096	21-May-2004	1	90885	1.095	0.074	0	0.156	0	37.79	1.523	0.097
SA1097	25-May-2004	1	91051	1.601	0.226	0	0.296	0.123	41.572	7.348	0
SA1097	3-Aug-2004	2	91051	0.877	0.073	0	0.05	0.018	17.436	0	0
SA1098	25-May-2004	1	91134	1.454	0.63	0	0.186	0	69.534	1.961	0
SA1098	22-Jul-2004	2	91134	1.498	0.135	0.011	0.062	0.011	53.725	1.782	0
SA1099	25-May-2004	1	91217	8.288	13.26	0.455	2.34	0.046	7.402	0.97	0
SA1100	26-May-2004	1	91300	1.195	0.305	0	0.18	0	67.915	4.566	0
SA1100	27-Jul-2004	2	91300	0.835	0.2	0	0.08	0	27.916	0.884	0.164
SA1101	31-May-2004	1	91217	1.644	0.362	0	0.348	0.056	7.138	0.404	0
SA1102	31-May-2004	1	91466	0.465	0.189	0	0.006	0	61.221	7.329	0
SA1102	3-Aug-2004	2	91466	1.195	0.378	0	0.201	0	61.989	9.935	0
SA1103	2-Jun-2004	1	91549	1.079	0.071	0	0.131	0.063	22.853	1.384	0
SA1103	4-Aug-2004	2	91549	0.821	0.115	0	0.083	0.032	20.933	0.832	0
SA1104	3-Jun-2004	1	91632	2.114	1.212	0	0.368	0.156	21.547	0.661	0
SA1104	4-Aug-2004	2	91632	0.835	0.149	0	0.054	0.01	19.23	0.13	0.13
SA1105	9-Jun-2004	1	91300	0.906	0.263	0.009	0	0	48.907	16.799	0
SA1105	13-Aug-2004	2	91300	0.95	0.065	0.005	0.067	0.004	13.064	0.079	0
SA1106	9-Jun-2004	1	91798	3.642	0.771	0.03	1.259	0.025	40.328	4.925	0.063
SA1106	16-Aug-2004	2	91798	3.089	0.949	0.011	0.831	0.012	32.678	3.287	0
SA1107	16-Jun-2004	1	91881	1.44	0.676	0	0.207	0.052	19.951	1.897	0.441
SA1107	20-Aug-2005	2	91881	0.593	0.138	0	0.048	0.005	6.581	0.233	0
SA1108	18-Jun-2004	1	91964	2.532	1.15	0.139	0.766	0.037	2.884	0.294	0.452
SA1109	19-Jun-2004	1	92047	2.893	0.361	0	0.622	0.01	28.97	2.43	0.195
SA1109	19-Aug-2004	2	92047	1.266	0.155	0.055	0.082	0.002	22.853	1.99	0
SA1110	21-Jun-2004	1	92130	2.959	1.03	0.071	0.039	0.092	5.024	7.123	0
SA1111	22-Jun-2004	1	91134	1.05	0.279	0	0.128	0.014	12.668	0.92	0
SA1112	23-Jun-2004	1	91466	1.923	0.625	0.061	0.125	0.029	73.393	8.256	0.206
SA1112	30-Aug-2004	2	91466	1.702	0.327	0.002	0.077	0.015	25.552	2.061	0

SA1113	24-Jun-2004	1	92379	3.532	0.156	0.085	0.064	0.037	4.23	3.877	0
SA1113	20-Sep-2004	2	92379	1.827	0.18	0.013	0.013	0.024	27.861	4.711	0
SA1114	25-Jun-2004	1	90387	4.082	0.256	0.114	0.019	0.071	73.609	7.178	0.346
SA1115	29-Jun-2004	1	91051	1.338	0.337	0	0.05	0.019	28.642	0.012	0
SA1115	30-Aug-2004	2	91051	1.353	0.248	0	0.121	0.02	9.865	0	0
SA1116	30-Jun-2004	1	91881	4.078	0.174	0.037	0.054	0.017	15.898	1.435	0
SA1116	2-Sep-2004	2	91881	3.166	0.131	0.062	0.026	0.016	17.264	0.902	0.028
SA1117	6-Jul-2004	1	91964	2.71	0.638	0	0.254	0.03	42.812	0.318	0.373
SA1117	17-Sep-2004	2	91964	3.135	0.687	0	0.325	0.005	40.922	0.575	0.611
SA1118	15-Jul-2004	1	91798	2.19	1.022	0	0.341	0.003	12.183	0.079	0
SA1118	15-Sep-2004	2	91798	1.952	0.387	0	0.193	0.01	30.88	1.196	0.195
SA1119	21-Jul-2004	1	92877	4.323	1.535	0.1	0.045	0.257	19.431	6.663	0
SA1119	27-Sep-2004	2	92877	3.214	0.583	0	0	0.048	29.223	4.756	0
SA1120	9-Nov-2004	2	92960	7.632	0.161	0.038	0.755	0.092	38.974	4.85	0.179
SA1121	2-Aug-2004	1	91549	4.249	0.935	0.259	0.36	0.005	52.838	8.262	0.483
SA1121	6-Oct-2004	2	91549	1.76	0.159	0.153	0.21	0	21.804	1.159	0
SA1122	4-Aug-2004	1	93126	1.491	0.13	0.057	0	0.026	6.332	4.247	0.033
SA1122	14-Oct-2004	2	93126	2.242	0.198	0.1	0.02	0.016	4.994	1.75	0
SA1123	11-Aug-2004	1	93209	2.819	0.187	0.042	0.061	0.081	18.67	5.22	0
SA1124	24-Aug-2004	1	93126	3.867	0.149	0.038	0.102	0.028	76.161	18.725	0.009
SA1124	15-Dec-2004	2	93126	1.795	0.05	0.042	0.019	0.072	15.856	2.858	0.009
SA1125	24-Aug-2004	1	92877	4.061	0.318	0	0.086	0.063	5.854	4.396	1.039
SA1125	10-Nov-2004	2	92877	2.242	0.087	0	0.045	0.037	11.282	2.673	0.178
SA1126	25-Aug-2004	1	90636	1.504	0.031	0	0	0.02	22.265	2.242	0.285
SA1126	26-Oct-2004	2	90636	3.34	0.056	0.008	0.185	0.047	41.079	2.711	0.291
SA1127	27-Aug-2004	1	93209	2.538	0.106	0.018	0.076	0.033	37.62	10.382	0
SA1127	27-Oct-2004	2	93209	1.55	0.068	0.086	0.013	0.055	9.592	3.413	0
SA1128	2-Sep-2004	1	92047	2.368	0.523	0.424	0.11	0.115	11.608	1.126	0.164
SA1128	2-Nov-2004	2	92047	1.449	0.09	0.309	0.019	0.028	11.289	0.952	0.028
SA1129	22-Sep-2004	1	93707	3.722	1.044	0.162	0.125	0.042	7.993	1.98	0
SA1130	27-Sep-2004	1	93790	3.07	0.231	0.114	0.096	0.119	50.89	9.822	0.426
SA1130	29-Nov-2004	2	93790	2.149	0.161	0.086	0.086	0.075	48.256	5.963	0.317
SA1131	7-Oct-2004	1	93873	2.335	0.239	0.071	0.121	0.032	5.083	2.488	0.203
SA1131	15-Dec-2004	2	93873	2.584	0.407	0.027	0.042	0.014	19.147	13.763	0.058
SA1132	16-Nov-2004	1	93956	3.134	0.112	0	0.191	0.077	35.3	2.534	0.009
SA1132	21-Jan-2005	2	93956	4.465	0.187	0.047	0.138	0.081	23.525	2.72	0
SA1133	19-Nov-2004	1	92379	4.909	1.363	0.217	0.08	0.042	7.144	4.108	0
SA1134	23-Nov-2004	1	93873	2.959	0.18	0.067	0.143	0.032	36.071	2.673	0.033
SA1134	24-Jan-2005	2	93873	1.55	0.2	0.081	0	0.021	28.595	2.35	0.131
SA1135	11-Jan-2005	1	94205	9.604	0.481	0.013	0.105	0.026	10.779	3.691	0.226
SA1135	11-Mar-2005	2	94205	1.186	0.07	0.096	0.033	0.04	11.535	2.534	0.361
SA1136	11-Jan-2005	1	93707	7.161	1.29	0.785	0.226	0.431	11.98	5.776	0.226
SA1136	14-Mar-2005	2	93707	6.028	0.224	0.605	0.162	0.242	64.912	10.8	0.281
SA1137	13-Jan-2005	1	94371	9.245	2.378	0.229	1.693	0.158	92.061	13.509	0.225
SA1137	14-Mar-2005	2	94371	17.447	13.606	0.736	2.127	1.327	11.283	4.617	0.317
SA1138	13-Jan-2005	1	93956	4.5	1.206	0.212	0.265	0.133	30.944	5.591	0.249
SA1138	5-Apr-2005	2	93956	4.273	0.299	0.22	0.179	0.138	25.642	3.6	0.131
SA1139	14-Jan-2005	1	92130	3.228	0.256	0.22	0.083	0.065	19.009	4.109	0
SA1139	15-Mar-2005	2	92130	4.303	0.538	0.217	0.28	0.05	13.005	2.167	0
SA1140	15-Jan-2005	1	94620	6.116	0.069	0	1.267	0.006	13.105	6.369	0.234
SA1140	17-Mar-2005	2	94620	12.755	0.095	0.048	3.512	0.087	56.935	10.36	0.132
SA1141	14-Jan-2005	1	94703	1.847	0.042	0	0.22	0.17	7.79	3.296	0
SA1141	6-Apr-2005	2	94703	2.741	0.013	0.023	0.229	0.223	30.4	3.475	0.1
SA1142	17-Jan-2005	1	94205	5.925	0.651	0.275	0.286	0.037	43.685	29.074	0.339
SA1143	17-Jan-2005	1	94703	1.913	0.045	0.038	0.267	0.098	26.59	4.995	0.1
SA1143	18-Mar-2005	2	94703	2.013	0.038	0.008	0.235	0.08	37.381	5.388	0.056
SA1144	19-Jan-2005	1	94952	2.617	0.051	0.078	0.247	0.059	22.197	6.77	0
SA1144	4-Apr-2005	2	94952	2.529	0.108	0.227	0.154	0.096	39.287	9.55	0.18
SA1145	19-Jan-2005	1	95035	1.891	0.06	0.133	0.23	0.067	38.076	6.904	0.077
SA1145	22-Mar-2005	2	95035	2.777	0.09	0.168	0.253	0.054	39.01	6.458	0.153
SA1146	19-Jan-2005	1	95118	1.362	0.045	0.018	0.17	0.022	5.196	1.217	0.11
SA1146	8-Apr-2005	2	95118	1.286	0.02	0	0.144	0.028	14.74	0.82	0.142
SA1147	18-Apr-2005	2	95201	2.258	0.041	0.177	0.127	0.056	52.834	6.1	0.174
SA1148	21-Jan-2005	1	92960	2.678	0.062	0.042	0.106	0.078	38.301	10.987	0
SA1149	25-Jan-2005	1	94371	1.581	0	0.137	0.184	0.13	21.287	5.081	0.131
SA1149	13-Apr-2005	2	94371	0.669	0	0.048	0.015	0.078	17.331	3.506	0.033
SA1150	21-Feb-2005	1							57.916	8.394	0.056
SA1152	4-Feb-2005	1							18.853	6.636	0.077
SA1153	25-Jan-2005	1	95118	2.982	0.093	0.117	0.988	0	84.691	17.264	0.012
SA1153	4-Apr-2005	2	95118	2.472	0.121	0.142	0.181	0.055	60.954	7.173	0.033

SA1154	3-Feb-2005	1							38.308	15.515	0.143