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COMPLEMENT REGULATORY PROTEIN LEVELS IN CHILDREN WITH SICKLE CELL TRAIT

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A thesis submitted in fulfilment for the award of the degree of Doctor of Philosophy in the Department of Medical Microbiology, University of Nairobi.

May 2009

DECLARATION

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

Signed.....Date.....

DECLARATION BY SUPERVISORS

This thesis has been submitted for examination with our approval as University

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DEDICATION

To My children Duncan, Paul and Grace and to my wife Pamela for their unwavering support during the long periods of absence while running the assays, during the analysis and write up

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LIST OF ABBREVIATIONS

CBC	Complete Blood Count
CR1	Complement Regulatory Protein 1
DAF	Decay Accelerating Factor
HbAA	Haemoglobin AA
HbAS	Haemoglobin AS
HIV	Human Immunodeficiency Virus
IC	Immune Complex
IFN-γ	Interferon-gamma
IgG1	Immunoglobulin G1
IgG2	Immunoglobulin G2
IgG3	Immunoglobulin G3
IgM	Immunoglobulin M
IL-10	Interleukin-10
IL-4	Interleukin-4
MFI	Mean Fluorescence Intensity
MIRL	Membrane Inhibitor of Reactive Lysis
MOH	Ministry of Health
NO	Nitric Oxide
PfEMP1	*P. falciparum* Erythrocyte Membrane Protein 1
RBCs	Red Blood Cells

SA	Severe Anaemia
SCT	Sickle Cell Trait
TNF-α	Tumor Necrosis Factor -α
WHO	World Health Organization

ACKNOWLEDGEMENTS

I am deeply grateful to my University supervisor, Prof Benson BA Estambale, the Chairman, University of Nairobi Institute of Tropical Diseases for taking time off his busy schedule to guide me through this work. I highly appreciate his detailed, constructive comments, the thorough guidance chapter by chapter during the write up and his support throughout this work.

I owe my most sincere gratitude to my University supervisor Prof JR Aluoch, The Chairman, Department of Internal Medicine, Moi University, Eldoret for sharing his vast knowledge and experience in the field of sickle cell anaemia, especially his help with the interpretation of some of the results and his keen interest in this work. I especially thank him for the many hours he put in this work and the encouragement, not forgetting his useful criticism of this work.

I would like to express my deep and sincere gratitude to my supervisor Prof José A Stoute for introducing me to the world of molecular medicine, allowing me to use his study subjects and getting the funds to bring this work to its conclusion. His wide knowledge in molecular medicine has been of great value to me. His understanding, encouragement and personal guidance is highly appreciated

Many people have helped me in the course of this work and I owe a lot to them. Special thanks to my colleagues Mike Odera, Collins Odhiambo (for their help with standardization of the assays and consultation whenever things did not work), Peter Mucheru (for flow work) and Lilian Ogonda, Rachel Ochola and Sammy Anyona for help with the statistical analysis and their useful criticism of the manuscript.

My sincere gratitude to Dr Bernhards Ogutu and Dr Njenga for their keen interest and encouragement during the course of this work. Special thanks to these gentlemen for encouraging me to join the world of research, it has been very rewarding!

To the Kombewa Clinical Research Centre team led by Mama Agnes and my very able Clinical Research Coordinator Stacey Maureen Okallo Gondi, you are one of the very best team to work with. It was an honor working with you.

To all those I have not mentioned by name but in one way or another contributed to this work I say thank you.

This work would not have been possible without the cooperation of the children and their parents from Kombewa District who willingly took part in this great scientific endeavor. A big thank you to all of you!

This work was supported by a training grant from the Fogarty International Center (1 D43 TW06239)

ABSTRACT

Sickle cell trait tends to have a similar distribution pattern as malaria in the malaria holoendemic areas of the tropics. This mirrored distribution pattern between malaria and sickle cell trait has been attributed to the relative protection conferred by the sickle cell trait against severe manifestations of malaria namely; cerebral malaria and severe anaemia. The mechanism of protection is not well understood but may be partly due to enhanced immune response and impaired parasite growth in the HbAS compared to HbAA. The Complement Receptor 1 (CR1/CD35) and Decay Accelerating Factor (DAF/CD55) are complement regulatory proteins (CRPs) that play an important role in the protection of red blood cells (RBCs) from complement mediated destruction and clearance of immune complexes (ICs) that occur during malaria and their acquired deficiencies have been associated with severe *P. falciparum* malaria. In this study, we hypothesized that sickle cell trait could be associated with increased expression of CR1 and DAF leading to increased rate of clearance of immune complexes from the circulation and resistance to complement attack. If this is true, then this could partially explain why people with HbAS are protected from severe malarial anaemia. We compared the mean parasite density in participants were well but parasitemic between those with HbAS and HbAA and using cytofluorometric techniques, we measured the level of CR1 and CD55 expression, immune complex binding capacity, and susceptibility to complement activation under normal and deoxygenated conditions of RBCs from individuals with HbAS or HbAA identified from a cohort of individuals living in a malaria endemic region of western Kenya.

The study design was a nested case-control study and was part of a cross-sectional study entitled “Erythrocyte Immune Complex Binding Capacity and Complement Sensitivity in Populations with Different Malaria Risks”. It was open to healthy male and females of aged 0 to 45 years who were residents of Kombewa Division of Kisumu West District. The potential participants were assessed for any acute or chronic illness which could interfere with the parameters under investigation. In cases of an acute illness, the potential participants were assessed, treated and asked to come again for re-evaluation. At

re-evaluation, the potential participants were enrolled when they were deemed well. Haemoglobin electrophoresis was carried on blood from all participants. Individuals who were less than 16 years and HbAS positive were identified and matched by age (± 2 months or ± 2 years for those below or more than 8 years, respectively) at a ratio of 1:1 or 1:2 with those with HbAA and these formed the nested case control cohort.

Because many conditions including malaria, HIV infection, and various other chronic conditions can alter the level of red cell complement regulatory proteins, participants with features of malnutrition manifested by marasmus or kwashiorkor; immune compromised status manifested by weight loss, thrush, or diffuse adenopathy; severe anaemia (Haemoglobin ≤ 5.0 g/dl); bacterial infection such as pneumonia; malignancy; and blood transfusion within 3 months preceding the study were excluded from the study.

In total, 402 were screened of which 342 were enrolled. Of the participants who were enrolled, 280 (81.4%) had haemoglobin AA, 60 (17.4%) had haemoglobin AS and 2 (0.6%) had haemoglobin SS. Those with HbAA and HbAS were included for the analysis bringing the total number 340 participants.

For the nested case control study 47 heterozygous sickle cell trait individuals aged 0-16 years were matched to 70 individuals with normal haemoglobin of similar age as stated above.

The Results from this Study showed that:

1) In asymptomatic individuals in the community without any signs of an acute or chronic illness and who are malaria parasites positive, the mean parasite count/μL for HbAS [4064.0 (95% CI 1858.0 – 6270.0)] is significantly lower than for the HbAA [11,067.9 (95% CI 7616.0 – 14520.0)]. ***P* = 0.001**
2) The nadir of haemoglobin in the community was observed in the >12 -24 months of age cohort and this corresponds to the age when these children have the highest mean parasite counts. This may explain the high prevalence of anaemia in this age group

3) The mean CR1 copy numbers per red blood cell was higher in the HbAS group than in the HbAA group, this difference was however not statistically significant ($P = 0.250$). This was also true under reduced oxygen saturation ($P = 0.400$).
4) Beyond the age of 96 months, the CR1 copy numbers per erythrocyte was significantly higher in the HbAS than in the HbAA participants, (***P* = 0.009**).
5) There were no significant differences in the mean CD55 antibody binding capacity between RBCs of individuals with HbAS and those with HbAA. Under reduced oxygen saturation, mean CD55 antibody binding capacity for the HbAS red blood cells were higher than for HbAA red blood cell but this again did not reach statistical significance ($P = 0.058$).
6) The mean immune complex binding capacity for the HbAS cells was higher than HbAA cells both under normal and reduced oxygen saturation (***P* = 0.017** and **0.003** respectively).
7) The mean immune complex binding capacity was lowest in the >6-12 months age group for both HbAS and HbAA; however, the overall picture showed that HbAS individuals had higher immune complex binding capacity than HbAA in all the age cohorts
8) In general, there was a weak positive correlation between the immune complex binding capacity and the age cohort r (117) = 0.198, ***P* = 0.034**.
9) Although the % C3b deposition was similar in both HbAS and HbAA cells under normal and reduced oxygen saturation, the highest incidence of % C3b deposition was in the 6 to < 24 and lowest in the 0-6 month's age cohort for both the HbAS and HbAA.
10) There was significant negative correlation between %C3b deposition and previous malaria infection in the children r (117) = -0.198, ***P* = 0.033**.

CHAPTER 1

INTRODUCTION

1.1 Sickle Cell Disorders and the Malaria Hypothesis

Sickle cell disease refers to a collection of autosomal recessive genetic disorders characterized by the presence of HbS variant of the globin chain while "sickle cell anaemia" refers to individuals with two copies of the variant (HbSS) and the primary haemoglobin present in their blood is the sickle haemoglobin. Individuals possessing one copy of the HbS variant plus one copy of another β-globin gene variant, such as HbC or Hb β thalassemias are referred to as compound heterozygotes (Ashley-Koch *et al.*, 2000).

Sickle cell trait (HbS) is the heterozygous form of sickle cell disease where the individual has normal haemoglobin HbA (normal) and HbS (sickle cell) gene. HbS carriers are protected from malaria infection and this is thought to have led to high frequency of HbS in individuals of African and Mediterranean ancestry (Ashley-Koch *et al.*, 2000). Despite this protection in the carriers, individuals with sickle cell disease have significant morbidity and mortality. Symptoms include chronic anaemia, acute chest syndrome, stroke, splenic and renal dysfunction, pain crises and susceptibility to bacterial infections (Ashley-Koch *et al.*, 2000; Platt *et al.*, 1994)

1.2 The Haemoglobin

Haemoglobin is the iron containing oxygen transport metalloprotein in the red cells of vertebrates. It is an assembly of four globular protein subunits, each composed of a protein chain tightly associated with a non-protein heme group (Bunn, 1987). Haemoglobin is synthesized in a complex series of steps; the heme part is synthesized in the mitochondria and the cytosol of immature red blood cells while the globin proteins are synthesized by ribosomes in the cytosol (Bunn, 1987)

The most common haemoglobin type in human adults is haemoglobin A, consisting of two α and two β sub units non covalently bound, each made of 141 and 146 amino acid

residues respectively and denoted α_2 and β_2. The α chains are controlled by α genes that lie next to each other on chromosome 16, while the β chains are controlled by β genes that reside on chromosome 11 (Bunn, 1987).

1.2.1 Disorders of Haemoglobin (Haemoglobinopathies)

Haemoglobinopathies are disorders that result from mutations in the globin genes and can be divided into two distinct types: those that cause quantitative abnormalities (the thalassemias) and those that cause qualitative abnormalities (e.g. sickle cell anaemia) (Giordano *et al.*, 2009; Lopez-Escribano *et al.*, 2009).

1.2.1.1 Thalassemias

Deficiencies in β-globin synthesis result in the β thalassemia whereas deficiency in α-globin synthesis causes the α-thalassemias. In normal individuals an equal amount of both α- and β-globin proteins are made allowing them to combine to form the correct haemoglobin tetramers. In thalassemias, this normal α/β globin chain ratio is modified leading to various clinical manifestations depending on the severity (Sripichai *et al.*, 2008; Weatherall and Clegg, 2001).

In α-thalassemias, there is inactivation of 1 to all 4 α-globin genes with the level of α-globin production ranging from very nearly normal to none. This results in a wide α-thalassemia clinical spectrum which ranges from a silent trait to very severe anaemia. Lack of α-globin chains synthesis designated as --/-- leads to haemoglobin Bart's which is incompatible with survival (Weatherall *et al.*, 2002).

Individuals heterozygous for β-thalassemia have what is termed thalassemia minor. Afflicted individuals harbor one normal β-globin gene and a mutation leading to production of reduced or no β-globin. Individuals that do not make any functional β-globin protein from 1 gene are referred to as β^0 heterozygotes whereas as β^+ heterozygotes have reduced production of β-globin. Thalassemia minor individuals are generally asymptomatic. Both α-thalassemia and β-thalassemia heterozygotes are extremely common in certain regions where malaria is hyper endemic and this has been

attributed to the protection it offers against severe malaria (Allen *et al.*, 1997; Weatherall, 1987; Weatherall and Clegg, 2001; Weatherall *et al.*, 2002)

1.2.1.2 Sickle Cell Disease and Sickle Cell Trait

The sickle cell genotype is due to a mutation in the beta globin sub unit located on the short arm of chromosome 11. A point mutation substituting thymine for adenine at the sixth codon of β gene, (GAG→ GTG), leads to valine incorporation, rather than glutamine and results in haemoglobin tetramers that aggregate into arrays upon deoxygenation in the tissues. This aggregation causes deformation of the red blood cell making it relatively inflexible and unable to traverse the capillary beds. Repeated cycles of oxygenation and deoxygenation lead to irreversible sickling resulting in clogging of the fine capillaries. Because bones are particularly affected by the reduced blood flow, frequent and severe bone pain results. This is the typical symptom during a sickle cell "crisis". Primary morbidity in sickle cell disease arises from vasoocclusive events or tissue damage resulting from obstructed blood flow. Common symptoms include pain crises, acute chest syndrome, cerebrovascular accidents and splenic and renal dysfunction. After infancy, individuals with sickle cell disease become susceptible to bacterial infections due to asplenia and disordered humoral immunity (Platt *et al.*, 1994). Long term recurrent clogging of the capillary beds leads to damage to the internal organs, in particular the kidneys, heart and lungs. The continual destruction of the sickled red blood cells leads to chronic anaemia and episodes of hyperbilirubinemia (Juwah *et al.*, 2003).

The severity of sickle cell disease is determined by the genotype. HbSS individuals are more severely affected followed by HbS/β°-thalassemia. HbSC and HbS/β^+ thalassemias tend to have a benign course of the disease. HbSS individuals have higher episodes of cerebrovascular accidents, HbSS and HbS/β°-thalassemias suffer from acute chest syndrome and pain crises more than the other genotypes while those with HbSC have increased risk of thromboembolic complications, retinopathy and renal papillary necrosis (Platt *et al.*, 1994)

Red blood cells in sickle-cell-trait individuals contain both normal haemoglobin (HbA) and sickle haemoglobin (HbS). Variable proportions of HbS (20% - 45%) with a mean value of 38 ± 5% have been reported in the sickle cell trait conditions, (Al-Shakour, 2000).

Sickle cell trait is generally a benign condition but has been associates with renal abnormalities especially urinary tract infections, hematuria and hyposthenuria, splenic sequestration or infarction at high altitudes and unexpected sudden deaths during strenuous exercise (Heller *et al.*, 1979; Kirk, 1987)

1.3 Haemoglobinopathies and Malaria

Glucose -6-phosphate dehydrogenase (G6PDH) deficiency, HbS and thalassemia are the most common metabolic disorders of the red blood cell. Inherited abnormalities of haemoglobin synthesis are divided into two groups. Those characterized by structurally haemoglobin variant are called haemoglobinopathies. Diseases in which one or more of the normal peptide chains of haemoglobin are synthesized at a lower rate are the thalassemias. HbS is the most common structurally abnormal haemoglobin; the other common ones are HbC, HbD, HbE and HbO (Weatherall and Clegg, 2001).

Sickle cell disorders refer to those states in which the red cell undergoes sickling when deoxygenated. The sickle cell disease include those that produce prominent clinical manifestations as seen in sickle cell anaemia, sickle cell HbC disease, sickle cell α-thalassemia and sickle cell HbD disease. Sickle cell anaemia is reserved for the homozygous disease while the trait has one abnormal gene (Weatherall and Clegg, 2001).

In 1949, JBS Haldane hypothesized that the high gene frequency of haemoglobinopathies in malaria endemic areas may have resulted from protection conferred against malaria (Lederberg, 1999). The geographical distribution of these haemoglobinopathies is very similar to that of malaria and it has been found that these disorders confer some resistance to malaria. Because of this protection, these disorders have reached a balanced polymorphism where malaria is hyper endemic.

It is thought that the haemoglobinopathies make the cell susceptible to haemolysis at reduced thresholds. Any internal or external stressors on the RBC can easily make them haemolyse. Invasion of the cells by merozoites represent such a stressor and would make the cells to haemolyse before the parasites have a chance to reproduce. (Carlson *et al.*, 1994) It has also been observed that individuals with these conditions although get invasion by the malaria parasite suffer less morbidity and fatality (Carlson *et al.*, 1994)

1.4 Genetics of Sickle Cell Disorders

Haemoglobin S arises from a mutation substituting thymine for adenine in the 6^{th} codon of the beta-chain gene, GAG to GTG. This causes coding of valine instead of glutamine in position 6 of the haemoglobin beta chain. The resulting haemoglobin has the physical properties of forming polymers under deoxygenated conditions. It also exhibits changes in solubility and molecular stability. These properties are responsible for the profound clinical expressions of the sickling syndromes (Conran *et al.*, 2009).

Under deoxygenated conditions, haemoglobin S undergoes marked decrease in solubility, increased viscosity and polymer formation. It forms a gel-like substance containing haemoglobin crystals called tactoids. The gel-like form is in equilibrium with its liquid form. A number of factors influence this equilibrium including oxygen tension, concentration of HbS and the presence of other haemoglobins (Buchanan *et al.*, 2004).

1.5 The Origin of Sickle Cell Disease

Sickle cell trait (SCT) and the sickle cell disease (SCD) are found primarily but not exclusively in people of black origin. The highest prevalence is in Africans and Afro-Americans and also in the Mediterranean areas (Aluoch, 1995). The HbS mutation was thought to have started around the Arabian Peninsula, after which the gene is thought to have spread to the Mediterranean basin, the Indian subcontinent and the tropical Africa This single mutation theory has since been discarded and it is now clear that the sickle cell mutation has occurred as several independent events. In Africa, the HbS is gene is associated with at least three haplotypes representing independent mutations. They are the Benin, the Senegal and the Central African Republic or the Bantu haplotypes found in

the central West Africa, the African west coast and the Central Africa (Bantu speaking Africa) respectively. A fourth one, the Asian haplotype is found in the eastern province of Saudi Arabia and central India.

It appears that the sickle cell mutation has occurred on at least three occasions in the African continent and at least once in either the Arabian Peninsula or the Central India and from the primary sites the migration to the other regions has occurred. Using restriction endonuclease haplotype analysis for the β-globin-like gene cluster from four separate geographic areas of Africa all of which have sickle cell gene, Pagnier and colleagues have shown that the sickle cell gene arose independently more than once. They confirmed more than three different haplotypes arose in Africa in at least three independent historical instances and then proceeded to expand through selection (Pagnier *et al.*, 1984).

1.6 Relationship between Sickle Cell Trait and Malaria

Children with sickle cell trait are protected not only from the severe manifestations of malaria for example severe malarial anaemia in malaria endemic areas (Aidoo, 2002; Dvorak *et al.*, 1975b; Ganczakowski *et al.*, 1995a; Ruwende *et al.*, 1995) but also from the early risk of chronic malnutrition (Kreuels *et al.*, 2009)

Recent studies show that sickle cell trait individuals harbor a wide range of *P. falciparum* genotypes and this multiplicity of infection may influence rapid acquisition of premunition by creating a reservoir of variant parasites in the host and this leads to early protection in carriers of the sickle cell gene (Kiwanuka *et al.*, 2009). The parasitized red blood cells of HbAS individuals also rosette less efficiently than the HbAA cells. This altered binding correlates with altered display of *P. falciparum* Erythrocyte Membrane Protein-1 (*Pf*EMP-1), the parasite's major adherence ligand and virulence factor on the red cells and this therefore protects them from severe manifestations of malaria (Cholera *et al.*, 2008). Other mechanisms of protection include impaired entry and growth of the parasites during the erythrocytic stage of development (Chippaux *et al.*, 1992a; Hill *et al.*, 1991; Pasvol *et al.*, 1978; Shear *et al.*, 1993) and enhanced removal of the parasitized

variant RBCs and enhanced probability of infection early in life (Weatherall and Clegg, 2001).

1.7 Complement Regulatory Proteins and Malaria

The erythrocyte complement regulatory proteins, Complement Receptor-1 (CR1), Decay Accelerating Factor (DAF/CD55) and Membrane Inhibitor of Reactive Lysis (MIRL/CD59) are important in protecting RBCs from complement mediated damage and in controlling the complement activation cascade. Erythrocyte CR1 and CD55 are important determinants of malaria susceptibility (Rowe *et al.*, 1997; Stoute *et al.*, 2003; Waitumbi *et al.*, 2000). The interaction of *P.falciparum* Erythrocyte Membrane Protein-1 (*Pf*EMP-1) expressed on *P. falciparum* infected cells with CR1 on uninfected erythrocyte results in rosetting leading to obstruction of blood flow and hence cerebral malaria (Rowe *et al.*, 1997). Rosetting has been shown to be more common in isolates from children with complicated malaria and its frequency is lower in isolates from blood group O patients than from blood group A or B (Cserti and Dzik, 2007; Rowe *et al.*, 1995). RBC CR1 deficiency is common in malaria –endemic region of Papua New Guinea and heterozygotes for the CR1 low-expressions allele (HL) are significantly protected from severe malaria possibly through reduced rosetting (Cockburn *et al.*, 2004). The selection of rosette reducing polymorphisms in populations with high malaria mortality is strong evidence that CR1-mediated rosetting plays a causal role in the pathogenesis of severe malaria (Cockburn *et al.*, 2004).

Studies carried out in Western Kenya where malaria is holoendemic and malarial anaemia is the main manifestation of severe malaria in children have suggested that low CR1 and CD55 levels on red cells of severe malarial anaemia cases increase their susceptibility to phagocytosis and complement mediated lysis (Stoute *et al.*, 2003; Waitumbi *et al.*, 2000). The CR1 and CD55 levels were shown to be corrected with treatment and transfusion and remained high long after the maximum expected lifespan of the donor RBCs suggesting that these changes were acquired (Stoute *et al.*, 2003; Waitumbi *et al.*, 2000). In Thai patients with severe malaria, a significant higher frequency of the Low expression genotype [LL] was observed. The LL genotype is

associated with decreased CR1 copy numbers per erythrocyte and this may result in decreased clearance rate of immune complexes from the circulation which then predisposes one to malaria through increased deposition of immune complexes on red cells (Nagayasu *et al.*, 2001).

1.8 Sickle Cell Trait and Malaria: Importance in Clinical Practice

The frequency of the mutant gene for sickle cell is widely distributed in the sub-Saharan Africa, the Middle East and the Indian subcontinent. Its prevalence in the sub-Saharan Africa is between 20-25% but can be as high as 40% in some regions. The World Health Organization (WHO) estimates that there are 2-32% carrier frequency of the sickle cell gene, and most of these are found in areas where malaria is hyper endemic (Weatherall and Clegg, 2001).

There is epidemiologic evidence that children with sickle cell trait are protected from the severe manifestations of malaria in malaria endemic areas (Dvorak *et al.*, 1975a; Ganczakowski *et al.*, 1995a; Ruwende *et al.*, 1995). HbAS has been shown to protect against severe malarial anaemia, high density parasitemia and to be associated with reduced all cause mortality between the ages of 2 to 16 months (Aidoo, 2002). This also happens to be the age when children have the highest episodes of severe malarial anaemia (Aidoo, 2002). The balanced polymorphism in the HbS-malaria relationship is thought to be maintained by higher mortality risk of HbAAs due to malaria and high mortality risk of HbSSs caused by complications of HbSS (Aluoch, 1997). The HbS allele apparently does not prevent infection but results in impaired entry and growth of the parasites during the erythrocytic stage of development (Chippaux *et al.*, 1992a; Hill *et al.*, 1991; Pasvol *et al.*, 1978; Shear *et al.*, 1993), enhanced removal of the parasitized variant RBCs (Weatherall and Clegg, 2001) and reduced rosette formation (Carlson *et al.*, 1994). When heterozygotes are infected, the merozoites, which have a high metabolic rate, consume a lot of oxygen and this leads to sickling under low oxygen tension. The spleen removes these sickled cells before the micro-organisms have a chance to produce a large infectious population in the body. It is this selective heterozygote advantage that maintains HbS gene at a higher level in malarial than in non-malarial environments

(Aluoch, 1997; Weatherall and Clegg, 2001). The prevalence of the sickle cell trait in Western Kenya is estimated to be approximately 17.4% (Aidoo, 2002). This high level of sickle cell carrier state in this region is thought to be maintained by the high malaria prevalence (Aluoch, 1997).

1.9 Malaria, Sickle Cell Trait and the Complement System

Erythrocyte complement regulatory proteins, CR1, CD55, and CD59 may protect RBCs from complement mediated damage that occur when immune complexes are formed during malaria infection (Jhaveri *et al.*, 1997; Waitumbi *et al.*, 2000). Children with severe malaria associated anaemia have been shown to have reduced immune complex binding capacity and increased susceptibility of their red cells to immune complex deposition and this may predispose their erythrocytes to complement mediated damage and phagocytosis (Owuor *et al.*, 2008). On the other hand, HbAS individuals have been shown to have fewer episodes of severe malaria associated anaemia, reduced risk of anaemia episodes and reduced risk of high density parasitemia than their counterparts with HbAA (Aidoo, 2002).

1.10 Gaps in the understanding of the way Sickle Cell Trait Protects from Malaria

Although several factors have been proposed to explain the mechanism underlying the protective effect of sickle cell carrier status against severe malaria manifestations (Carlson *et al.*, 1994; Chippaux *et al.*, 1992a; Hill *et al.*, 1991; Pasvol, 1980; Shear *et al.*, 1993; Weatherall and Clegg, 2001), none of these can fully explain why individuals with sickle cell trait are protected from severe manifestations of malaria especially severe anaemia.

Severe *P. falciparum* malaria leads to the formation of immune complexes in the peripheral circulation and increased immune complexes in the peripheral circulation have been associated with the development of severe anaemia (Mibei *et al.*, 2005; Stoute *et al.*, 2003). The complement receptor proteins CR1 and CD55 protect RBCs from

complement mediated damage that can occur when immune complexes are formed during malaria infection (Jhaveri *et al.*, 1997).

Given the important role of RBCs in removal of immune complexes from circulation and in regulation of complement activation, it is therefore important to consider the competence of sickle cell trait RBCs in these functions. So far, the level of CR1 and CD55, the immune complex binding capacity and the susceptibility to complement in heterozygous carriers of the sickle cell gene has not been studied. There is strong evidence that the complement regulatory system machinery is important in the protection from the severe manifestations of malaria. The aim of this study was therefore, to study the same machinery in sickle cell trait to determine whether this could explain the protection from severe malaria.

The mechanism of protection from the severe manifestations of malaria afforded by sickle cell trait is especially important because its understanding could hold the key to future novel therapeutic interventions and prophylactic measures against severe malaria.

1.11 Justification for the Study

Severe *P. falciparum* malaria complications for example malarial anaemia is a major cause of morbidity and mortality of children under the age of five years living in malaria endemic areas in Western Kenya. The development of effective preventive measures such as malaria vaccine will be hastened by a better understanding of factors that contribute towards protective immunity and pathological events associated with severe disease. It has been observed that children with sickle cell trait are protected from the severe manifestations of malaria (Aidoo, 2002). A number of mechanisms have been proposed to explain this protection (Carlson *et al.*, 1994; Chippaux *et al.*, 1992b; Hill *et al.*, 1991; Pasvol *et al.*, 1978; Shear *et al.*, 1993; Weatherall and Clegg, 2001), but these do not fully explain the degree of this protection.

Other investigations have implicated low levels of RBC complement regulatory proteins (CR1 and CD55) in the pathogenesis of severe anaemia (Stoute *et al.*, 2003; Waitumbi *et al.*, 2000) and also associated their acquired loss with increased circulating immune

complexes (Waitumbi *et al.*, 2000). Moreover CR1 molecule expression in normal individuals is genetically determined and variations of up to 10-fold in the level of expression have been demonstrated in different normal individuals (Moulds, 2002).

Although the importance of red cell complement regulatory proteins in protection against severe malarial anaemia have been demonstrated, no studies have been carried out to determine whether these molecules contribute to the protective effects of sickle cell trait against severe malarial anaemia. The purpose of the present study was to investigate whether there are differences in the level of expression of CR1 and CD55, immune complex binding capacity and susceptibility to complement activation between red cells of individuals with HbAA and HbAS. In addition, the possible association between these differences with age was also examined. The current study has demonstrated significant higher mean immune complex binding capacity on red cells of HbAS individuals than HbAA individuals. This may partly contribute to the protective effect of sickle cell trait against severe malarial anaemia.

1.12 Research Questions

1) Do HbAS red cells have higher levels of complementary regulatory proteins (CR1 and CD55), higher immune complex binding capacity and higher susceptibility to complement activation than those of HBAA red cells?
2) What are the effects of varying the haemoglobin oxygen saturation on the above parameters?

1.13 Hypothesis

RBCs of individuals with sickle cell trait express higher levels of CR1 and CD55, have higher immune complex binding capacity and are less susceptible to complement activation than RBCs of individuals with normal adult haemoglobin.

1.14 General Objective

To compare the level of CR1 and CD55 expression, immune complex binding capacities and complement susceptibility of red blood cells of individuals with sickle cell trait versus those who are homozygous for haemoglobin A.

1.14.1 Specific Objectives

1) To determine differences in the expression levels of CR1 and CD55 on RBC of children with sickle cell trait and those with homozygous haemoglobin A under normal and reduced oxygen saturation.
2) To determine differences in immune complex binding capacities of RBCs of children with sickle cell trait and those who are homozygous for haemoglobin A under normal and reduced oxygen saturation.
3) To determine differences in the complement susceptibility of RBCs of children with sickle cell trait and those homozygous for haemoglobin A under normal and reduced oxygen saturation.

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CHAPTER 2

GENERAL LITERATURE REVIEW

2.1 Etiology of Human Malaria

Malaria is an acute disease caused by a unicellular parasite of the genus *Plasmodia* and spread by the female *A*nopheles mosquito. Four species of *Plasmodia* cause human malaria namely: *P. falciparum, P. ovale, P.vivax* and *P. malariae* (Bray and Garnham, 1982; Schofield and Grau, 2005; Tuteja, 2007). *P. falciparum* is the most virulent plasmodia species infecting man and accounts for the bulk of morbidity and mortality (WHO, 2005). The reasons for this parasites virulence are not fully understood but are thought to be partly due to the fact that it has a short pre-erythrocytic period, pre-patent period and incubation period (Bray and Garnham, 1982; Schofield and Grau, 2005; Tuteja, 2007). Unlike the other species of Plasmodia, *P. falciparum* invades a large percentage of red blood cells (RBCs) producing more merozoites which thus leads to high parasitaemia. On the other hand, *P. vivax* and *P. ovale* are restricted to reticulocytes while *P vivax* invades mainly the old red cells (Bray and Garnham, 1982; Schofield and Grau, 2005; Tuteja, 2007).

Other factors attributed to its virulence include cytoadherence, rosette formation and, the ability of the parasites to sequester in the cerebral microvasculature of the microvasculature of the deeper organs, cytokine production and immune evasion mechanisms (Carlson *et al.*, 1994).The malaria vector, *Anopheles gambiae*, the most widespread and the most difficult to control preferentially feeds on humans and is long lived, thus making it very effective in malaria transmission (Greenwood and Mutabingwa, 2002).

The entomological inoculation rate for *A. gambiae* is as high as 1000 in some areas of Africa making transmission intense (Greenwood and Mutabingwa, 2002).

2.2 The Epidemiology and Economic Burden of Malaria in Africa

Annually an estimated 300-500 million clinical episodes of malaria are observed resulting in 1-3 million deaths. The true burden of malaria is however difficult to assess because of poor disease surveillance in malaria endemic areas, many febrile illnesses have similar clinical presentation as malaria and parasitological diagnosis is lacking in many centres (Breman, 2001).

Approximately 90% of all the malaria deaths occur in Africa South of the Sahara. This is partly because the majority of infections are caused by *P. falciparum*, the most dangerous malaria parasite. Children and pregnant women bear the brunt of the disease. Malaria contributes to childhood mortality in several ways. Acute malaria infection may lead to cerebral malaria, respiratory distress and hypoglycemia, while chronic repeated infections can lead to severe malarial anaemia (Marsh *et al.*, 1995). In pregnant women, malaria is a major cause of pre term delivery and low birth weight (Feresu *et al.*, 2004; Tako *et al.*, 2005).

In malaria endemic countries of Africa, 25-40% of all outpatient clinic visits are due to malaria and in some countries 20-50% of all hospital admissions are because of malaria. A high number of deaths among the hospitalized patients are due to malaria and this is partly attributable to late presentation, inadequate management and unavailability of effective drugs (Breman, 2001).

Most of the malaria deaths in the sub-Saharan Africa occur mainly in children under the age of 5 years. The prevalence of severe forms of malaria is high in children admitted to hospital with cerebral malaria affecting 575,000 children a year in Africa and killing 10-40% of the patients. Of those who survive 5-20% experience neurological sequelae including behavioral disorders, intellectual impairment and seizure disorders (Murphy and Breman, 2001). Severe malarial anaemia is estimated to occur 1.42-5.66 million times annually and kills 190,000-974,000 children < 5 years annually (Murphy and Breman, 2001). Respiratory distress, hypoglycemia and overlapping clinical

manifestations affect an additional 1.12-1.99 million cases resulting in 225,000 deaths annually (Marsh *et al.*, 1995; Marsh and Snow, 1999).

Malaria is a disease of poverty stricken and underdeveloped countries and in general, where malaria prospers most, human societies prospered least (Sachs and Malaney, 2002). The global distribution of per capita Gross Domestic Product (GDP) shows a striking correlation between malaria and poverty (Sachs and Malaney, 2002). Endemic areas which are mainly the tropics and subtropical zones have therefore lower rates of economic growth. It impedes development by its effect on fertility, population growth, worker productivity, absenteeism, premature mortality and medical costs (MOH, 2008; Sachs and Malaney, 2002).

Majority of malaria afflicted countries are in the Sub Saharan Africa, the poorest regions of the world. The average annual gross domestic production (GDP) growth rate for malaria ridden countries (defined as a country with index above 0.5) was 4% per annum as compared with average of 2.3% increase yearly in the countries without significant malaria presence. The average GDP per capita for high malaria countries as measured in US dollars was $1526 and the average for the rest of the countries was $8268. This indicates that non malaria countries' economies are five times more productive and growing at five times higher rate than those of malaria countries (Sachs & Jeffrey, 2002). Malaria inhibits economic growth and lack of economic growth allows malaria to be unchecked. The cost of malaria treatment has been estimated to reduce African continents' GDP by $12 billion, which is larger than the entire GDP of many Sub-Saharan countries. Without malaria, the GDP of some African countries would be around 30% greater than it is today. Malaria reduces the GDP of African countries by $12 billion a year (Sachs & Jeffrey, 2002). The level of international spending on malaria control in poor regions has however been dismal; less than $100 million per year. It was estimated that by 2007, effective treatment and prevention should have required $2.5 billion and this figure should increase to $4 billion by 2015 (Sachs and Malaney, 2002). The WHO estimates that the cost of even basic control programmes that will cover the whole population is perhaps as much as US$ 2 billion per year for an indefinite period (Sachs and Malaney, 2002)

2.3 The Epidemiology and Economic Burden Malaria in Kenya

Malaria is still a major cause of morbidity and mortality in Kenya and was the leading cause of outpatient and inpatient mortality from 1989-2000 in Kenya (MOH, 1989-2000). It is estimated that 25 million of 34 million Kenyans are at risk of infection; 30-50% of all outpatient attendances and 20% of the admissions are due to malaria. It claims 34,000 lives annually, pregnant women and their unborn children are particularly vulnerable to malaria which is a major cause of pre-natal deaths and low birth weight. It is also estimated to cause 20% of the deaths in children. Many children who survive severe disease may suffer from learning impairment and brain damage (MOH, 2008).

In 2001-2002, the operation cost for running the malaria programme for the Kenyan Government was projected at US$ 17 million and it was estimated that the Ministry of Health required approximately US$ 107 million for its malaria control budget (MOH, 2008), In addition, it is estimated that 170 million working days are lost to the disease per year (MOH, 2008). There has been however, a downward trend in malaria cases from the year 2002 attributable to scaling up of malaria control interventions, notably distribution of insecticide treated bed nets especially the long lasting insecticide treated bed nets (MOH, 2008).

2.4 Clinical Manifestations and Outcome of Malaria

The clinical signs and symptoms of malaria are non specific and can be confused with any febrile illnesses which are common in malaria endemic areas. The general clinical features are characterized by acute febrile paroxysms. The manifestation and severity depends on species, host immunity, general health, nutritional status and age, length of exposure, the transmission pattern and the presence or absence of certain genetic factors known to confer protection (Aidoo, 2002; Aluoch, 1997; Marsh *et al.*, 1995; Marsh and Snow, 1999).

In areas where endemicity is relatively low due to infrequent transmission, malaria tends to occur in epidemics. Compared to areas of stable transmission where the risk of death

from malaria infection is 2-3%, in epidemic outbreaks, case fatalities of up to 10 times greater can occur (Kiszewski and Teklehaimanot, 2004).

Severe malaria becomes less likely as children grow older but when it does occur, the children and adults are likely to develop life threatening cerebral manifestations (Kiszewski and Teklehaimanot, 2004). Stability of transmission also affects pregnancy outcome. Premature deliveries are common in epidemic prone regions while intra uterine growth retardation predominate where transmission are stable (Kiszewski and Teklehaimanot, 2004).

In a large prospective study of children admitted to the pediatric ward of Kilifi district hospital with a primary diagnosis of malaria, four key prognostic indicators: impaired consciousness, respiratory distress, hypoglycemia and jaundice were found to identify those at the greatest risk of dying (Marsh, 1995). In children with malaria with impaired consciousness or malaria with severe respiratory distress, a big percentage was also found to have severe malarial anaemia. Severe malarial anaemia in combination with either of the two syndromes was noted to have a higher mortality rate. The concurrent occurrence of all the three resulted in mortality rate of up to 34.7% (Marsh, 1995). Children with severe malarial anaemia and respiratory distress were found to have an underlying lactic acidosis. The lactic acidosis identified those at the greatest risk of death and was due to inadequate tissue oxygenation. This lactic acidosis often resolves rapidly during transfusion (English *et al.*, 1997).

In Western Kenya, malaria transmission is very high with entomological inoculation rates of 50-300 (Beier *et al.*, 1994a). The commonest manifestation of severe malaria in this region is severe malarial anaemia. The susceptibility to getting severe malarial anaemia is highest between the ages of 6-24 months (Stoute *et al.*, 2003; Waitumbi *et al.*, 2000) and the mortality among the hospitalised children range from 20-30% (Lackritz *et al.*, 1992; Lackritz *et al.*, 1997). The carriage of sickle cell gene is equally high with prevalence of up to 20% (Aidoo, 2002). The high level of sickle cell carrier state is thought to be maintained by the high malaria prevalence (Aluoch, 1997).

2.5 Pathogenesis of Malarial Anaemia

The pathogenesis of severe malarial anaemia remains poorly understood despite much work (Abdalla *et al.*, 1980; Weatherall *et al.*, 1983; Wickramasinghe and Abdalla, 2000). It is however thought to be multifactorial resulting from both increased RBC destruction and decreased RBC production (Abdalla *et al.*, 1980; Wickramasinghe and Abdalla, 2000). Increased RBC destruction may result from erythrophagocytosis, splenic removal and autoimmune haemolysis (Abdalla *et al.*, 1980; Wickramasinghe and Abdalla, 2000). On the other hand, decreased RBC production can result from suppressed erythropoeisis by TNF-α (Akanmori *et al* 2000), bacterial infections (Luzzatto, 1979), dyserythropoeisis (Abdalla, 1988; Abdalla, 1990; Kurtzhals *et al.*, 1999), and erythroid hypoplasia and cytokine imbalance (Abraham and Kroeger, 1999; Akanmori, 2000; Luty *et al.*, 2000; Othoro *et al.*, 1999).

Many patients in malaria endemic areas display multiple pathology including infections (intestinal helminthes, HIV), nutritional deficiencies (iron, folate, vitamins A, B and C) and genetic factors such as haemoglobinopathies, thalassemias and glucose-6- phosphate dehydrogenase (G6PD) deficiency. Numerous studies undertaken to elucidate the pathogenesis of severe malarial anaemia do not include the presence or absence of red cell abnormalities and enzyme deficiencies. The contribution of these genetic factors to the pathogenesis or protection from severe malaria remains largely unknown. However, it has been observed that sickle cell trait offers protection from the severe manifestations of malaria but the exact mechanisms are not yet known fully known. (Carlson *et al.*, 1994; Chippaux *et al.*, 1992a; Hill *et al.*, 1991; Pasvol *et al.*, 1978; Shear *et al.*, 1993; Weatherall and Clegg, 2001).

2.5.1 The Role of Cytokines in the Pathogenesis of Severe Malarial Anaemia

The balance in the production of the Th-1 type pro-inflammatory cytokines such as TNF-α and IFN-γ and the Th-2 type anti-inflammatory cytokines such as IL-10 and IL-4 may be critical in the pathogenesis of severe malarial anaemia (Othoro *et al*, 1999, Perkins *et al*, 2000). High plasma TNF-α concentration have been found to correlate strongly with

increasing disease severity (Perkins *et al*, 2000), whereas, low plasma IL10/ TNF-α ratio have been associated specifically with SA (Othoro *et al.*, 1999; Perkins *et al.*, 2000). The production of TNF-α not only induces fever but also has been implicated in suppression of erythropoeisis; reduced erythropoietin production and increased erythrophagocytosis (Akanmori, 2000). High levels of IL-10; a potent anti-inflammatory mediator, have been shown to protect against severe anaemia possibly by down-regulating the effects of TNF-α (Othoro *et al.*, 1999). Plasma levels of IL-10 have been found to be lower in children with SA than in those with uncomplicated malaria thus indicating further that SA may result from deficiency in the regulation of the pro-inflammatory cytokines (Kurtzhals *et al.*, 1998; Othoro *et al.*, 1999). Furthermore, the fever and elevated temperature induced by TNF-α can suppress parasitemia; prolonged exposure to TNF-α however promotes severe disease. Nussenblatt and colleagues (Nussenblatt *et al.*, 2001) found higher levels of TNF-α in association with higher levels of IL-10 levels at admission for malaria and on subsequent visits for older children with malaria. In younger children, this relationship was not maintained at subsequent visits. This observation suggests that younger children do not maintain IL-10 production in response to the inflammatory process and this mechanism may contribute to the more severe anaemia found in the younger children (Nussenblatt *et al.*, 2001). Thus, children with *P. falciparum* infection who produce balanced levels of IL-10 to regulate excessive TNF-α are better able to control severe anaemia (Kurtzhals *et al.*, 1998; Nussenblatt *et al.*, 2001; Othoro *et al.*, 1999) Higher TNF-α concentration correlate strongly with increasing disease severity and the balance between plasma IL-10 and TNF-α concentration or the IL-10/ TNF-α ratio was shown to be predictive of SA (Othoro *et al.*, 1999). TNF-α and IL -10 are also positively correlated with parasitemia (Kurtzhals *et al.*, 1998; Luty *et al.*, 2000; Othoro *et al.*, 1999). Production of suboptimal levels of TNF in response to any level of parasitaemia may lead to persistent TNF levels sufficient to interfere with erythropoeisis and increased erythrophagocytosis (Akanmori, 2000), in turn contributing to bone marrow suppression and disturbed iron metabolism (Kurtzhals *et al.*, 1998).

2.5.2 The Role of Nitric Oxide in the Pathogenesis of Severe Malarial Anaemia

Nitric oxide is synthesized in various nucleated cells by oxidative deamination of the amino acid L-arginine to L-citrulline in a reaction catalysed by nitric oxide synthase in the presence of various co-substrates (Burgner, 1999). Investigations of human malaria have demonstrated that NO plasma levels are elevated during *P. falciparum* malaria infection (Boutlis *et al.*, 2003).

The inducible NOS isoform (NOS2) represents the high output pathway to NO production and the NOS2G-954C (NOS2-Lambarene) has been associated with a degree of protection against the development of severe malaria in some studies (Kun *et al.*, 1998). TNF is a potent inducer of inducible nitric oxide synthase 2 (NOS2). NO-related species mediate inhibition of malaria parasite by hepatocytes and monocytes *in vitro* and NO production is inversely proportional to disease severity. Levels of NO metabolites which are used to demonstrate cytokine inducible NO synthesis in human and leukocyte NOS2 expression were decreased in both uncomplicated and cerebral malaria indicating suppression of NO synthesis rather than excessive production in clinical malaria in children and the suppression of NO synthesis increased with disease severity. The suppression of NO mediated protective immune response after infection with *P. falciparum* may result in inadequate control of parasite replication and may have contributed to the development of clinical malaria and cerebral malaria in these children (Anstey, 1996). Suppression of NO production in clinical malaria and cerebral malaria may have deleterious effects other than a possible impaired ability to suppress parasite development. TNF-α, IL-1 and other pro-inflammatory cytokines are known to increase a number of human endothelial molecules involved in cytoadherence and sequestration (e.g. ICAM-1, V-CAM-1, E-selectin). NO decreases this endothelial activation (Anstey, 1996). Therefore decreased NO synthesis in malaria could increase endothelial adhesive molecules, increased parasite sequestration and greater likelihood of developing cerebral malaria. Decreased NO production may also result in increased production of TNF-α. Asymptomatic malaria exposed children have higher levels of NO than children with severe disease and NO has been proposed as a mediator of malaria tolerance where children have parasitemia without fever. This was also found in Papuan adults (Craig SB

et al, 2003). On the other hand Nitric oxide decreases erythropoiesis and has been implicated in anaemia of chronic disease. High levels of NO have been found in Tanzanian children with low grade asymptomatic parasitemia with severe anaemia compared to those with acute uncomplicated malaria (Anstey *et al.*, 1999). These findings are consistent with those of Gyan and colleagues (Gyan *et al.*, 2002) who also demonstrated higher NO levels in children with SA than those with cerebral malaria and uncomplicated malaria and also found an association between elevated levels of NO with SA. Therefore, in addition to impaired ability to clear parasites, other deleterious effects of decreased NO production include increased TNF-α production, increased expression of endothelial adhesive molecules, increased parasite sequestration and greater likelihood of developing cerebral malaria (Nahrevanian, 2006)

2.5.3 The Role of Complement Regulatory Proteins in the Pathogenesis of Malaria

2.5.3.1 The Complement System

The complement system is a series of over 30 serum and cell surface glycoproteins that interact with other immune system molecules and with one another, normally in a regulated manner to perform many immune system functions such as solubilization and phagocytic clearance of immune complexes, killing of microbes and activation of inflammation (Abul *et al.*, 1994; Reinagel *et al.*, 1997; Walport, 2001a; Walport, 2001b). The system can be activated by antibody and forms a link between immunity and inflammation. It is also an effector mechanism that leads to destruction of micro-organisms.

The major pathways leading to complement activation are classical and alternative pathways. The classical pathway is triggered by binding of certain antibodies (notably IgM but also IgG1, IgG2 and IgG3) to an antigen while the alternative pathway is activated by bacterial cell surface molecules. C1q triggers the serine proteases C1r and C1s, the latter cleaving C4 to C4b, which exposes a specific binding site for C2. C1s then cleaves C2 and the resulting C3 convertase C4b2a cleaves C3 to C3b to form the C5 convertase C4b2a3b (Walport, 2001a; Walport, 2001b).

The alternative pathway occurs in the absence of specific immunity reactions but can be activated by aggregated IgA, some micro-organisms or virus infected cells and a variety of complex polysaccharides. The C3b formed by the cleavage of C3 forms a complex with factor B that is consequently cleaved by factor D. The resulting C3 convertase is stabilized by the binding of properdin. Cleavage of C3 and binding of an additional C3b to the C3 convertase give rise to the C5 convertase of the alternative pathway. Subsequent reactions are common to both pathways (Walport, 2001a; Walport, 2001b).

The classical, alternative and the Mannose Binding Pathways all converge to C3. C3 is split by C3 convertase into C3a and C3b. C3a is an anaphylotoxin while C3b is an opsonin. C3b together with C4b2b acts as C5 convertase. C5b binds to C6 and C7 leading to C5b67 which in turn binds C8 to form C5b678. The last step is the binding of C9 to form the membrane attack complex leading to cell lysis. Activation of the complement proteins leads to the coating of foreign agents with C3b and to a lesser extent, C4b, a process known as opsonization which is crucial for phagocytosis. Opsonization also leads to the formation of Membrane Attack Complex, leading to cell lysis (Walport, 2001a).

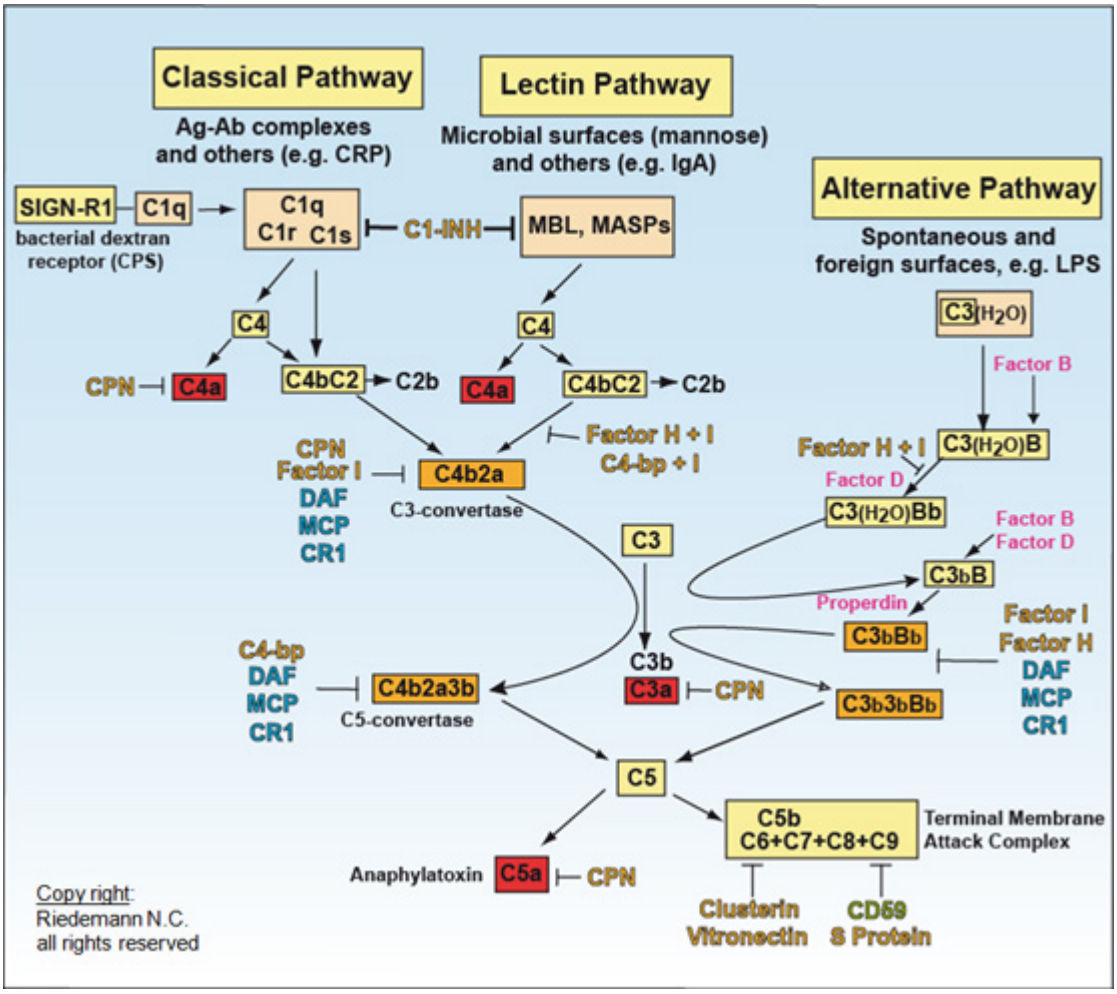

Figure 2.1: Complement System (*Image from inflarx.com images web site*)

2.5.3.2 The Complement in Health and Disease

The complement system is an essential component of the innate immune response to several infectious agents (Guo and Ward, 2005). However, excessive activation of complement system, especially formation of the potent pro inflammatory anaphylatoxin C5a, has been implicated in mediating deleterious host responses to bacterial infections and contributing to the development of sepsis, adverse outcomes, and death (Guo and Ward, 2005; Ward, 2004). The complement system has also been suggested to play a role in pathogenesis of many diseases with an immune component such as asthma, lupus

erythematosus, glomerulonephritis, autoimmune heart disease and multiple sclerosis (Iida *et al.*, 1982; Walport, 2001a; Walport, 2001b).

Moreover, deficiencies of the terminal pathway predispose to both autoimmune disease and infections, particularly Neisseria meningitis, due to the role that the C56789 complex plays in attacking Gram-negative bacteria (Walport, 2001a; Walport, 2001b). Mutations in the complement regulators factor H and membrane cofactor protein have been associated with atypical haemolytic uraemic syndrome whereas a common single nucleotide polymorphism in factor H (Y402H) has been associated with the common eye disease; age-related macular degeneration both of which are disorders thought to be due to aberrant complement activation on host surfaces (Abul *et al.*, 1994).

2.5.4 The Role of Complement System in the Pathogenesis of Cerebral Malaria and Malarial Anaemia

Various studies of complement levels in patients with *P. falciparum* infection have associated hypocomplementemia with the severe complications of malaria for example cerebral malaria (Adam *et al.*, 1981) and severe malarial anaemia (Nyakoe *et al.*, 2009) thus indicating a relationship between complement consumption and these clinical complications.

Immune complexes (ICs) formed during malaria infection and malarial antigens can activate complement either through the classical or the alternative pathways (Adam *et al.*, 1981; Wenisch *et al.*, 1997). In humans, the subsequent covalent binding of C3b or C4b to the complexes allows them to bind to complement receptor 1 (CR1, CD35) expressed on erythrocytes. Once bound to erythrocytes via CR1, the immune complexes are carried to the liver and spleen, where they are removed from the erythrocytes and phagocytosed (Schifferli, 1996). Thus, low CR1 expression levels on erythrocytes may result in poor ICs clearance capacity, which then predisposes one to IC-mediated complications through their deposition in various organs, such as the kidney (Allison *et al.*, 1969; Boonpucknavig and Sitprija, 1979) or the choroid plexus (June *et al.*, 1979). Deficiencies in red cell CR1 and CD55 in children with SMA have been reported to result in marked

decline in IC binding capacity and increased C3b deposition on red cells (Odhiambo *et al.*, 2008; Owuor *et al.*, 2008) which may predispose erythrocytes to complement-mediated damage and phagocytosis in vivo (Owuor *et al.*, 2008).

Complement activation also leads to generation of C5a and C3a fragments which are important inflammatory activators that induce vascular permeability, recruitment and activation of phagocytes (Gardinali *et al.*, 1992). Experimental infection with *Plasmodium berghei ANKA (PbA)* led to development of cerebral malaria in C5 sufficient mice whereas C5 -deficient strains were resistant and antibody blockade of C5a or C5a receptor (C5aR) rescued the susceptible mice from cerebral malaria (Patel *et al.*, 2008).

Additional *In vitro* studies supporting a role for C5/ C5a in the pathogenesis of CM showed that C5a-potentiated cytokine secretion induced by the malaria product *P. falciparum* glycosylphosphatidylinositol and C5aR blockade abrogated these amplified responses (Patel *et al.*, 2008). The C5aRs are constitutively expressed by CNS neurons, suggesting that they may be at risk in settings of CM associated inflammation and complement activation (O'Barr *et al.*, 2001). The C5a has also been shown to up-regulate several endothelial cell adhesion molecules including intercellular cellular adhesion molecule 1, vascular cellular adhesion molecule 1, and P- and E-selectins (Wada *et al.*, 2001) that are implicated in the sequestration of *P. falciparum* infected erythrocytes in cerebral microvasculature and hence may contribute to cerebral malaria pathogenesis (Patel *et al.*, 2008).

2.5.4.1 Complement Regulatory Proteins

The complement system is regulated by various complement regulatory proteins (CRPs). Complement Receptor 1 (CR1), Membrane Cofactor Protein, Decay Accelerating Factor, Factor H, C4-binding protein belongs to the regulators of complement activation (RCA) family (Hourcade *et al.*, 1989). The RCA are a multi-gene family which comprises six serum or membrane proteins that share a common functional activity of regulation of the C3 convertase and a common structural motif of 60 amino acid Short Consensus Repeat (SCR) domains. The RCA proteins control complement activation by promoting

irreversible dissociation of complement convertases and serving as co-factors in the factor-1 mediated cleavage of C3b and C4b (Hourcade *et al.*, 2002)

The CD55 catalyzes the degradation of C3 convertases and also promotes the inactivation of C3b. The CD59 inhibits the formation of the MAC by binding C5b678 and blocks the binding of C9 (Kinoshita *et al.*, 1986; Walport, 2001a; Walport, 2001b). CD55 and CD59 both play a role in regulating haemolysis due to deposition of immune complexes on the surface of RBCs (Terpos *et al.*, 2008; Waitumbi *et al.*, 2000).

Complement receptor-1 is found on the surface of erythrocytes and most leukocytes, glomerular podocytes, and follicular dendritic cells (Ahearn and Fearon, 1989). Erythrocyte CR1 promotes C3b and C4b decay, stimulates phagocytosis and binds immune complexes which are transported to the liver and the spleen for transfer to and ingestion by the macrophages leading to their elimination. Immune complex free RBCs then return to the circulation where they can continue to participate in immune complex clearance (Zorzetto *et al.*, 2002).

2.5.4.2 Complement Receptor 1 in Pathology and Protection in Malaria

Complement receptor 1 (CR1, CD35) is a 200 kDa immune regulatory molecule (figure5b) found on the surface of erythrocytes and most leukocytes including neutrophils, eosinophils, mononuclear phagocytes, follicular dendritic cells, B lymphocytes, a subset of T lymphocytes and glomerular podocytes (Ahearn and Fearon, 1989). The CR1 expressed on the surface of the phagocytic cells mediates adherence and ingestion of C3b/C4b-coated particles (Reinagel *et al.*, 1997). On B lymphocytes and follicular dendritic cells, these activities promote antigen localization and processing (Krych-Goldberg and Atkinson, 2001).

Erythrocyte CR1 promotes decay of C3 and C5 convertases, protein complexes that catalyze the cleavage of C3 and C5 into C3b and C5b respectively (Abul *et al.*, 1994) They also serves as co-factor for irreversible cleavage of C3b into iC3b and C3dg as well as C4b into C4c and C4d by Factor 1 (Ahearn and Fearon, 1989). The CR1 on red cells also bind C3b-opsonized immune complexes (ICs) and carry them to the liver and spleen

where they are removed from circulation (Hess and Schifferli, 2003; Zorzetto *et al.*, 2002) without the ingestion of the erythrocyte by the mononuclear phagocytes (Reinagel *et al.*, 1997). Therefore, CR1 prevents the deposition of C3b on cell surfaces and has a critical role in the removal of ICs from circulation (Odhiambo *et al.*, 2008; Waitumbi *et al.*, 2000).The rate of immune complex clearance from the circulation has been shown to be directly correlated to the number of CR1 molecules expressed on the erythrocytes (CR1/E ratio) (Zorzetto *et al.*, 2002). Variation in CR1 level and its expression is associated with a number of autoimmune disorders such as glomerulonephritis, systemic lupus erythematosus, immune haemolytic anaemia, rheumatoid arthritis and sarcoidosis (Zorzetto *et al.*, 2002). Erythrocyte CR1 has also been proposed to be an important determinant of malaria susceptibility in several investigations (Rowe *et al.*, 1997; Stoute *et al.*, 2003; Waitumbi *et al.*, 2000).

2.5.4.3 The Structure of Complement Receptor-1

CR1 is approximately a 200kDa, single-chain glycoprotein. The extracellular domain is comprised of 30 complement control proteins repeats (CCPs) or Short Consensus Repeats (SCR) of which the 28 N-terminal CCPs can be organized based on degree of homology into four long homologous repeats, in the most common CR1 allotype. These are designated LHR-A, LHR-B, LHR-C and LHR-D each composed of 7 CCPs.

There are two functionally distinct active sites in CR1. Site 1 is located in CCPs 1-3 of the LHR A. Site 2 is in CCPs 8-10 of LHR B and CCPs 15-17 of LHR C. Site 1 binds mainly C4b and has decay accelerating activity for the classical and alternative pathway C3 convertases (Krych-Goldberg *et al.*, 1999). Site 2 in both LHR B and LHR C binds C3b and C4b and possess co-factor activity for factor I mediated cleavage of C3b and C4b (Hourcade *et al.*, 2002). LHR D and /or SCRs 29+30, binds C1q and therefore may participates in immune complex formation (Tas *et al.*, 1999). The Knops blood group system antigens which shows marked differences in the African and Caucasian population and have been linked to protection against malaria (Moulds *et al.*, 2001) are located on CCP24 and 25 of LHR-D.

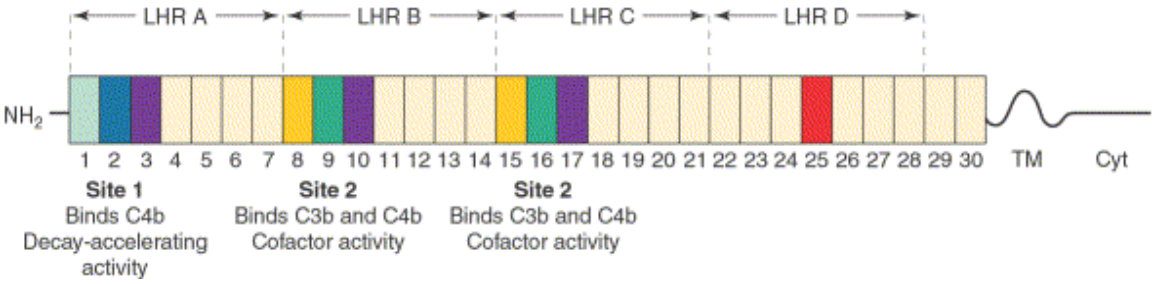

Figure 2.2: The Structure of Complement Receptor -1 (Krych-Goldberg *et al.*, 2002)

2.5.5 The Decay-Accelerating Factor (CD55)

Decay-accelerating factor is a 70-KDa glycoprotein composed of four SCR domains of approximately 60 amino acids each followed by a 67 amino acid serine/threonine-rich region that is heavily o-glycosylated and a carboxyl-terminal GPI anchor that attaches DAF to the cell membrane through direct insertion of the phosphatidyl moiety into the outer lipid bilayer (Lukacik *et al.*, 2004).

DAF binds C3bBb and C4B2a and dissociates the convertases thereby preventing the destruction of host tissue (Kuttner-Kondo *et al.*, 2003; Kuttner-Kondo *et al.*, 2001). DAF is widely distributed on human hematopoeitic, epithelial and endothelial tissues. A soluble form of DAF can be detected in body fluids including plasma, tears, saliva and urine (Lublin, 2005). The importance of these complement regulatory proteins is highlighted by the disease Paroxysmal Nocturnal Haemoglobinuria (PNH), an acquired hemolytic syndrome with three cardinal clinical features: intravascular hemolysis, venous thrombosis and a variable degree of bone marrow failure (Chrobak, 2000). The hemolytic phenotype of PNH arises because DAF, CD59 and C8 binding proteins are all absent from the cell surface because of a defect in the pathway for the biosynthesis of the GPI anchor. DAF is normally expressed as a glycosylated 70-kDa protein, although an 82-kDa form has been described (Hensel *et al.*, 2001). The functional sites of DAF are contained within four short consensus repeats (SCRs, also known as complement control protein motifs—CCPs—or ^{sushi} domains)

It has been shown that classical and alternative pathway regulatory activity of DAF is associated with SCRs 2 and 3 and SCRs 2, 3, and 4, respectively (Brodbeck *et al.*, 1996).

2.5.5.1 The Role of DAF in Health and Disease

DAF is expressed in all blood cells but over expressed in some malignant cells, making these cells more resistant to complement mediated hemolysis (Spendlove *et al.*, 2006). DAF also serves as a cellular receptor for uropathogenic E. coli and a number of viruses (Hasan *et al.*, 2002). Evidence from multiple disease models suggest that DAF plays an important role in protection against tissue damage in autoimmune diseases. The use of recombinant DAF as a therapeutic agent in autoimmunity and inflammation, and of DAF transgenic animals in xenotransplantation, is being actively investigated (Lublin, 2005).

In pregnancy, a fully active complement system is present at the placental level and its role is to protect the fetus and the mother against infections and other toxic agents. As the fetus is semi allogenic and allo-antibodies commonly develop in the mother, the placenta is potentially subject to complement mediated immune attack at the feto-maternal interface with the potential risk of fetal loss. Uncontrolled complement attack is prevented by three regulatory proteins, DAF, MCP and CD59 positioned on the surface of the trophoblast. Mice deficient in the complement regulator Crry normally suffer growth retardation or death (Girardi *et al.*, 2006).

Deficiency of CD55 has also been implicated in the pathogenesis of severe malarial anaemia (Waitumbi *et al.*, 2000).

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CHAPTER 3

MATERIALS AND METHODS

3.1 Study Site

The study site was Kombewa Division, in Kisumu West District, Nyanza Province of - Western Kenya. Kombewa borders Lake Victoria and has a population of about 65 000 people. Malaria transmission in this area occurs all year round with peak seasons following the long rains (March to May) and the short rains (October to December). The annual inoculation rates are estimated to be 300 infective bites per person per annum (Beier *et al.*, 1994b). This site is situated about 35 kilometres west of Kisumu town and has previously been used as a site for many epidemiological studies in both adults and children. The research assays were performed at the Walter Reed Project/KEMRI research laboratory in Kondele, Kisumu town.

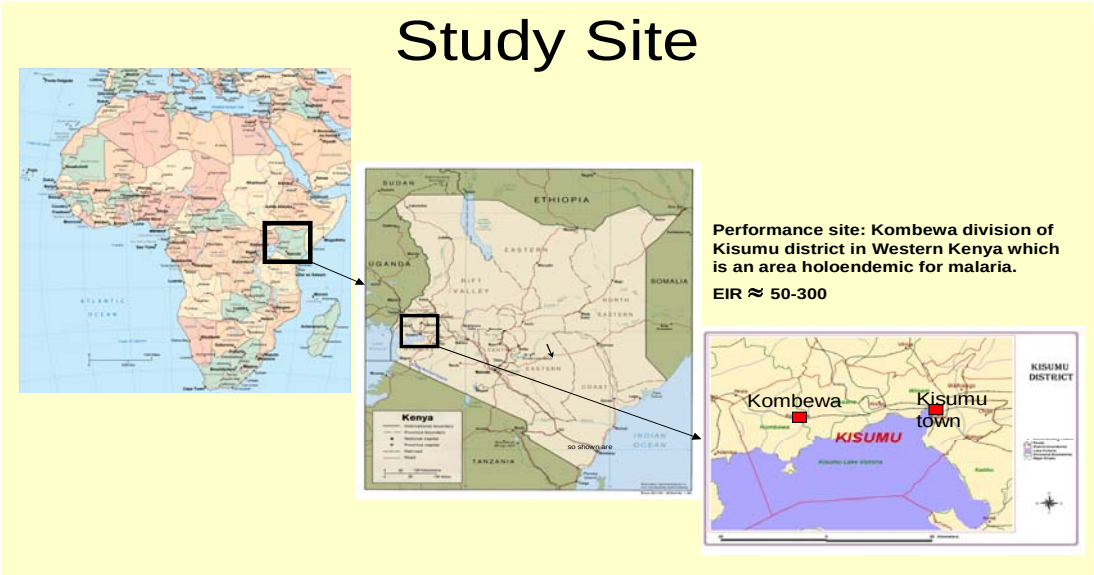

Figure 3.1: Study Site

3.2 Study Design and Patient Population

This was a cross-sectional survey with nested case-control study which was open to healthy male and females of aged 0 to 45 years who were residents of Kombewa Division of Kisumu West District. The potential participants were assessed for any acute or chronic illness which could interfere with the parameters under investigation. In cases of an acute illness, the potential participants were assessed, treated and asked to come again for re-evaluation. At re-evaluation, the potential participants were enrolled when they were deemed well. Haemoglobin electrophoresis was carried on blood from all participants. Individuals who were less than 16 years and HbAS positive were identified and matched by age (± 2 months or ± 2 years for those below or more than 8 years, respectively) at a ratio of 1:1 or 1:2 with those with HbAA and these formed the nested case control cohort.

3.3 Recruitment

All subjects were recruited by non-coercive means. Consent was obtained from the parent or guardian of the volunteers and a consent form was duly signed that attested to the willingness of the parent's agreement for the child to participate in the study. All subjects underwent a standardized clinical evaluation and physical examination by an experienced physician or physician assistant at enrollment and blood was collected. Minor illnesses such as upper respiratory tract infections were treated and if malaria was present, this was also treated. All subjects with non-severe malaria were given treatment for malaria if the Giemsa-stained blood smear was positive. Artemether/lumefantrine (Coartem®) was the first line. The dose of malaria treatment was in accordance with Kenyan Ministry of Health guidelines which currently recommends the use of Coartem as first-line therapy for the treatment of uncomplicated *P. falciparum* malaria. Individuals who were still parasitemic following treatment with Coartem were treated with quinine.

3.3.1 Inclusion Criteria

The study was open to healthy male and females up to 45yrs of age. For the nested case control, children up to the age of 16 years with HbAS on Hb electrophoresis were matched to those with HbAA as described above.

3.3.2 Exclusion Criteria

Volunteers were excluded when they had evidence of concomitant infection, severe malaria and other conditions which in the opinion of the clinician could affect the parameters being measured for example malignancy, protein energy malnutrition, severe anaemia or transfusion in the preceding three months.

3.4 Collection and Processing of Blood Samples

Blood for CBC and hemolysate preparation was collected by trained clinical personnel using sterile and disposable needles, syringes or lancets into a 2 ml EDTA vacutainer tube (Becton Dickinson, San Diego, CA) and kept at 4 °C until processed. Approximately 10 µl of blood was used for preparation of Giemsa-stained thick and thin smears. Blood smears were read by experienced microscopists to confirm the diagnosis of *P. falciparum* malaria.

3.5 Assays and Procedures

3.5.1 Blood Smears

At enrollment a Giemsa-stained thick and thin smears were prepared from finger prick or EDTA blood collected by venepuncture. After staining, a minimum of 50 high power fields (HPF) were scanned for a positive smear and 200 HPF for a negative smear. The number of asexual stage parasites was presented per 200 white blood cells (WBCs) counted. The parasite density per µl was calculated by multiplying the total WBCs by the number of parasites per 200 WBCs.

3.5.2 Complete Blood Picture

Complete blood count was done using a standard hematology analyzer (Coulter, Hialeah, FL).

3.5.3 Haemoglobin Electrophoresis

Haemoglobin electrophoresis on cellulose acetate plates was carried out under alkaline conditions using reagents and kits from Helena Laboratories (Beaumont, TX). Briefly, 100 μL of EDTA whole blood was placed in a micro-centrifuge tube and the packed red cell pellet were washed x3 with normal saline or phosphate buffered saline (PBS) pH 7.4. 10μL of packed cells was mixed with 60 μL of hemolysate reagent, mixed well by vortexing and allowed to stand for 5 min. 5μL of sample and controls was placed on each well of the electrophoresis chamber. After applying voltage for 25 min, the plate was soaked in Ponceau S stain for 5 min and destained in successive washes of 5% acetic acid for 2 min.

3.5.4 Deoxygenation of the RBCs for Assay

An equal amount of RBCs in wash buffer was added to freshly prepared disodium hydrogen phosphate (Na_2HPO_4, FW 142g) 0.114M and sodium dithionite ($Na_2S_2O_4$ FW 174.1g) 0.114M at a ratio of 2:3, filter sterilized through a 0.22um filter. The disodium hydrogen phosphate was prepared from a stock solution while the sodium dithionite was prepared fresh every day. The cells were incubated at 37ºC for 1 hour and then washed twice with wash buffer before running the assays side by side. This duration of treatment with the dithionite was found to give the maximal sickling for HbAS RBCs as depicted in the picture below but to had no effect on HbAA RBCs.

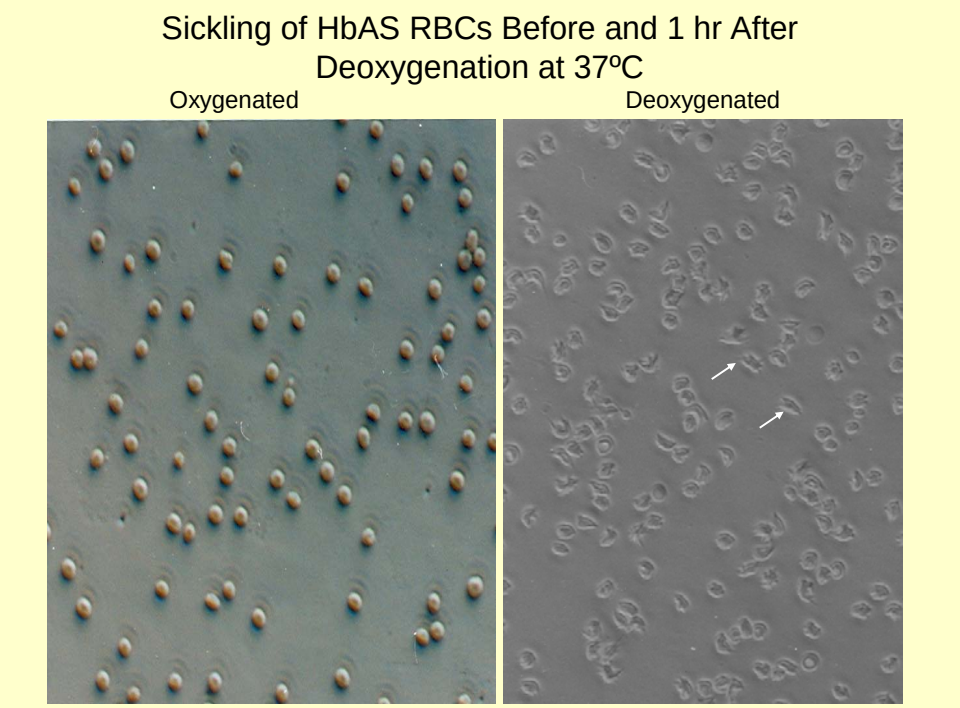

Figure 3.2: Morphologic changes when HbAS cells are treated with sodium dithionite.

This photo micrograph shows changes in red blood cell morphology after treatment of the HbAS cells with sodium dithionite and incubating at 37ºC for 1 hr. [Note the sickling of the red cells (arrowed)].

3.6 Sample Size Calculation and Data Analysis

3.6.1 Sample Size Calculation

Based on a prevalence of sickle cell trait in Western Kenya of 15%, we expected to identify about 36 cases between the ages of 6 months and 8 years. For a paired sample t test, this number and the same number of controls provided at least a 80% power to detect a difference in the level of CR1 of 45 between cases and controls with $\alpha = 0.05$, two tailed.

3.6.2 Data Analysis

Statistical analysis was done using Statistical Package for Social Sciences (SPSS V 11.0 and V 15.0). Demographic data pertaining to the volunteers were presented in a descriptive fashion. The independent samples *t*-test was used for comparisons of normal continuous data between two groups. Chi-square ($\chi 2$) and Mann-Whitney U tests were used to examine differences between proportions and for pairwise comparisons of medians, respectively. Pearson's correlation coefficient was used to examine relationship between any two numerical variables. Analysis of variance (ANOVA) was used to detect differences across age groups adjusting for factors and covariates. Bivariate logistic regression analysis was used to determine the Odds Ratio (OR) and the 95% confidence interval (CI) for haemoglobin level, mean CR1 copy numbers per erythrocyte, mean CD55 antibody binding capacity, Cb3 deposition and the presence of *P. falciparum* parasitemia. Multivariate logistic regression was used to determine the differences in mean CR1 copy numbers per erythrocyte, mean antibody binding capacity under deoxygenated conditions and immune complex binding capacity between the two groups (HbAA and HbAS). All tests were two-tailed with $\alpha \leq 0.05$.

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CHAPTER 4

PREVALENCE AND AGE SPECIFIC DISTRIBUTION OF MALARIA AND SICKLE CELL TRAIT IN THE STUDY POPULATION

4.1 Introduction

P .falciparum malaria has had huge impact on the human genome as evidenced in relative resistance to malaria by numerous hereditary red cell disorders including membrane protein disorders, enzyme deficiencies and haemoglobinopathies (Durand and Coetzer, 2008).

Haldane, (Haldane, 1949), observed that several red blood cell disorders such as sickle cell anaemia are common in areas where malaria was endemic and hypothesized that malaria was the selection pressure for the occurrence of some of these genetic polymorphisms. A recent investigation by Crompton and colleagues (Crompton *et al.*, 2008) reported that sickle cell trait was associated with a delay in time to first malaria episode between the ages of 2-10 years. It has been suggested that malaria protection by HbAS involves enhancement of both innate and acquired immunity to the parasite (Williams *et al.*, 2005a). The protective effect of HbAS was found to be remarkably specific for *P. falciparum* malaria (Williams *et al.*, 2005b) which is the major cause of devastating illness especially in the pediatric age group (Marsh *et al.*, 1995). Williams and colleagues (Williams *et al.*, 2005b) found that HbAS had no effect on the prevalence of symptomless parasitemia but was protective against mild clinical malaria, admission to hospital for malaria and approximately 90% protective against severe or complicated malaria. Although malaria is often fatal in individuals with sickle cell anaemia (HbSS), the protection from infection appears to operate in HbS dose dependent manner and individuals with HbSS have an even lower risk of infection than those with HbAS (Aluoch, 1995).

This was a nested case-control study and was part of a cross-sectional study entitled "Erythrocyte Immune Complex Binding Capacity and Complement Sensitivity in Populations with Different Malaria Risks" as described earlier (Chapter 3). In the nested case-control study of children, we quantified and determined the role of CR1 and CD55 levels, the immune complex binding capacity and the complement susceptibility of RBCs of participants with sickle cell trait and those homozygous for haemoglobin A as detailed in Chapters 5-8. For all the participants, we compared those with HbAS and HbAA to see whether there were any differences in the haemoglobin levels, the presence of malaria parasitemia and parasite densities between the two groups at a time when they were well, a febrile and had no signs of any acute or chronic illness.

4.2 Materials and Methods

This was as described earlier (Chapters 3).

4.3 Results

4.3.1 Recruitment of Volunteers

402 volunteers were screened of which 190 were enrolled on the first visit. 196 volunteers had malaria and other intercurrent illnesses and were treated and told to come after two weeks while 16 were excluded for various reasons like chronic illnesses. During the second visit, 152 volunteers were enrolled, ten were given treatment a second time and thirty four were lost to follow up. Three volunteers were enrolled on the third visit and seven were excluded after meeting the recruitment target. After meeting the target for the enrollment, haemoglobin electrophoresis was done for all the study volunteers and for those subjects who had sickle cell trait (HbAS), they were matched with those who had normal haemoglobin. In total we had 58 volunteers with sickle cell trait who were matched to 89 volunteers with normal haemoglobin at a ratio of 1:1 or 1:2. For those < 8years, they were matched at ±2 months while for those > 8years, they were matched at ± 2 years.

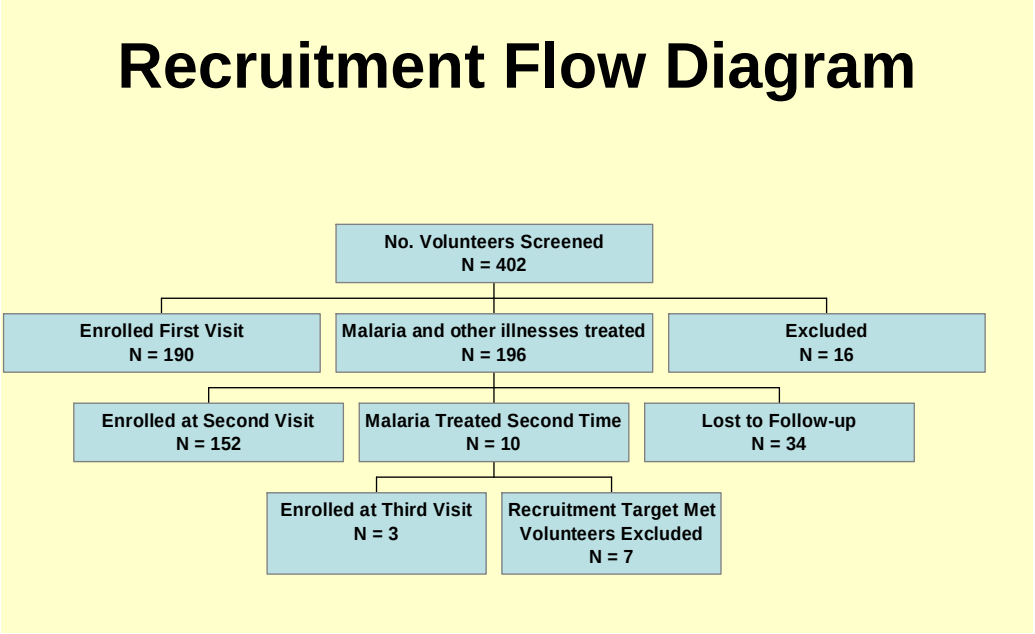

Figure 4.1: Recruitment of Study Volunteers

4.3.2 Demographic and Laboratory Characteristics of the Study Population

342 participants were enrolled for this study of which, 280 (81.4%) had haemoglobin AA, 60 (17.4%) had haemoglobin AS and 2 (0.6%) had haemoglobin SS. Those with HbAA and HbAS were included for the above analysis. This study showed that gender (P = 0.828), median age in months (P = 0.726) and presence or absence of malaria parasitemia (P = 0.638) were not significantly different between the two groups. The mean parasite count/µL for the HbAA [mean = 4822.4 (95% CI 3196.30 – 6448.60)] was more than twice as high as those with HbAS [mean = 1693.3 (95% CI 669.70 – 2717.00)]. This did however not reach significance (P = 0.406) see Table 4.1

Table 4.1 Demographic and Laboratory Characteristics of the Study Participants

Characteristic	HbAA (n =280)	HbAS (n = 60)	*P*
Gender			
Male n (%)	145 (51.8)	32 (53.3)	0.828[a]
Female n (%)	135 (48.2)	28 (46.7)	
Age in months (Median; 25th -75th percentiles)	36.50 (14.00 – 130.25)	30.0 (12.25 – 128.25)	0.726[b]
Haemoglobin levels (Median; 25th -75th percentiles)	11.00 (9.80 – 12.58)	11.15 (10.23 – 12.10)	0.825 [b]
Mean parasite count (95% C I)	4822.4 (3196.30 – 6448.60)	1693.3 (669.70 – 2717.00)	0.406[b]
Numeric *P. falciparum* read			
Negative n (%)	154 (55.0)	35 (58.3)	0.638[a]
Positive n (%)	126 (45.0)	25 (41.7)	

[a]Chi-square (χ^2) test, [b]Mann-Whitney U test

4.3.3 Demographic and Laboratory Characteristics of Children with Parasitemia within the Study Groups

For HbAS with parasitemia, there was no significant difference in gender (P = 0.326), median age in months (P = 0.707) and median haemoglobin levels (P = 0.602). The mean parasite count/μL for HbAS was 4064.0 (95% CI 1858.0 – 6270.0) and for the HbAA with parasitemia, the mean parasite count/μL was 11,067.9 (95% CI 7616.0 – 14520.0). This difference was statistically significant ***P* = 0.001** (independent sample T-test) see Table 4.2

Table 4.2 Demographic and Laboratory Characteristics of Children with Parasitemia within the Study Groups

Characteristic	**HbAA (n = 122)**	**HbAS (n = 25)**	***P***
Gender			
Male n (%)	61 (50.0)	15 (60.0)	0.326[a]
Female n (%)	61 (50.0)	10 (40.0)	
Age in months (Median; 25th -75th percentiles)	37.50 (13.00 – 104.50)	30.00 (10.00 – 95.50)	0.707[b]
Haemoglobin levels (Median; 25th -75th percentiles)	11.00 (9.86 – 12.50)	11.30 (10.60 – 11.85)	0.602 [b]
Mean parasite count (95% C I)	11,067.87 (7615.95 – 14519.79)	4,064.00 (1857.93 – 6270.07)	**0. 001**[c]

Parasitemic children (n=147) were stratified according to their haemoglobin type into (a) HbAA (n=122) and (b) HbAS (n=25). Data is presented as either mean or median with appropriate intervals or percentiles respectively.

a - χ^2 analysis, b – Mann-Whitney U test, c – Independent sample T-test

4.3.4 Relationship between Haemoglobin Level and Presence of Parasitemia in HbAA and HbAS Red Cells.

For all the participants, there was no difference in haemoglobin level between HbAA and HbAS (OR; 1.065, 95% CI, 0.861 – 1.317; *P* = 0.564, Table 4c), *P. falciparum* count/μL (OR; 1.000, 95% CI, 1.000 – 1.000; *P* = 0.084, Table 4c) and the presence or absence of parasitemia (OR; 0.873, 95% CI, 0.496 – 1.536; *P* = 0.637, Table 4.3)

There was no difference in haemoglobin levels in the parasitemic group between HbAA and HbAS (OR; 0.964, 95% CI, 0.763 – 1.219; *P* = 0.761, Table 4c). There was a non significant protection of HbAS against high density parasitemia compared to HbAA (OR; 1.000 95% CI, 1.000 – 1.000; *P* = **0.065**, Table 4.3).

Table 4.3 Relationship between Haemoglobin Level and Presence of Parasitemia in HbAA and HbAS Red Cells

Characteristics	**Variable**	**HbAA**		
		Odd Ratio (OR)	95% CI	***P***
PARASITAEMIC AND APARASITAEMIC CHILDREN (340)	Haemoglobin levels (g/dL)	1.065	0.861-1.317	0.564
	P. falciparum count/μL	1.00	1.00-1.00	0.084
	Numeric *P. falciparum* read	0.873	0.496-1.536	0.637
PARASITAEMIC CHILDREN (147)				
	Haemoglobin levels (g/dL)	0.964	0.763-1.219	0.761
	P. falciparum count/μL	1.00	1.00-1.00	0.065

Bivariate logistic regression analysis to determine the Odds Ratio (OR) and the 95% confidence interval (CI)

4.3.5 Demographics, Hematological and Parasitological Characteristics of the Study Cohorts

The total number of study participants investigated was divided into 8 age group cohorts. The mean haemoglobin level, haemoglobin type, and number positive for parasitemia for each of the age cohorts are summarized in table 4.4 and figures 1, 2, 3 respectively.

The recruitment of study participants is detailed in Figure 4.1. Of the 196 volunteers who had malaria at first visit, majority of children in the >12-24 (20/60), >24-48 (36/60), >48-96 (33/60) and >96-192 (22/31) months age category with the highest number/percentage in the >48-96 month age category (see Table 4.4) There was no correlation between parasitemia and haemoglobin levels. Despite many participants being parasitemic in the >48-96 month age category, the mean parasite/μL was highest in the >12-24 months category (see Table 4.2 and Figure 4.4).

Table 4.4 Demographics, Hematological and Parasitological Characteristics of the Study Cohorts

Cohort (Months)	**No**	**Gender**		**Haemoglobin type**		**Haemoglobin Level**	***P. falciparum* positive**	
		F	M	AA	AS		Neg	Pos
0 - 6	31	11	20	27	3	11.6 (10.6 – 11.8)	25	6
>6 - 12	60	31	29	47	11	10.1 (9.8 – 10.5)	40	20
>12 - 24	60	32	28	48	11	9.6 (9.2 – 10.0)	24	36
>24 - 48	60	29	31	50	10	10.6 (10.2 – 11.0)	27	33
>48 - 96	31	16	15	26	5	12.3 (12.0 – 12.7)	9	22
>96 - 192	30	13	17	13	17	12.7 (12.2 – 13.2)	9	21
>192 - 384	42	23	19	38	4	13.4 (12.7 – 14.0)	30	12
384 - 540	30	12	18	21	9	12.9 (12.1 – 13.6)	26	4

NB: Two volunteers in the 0-6 and >6-12 months age category had Haemoglobin SS.

Out of the 344 volunteers, 14 had *P. Ovale* and 24 had *P. Malariae* infections.

4.3.6 Distribution of Malaria Parasite Density by Age Cohort

The mean parasite count/μL was highest in the >6 – 24 age group cohort after which there was a general reduction in the mean parasite count/μL for the general group. (Figure 4.4)

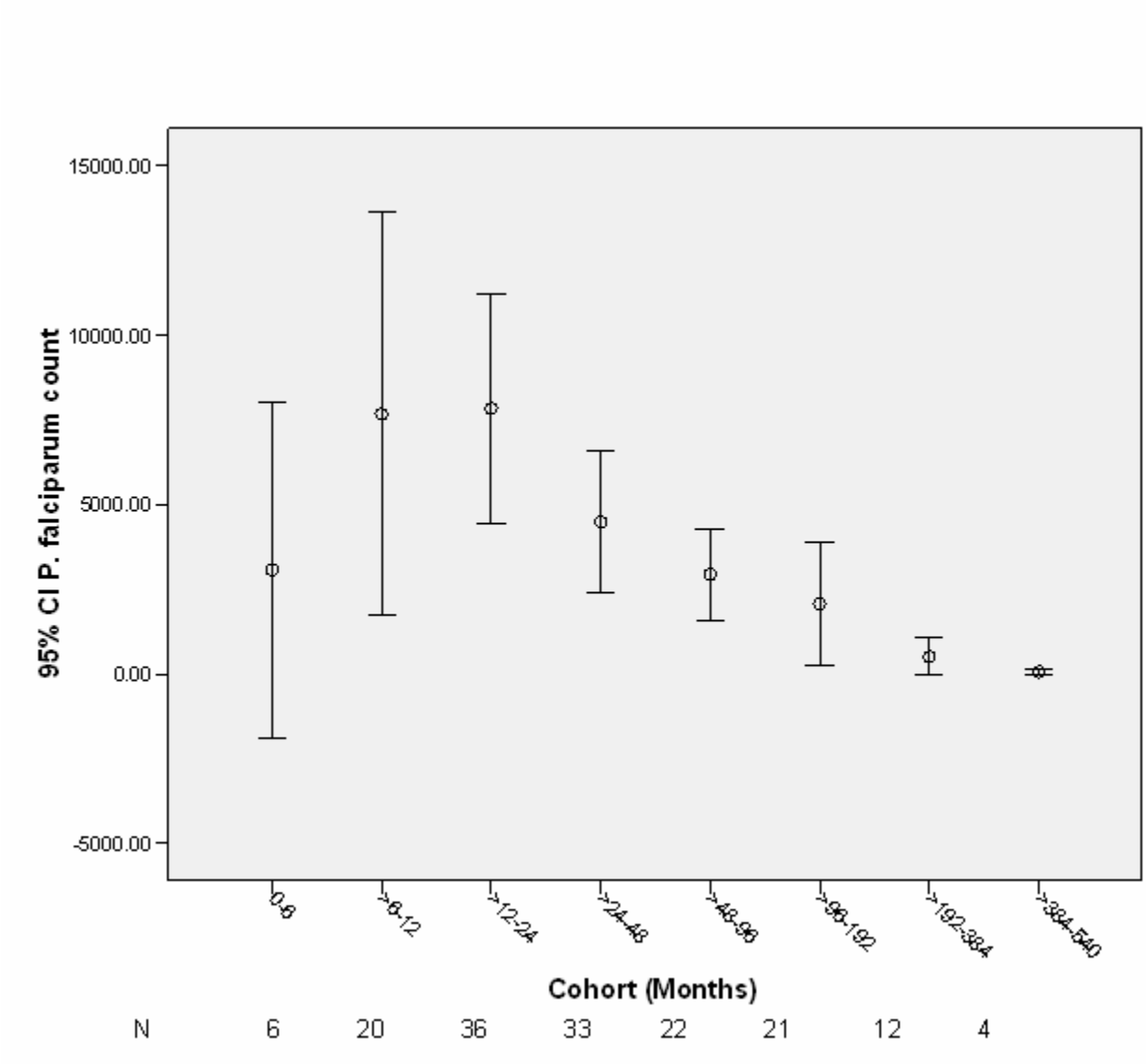

Figure 4.2: Mean *P. falciparum* Parasite Count/μL in the Age Cohorts

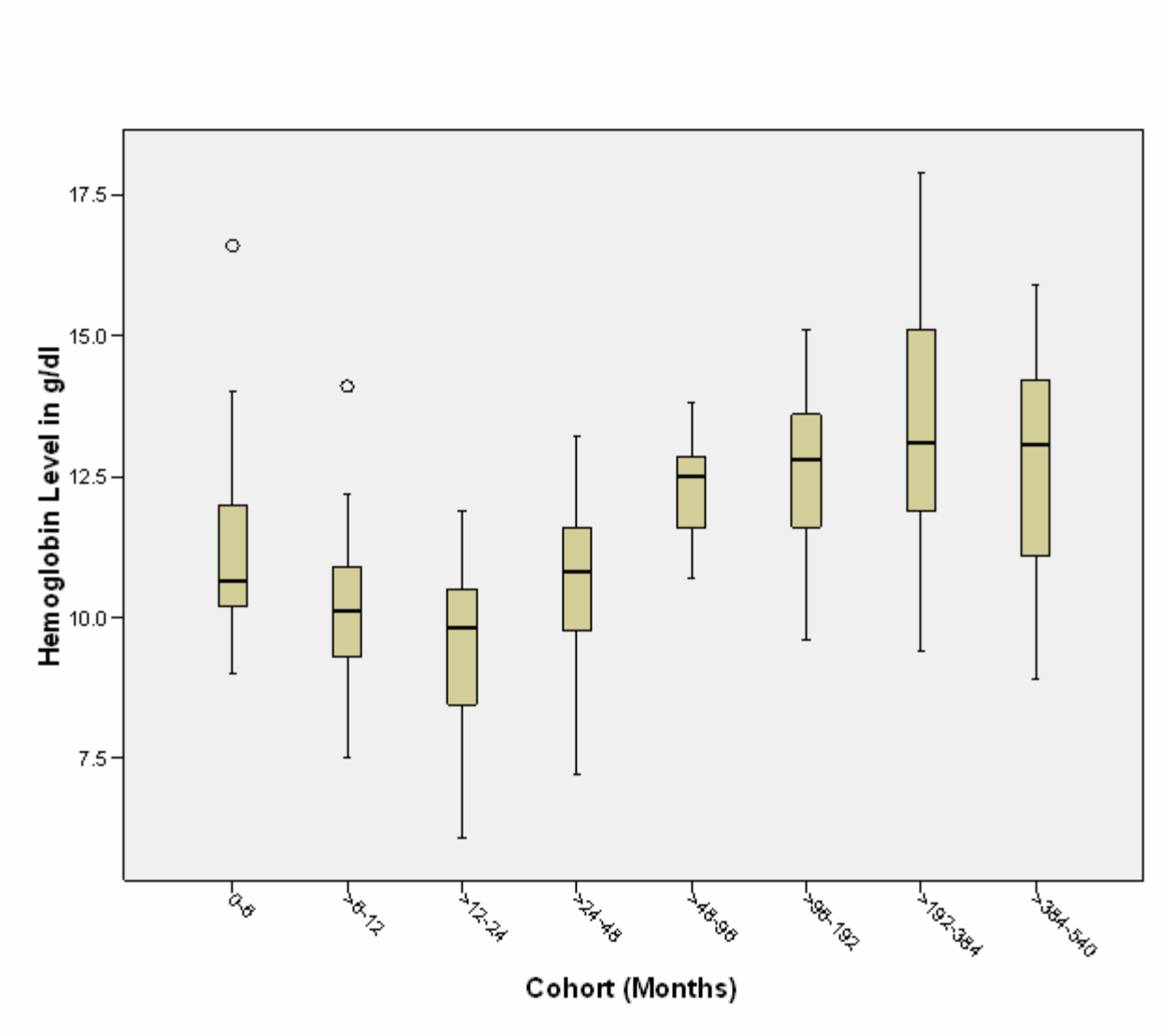

Figure 4.3: Mean Haemoglobin Levels of the Age Cohorts

4.4 Discussion

The mortality caused by malaria in high malaria endemic parts of the world has led to evolvement of sickle cell trait which has been shown to protect against severe manifestations of malaria (Williams *et al.*, 2005b). Haldane proposed that the sickle cell gene provides an advantage in malaria endemic regions (Haldane, 1949). Allison (Allison, 1954) presented evidence for the balanced polymorphism of sickle cell gene and the sickle cell trait (heterozygotes) has been suggested to provide resistance to *P. falciparum* malaria. Sickle cell trait has been shown to lead to delay in the first malaria episode leading to suggestion that haemoglobin typing be done as part of the clinical malaria vaccine and drug trials (Crompton *et al.*, 2008).

When a gene that has potential for reducing fitness achieves high frequencies in the population, it is assumed that the gene confers a survival advantage (Beutler, 1994).

This study showed that the mean parasite count/μL for HbAS was 4064.0 (95% CI 1858.0 – 6270.0) and for the HbAA, the mean parasite count/μL was 11,067.9 (95% CI 7616.0 – 14520.0). This difference was statistically significant (***P* = 0.001**, Independent sample T test). This study is in agreement with an earlier study which showed that the mean *P. falciparum* parasitemia were significantly lower in the HbAS than in the HbAA children and that sickle cell trait seemed to decrease the level of parasitemia (Chippaux *et al.*, 1992b). Other studies have found that individuals with HbAS have lower parasite densities compared to HbAA individuals in asymptomatic, mild and severe malaria (Aidoo, 2002; Williams *et al.*, 2005a) and time to reappearance of *P. falciparum* is lower in HbAS individuals after drug treatment (Sokhna *et al.*, 2000)

The nadir of haemoglobin for the various age cohorts was in the >12-24 months age category. In keeping with previous reports (Crawley, 2004), a significant correlation was observed between age and haemoglobin level r (340) = 0.550 $P < 0.01$. The >12-24 months age category also corresponded to high increase in the percentage parasitemia. This low peak in haemoglobin level has recently been associated with increasing malaria prevalence; low complement regulatory proteins, increased C3b deposition and therefore

development of malaria irrespective of the carrier status for sickle cell (Odhiambo *et al.*, 2008)

There were two peak age groups for malaria parasitemia namely: >12-24 months and >48-96 months age cohorts. The first peak corresponded to the lowest level of haemoglobin. There was no relationship between the presence of *P. falciparum* malaria parasitemia and haemoglobin level.

4.5 Conclusion

In asymptomatic individuals in the community without any signs of an acute or chronic illness, the mean malaria parasite density in HbAS is significantly lower than in HbAA individuals.

The nadir of haemoglobin in the community is >12 -24 months of age and this corresponds to the period where these children have the highest mean parasite counts. This may explain the high prevalence of anaemia in this age group.

4.6 Recommendations

Sickle cell carrier status is prevalent in this region and this study has shown that in the presence of carrier status for sickle cell (HbAS), these children are protected from getting high density parasitemia. Given the upcoming malaria vaccine trial and the fact that some studies have also shown similar results (Sokhna *et al.*, 2000), it is important to screen all potential malaria vaccinees for sickle cell trait and factor this in the analysis so as to tease out the protection from sickle cell trait from protection from the vaccine.

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CHAPTER 5

COMPLEMENT REGULATORY PROTEINS IN CHILDREN WITH SICKLE CELL TRAIT

5.1 Introduction

Complement activation plays an important role in immune defense against malaria infection (Nyakoe *et al.*, 2009; Wenisch *et al.*, 1997) but may contribute to the disease severity if activated in excess (Adam *et al.*, 1981; Nyakoe *et al.*, 2009) or if inappropriately controlled (Stoute, 2005). Indeed, complement factors have been associated with cerebral malaria (Adam *et al.*, 1981) and implicated in the pathogenesis of severe malarial anaemia through mechanisms leading to increased destruction of uninfected as well as infected red blood cells (Kai and Roberts, 2008; Stoute, 2005).

Red blood cells are normally protected from autologous complement attack through the action of various complement regulatory proteins, such as complement receptor 1 (CR1, CD35), decay accelerating factor (DAF, CD55) and membrane inhibitor of reactive lysis (CD59) expressed on these cells (Abul *et al.,* 1994). Recent studies have demonstrated age-related changes in the expression levels of the erythrocyte complement regulatory proteins; CR1 and CD55 (Waitumbi *et al.*, 2004) and associated deficiencies of these molecules with severe malarial anaemia (Stoute *et al.*, 2003). Although complement regulatory proteins play an important role in preventing complement mediated erythrocyte destruction, they have not been investigated for association with protection of sickle cell traits against severe malarial anaemia. In this study, it was hypothesized that erythrocytes of individuals with sickle cell trait may have increased complement receptor-1 per erythrocyte and over expression of DAF which in turn causes increased rate of immune complexes clearance from the circulation and hence resistance to complement attack. To test this hypothesis, RBCs of individuals with HbAS and those with HbAA were examined for differences in CR1 and CD 55 expression levels, immune complex binding capacity and susceptibility to complement deposition.

5.2 Materials and Methods

5.2.1 Staining of Red Blood Cells for Measurement of CR1 and CD55

Venous blood (2.5 ml) was collected in ethylenediaminetetraacetic acid (EDTA) vacutainers (Becton Dickinson, USA). Samples were kept at room temperature until arrival in the laboratory. A 1:50 dilution of RBC packed cell volume (PCV) in Alsevers' buffer was made, from EDTA anticoagulated blood samples, centrifuged at x500g for five minutes and resuspended in the same volume of fresh Alsevers' buffer. The cells were then stored at -70º C for later use.

Erythrocyte CR1 and CD55 levels were determined using frozen samples. In preliminary experiments, we observed no significant effect of freezing on the level of CR1 or CD55.

One hundred microlitres of thawed erythrocytes suspension was added to each of the U-bottom 96well plate (Nunclon, Denmark). The cells were washed twice using 100µl of Alsevers'wash buffer (PBS, 1%BSA, 0.1% Azide sterilized through a 0.22□m filter).The cells were then resuspended in 100µl of 1:50 dilution of primary Mouse anti-human CR1 (IgG1, Accurate clone E11, MAS441) reagent and incubated for 30 minutes at room temperature on an orbital shaker in the dark (Plate sealed and wrapped in aluminium foil). At the end of the incubation period the RBCs were washed twice using 200µl of wash buffer and then stained using 100µl of 1:50 dilution of the secondary reagent (Goat anti-mouse IgG FITC, BD349031) for 30 minutes at room temperature on an orbital shaker in the dark. The RBC's were subsequently washed twice and then resuspended in 200µl of 1% Para formaldehyde until acquisition within 24 to 48 hours after staining.

5.2.2 Flow Cytometric Analysis

Flow cytometry was carried out using a FACScan flow cytometer (Becton-Dickinson). Analysis was done using FCS Express v2.5 (De Novo Software, Los Angeles, CA). Red cells were gated on the basis of their forward and side scatter characteristics using logarithmic amplification. All the assays were done in parallel to red blood cells from a healthy aparasitemic subject who acted as a positive control for complement regulatory

proteins as an internal measure of consistency. The MFI values for CR1 and CD55 were normalized to the mean of the MFI of the red cell standard using the formula

CorrMFIs = MFIs x MFIcmean/MFIc,

Where "CorrMFIs and MFIs are the corrected and uncorrected sample MFI respectively, 'MFIcmean" is the mean of all the MFI values of the standard control, and "MFIc" is the MFI of the control obtained in parallel with the sample.

The number of molecules of CR1 per red cell was derived from a fluorescence standard curve created using cells with known CR1 numbers. Red cell anti-CD5 antibody binding capacities (ABC) were derived from a standard curve created using beads of known antibody binding capacity (Bangs Lab, Fishers, IN) (Schwartz *et al.*, 1994)

5.3 Results

5.3.1 Difference between Mean CR1 Copy Numbers between HbAS and HbAA Cells under normal and reduced Oxygen Saturation

The mean CR1 copy numbers before and after deoxygenation with sodium dithionite are presented as box-and-whisker plots. For each group, the horizontal line in the middle of the box marks the median of the sample. The box represents the interquartile range and the central 50% of the data falls within the range of the box. The whiskers are the vertical lines extending up and down from each box and they represent the upper and the lower 25% of the data. The mean CR1 copy numbers per red blood cell was higher (508, [SD=177]) in the HbAS group than in the HbAA group (471 [SD=165]), this difference was statistically not significant (P = 0.250). When the red blood cells were treated with sodium dithionite, the mean CR1 copy numbers per erythrocyte were higher in both groups, for HbAS (576, [SD=149] and for HbAA (603, [SD=184] but again, there was no statistically significant difference between the two groups (P = 0.400) (Figure 5.1)

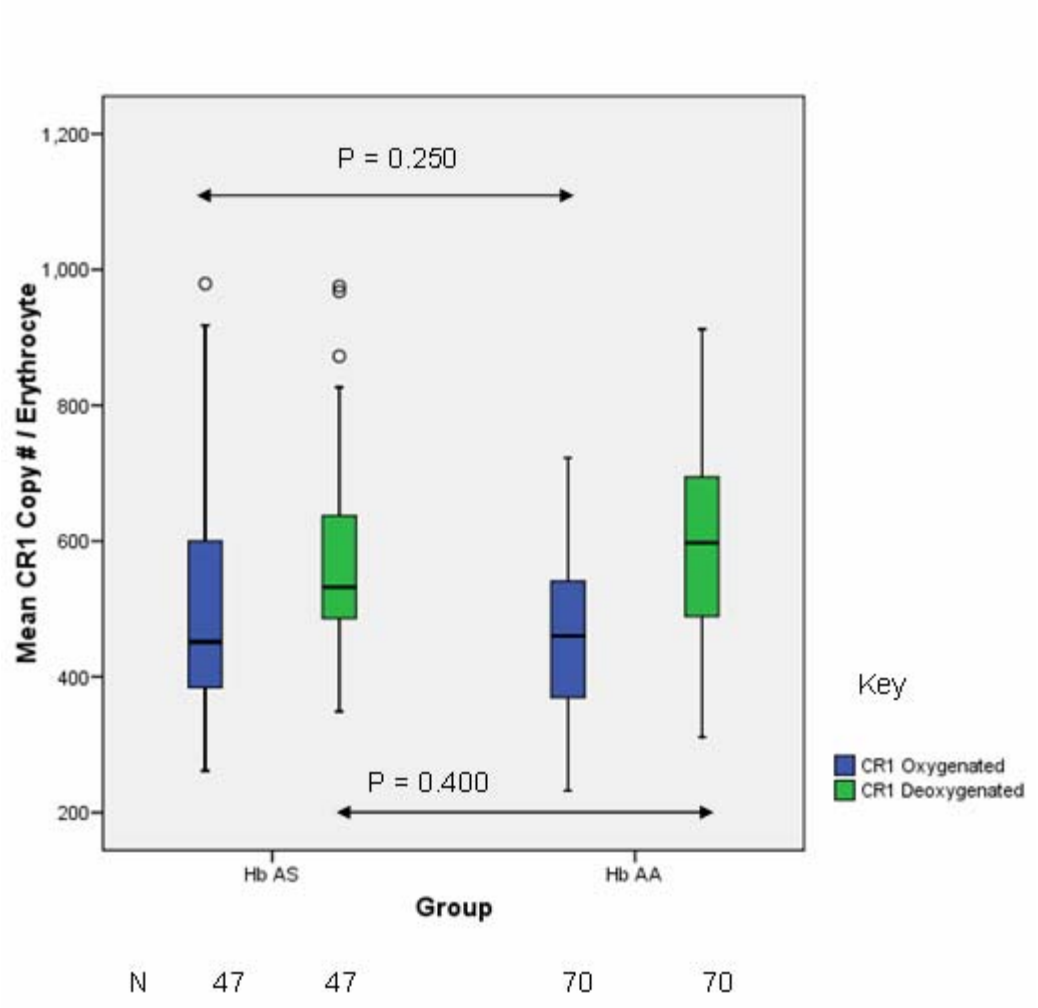

Figure 5.1: CR1 copy numbers between HbAS and HbAA red cells under normal and reduced oxygen saturation

5.3.2 Differences in CR1 Copy Numbers between HbAS and HbAA Red Cells in each Age Cohort

The CR1 copy numbers per erythrocyte (Figure 5.2) was observed to be significantly higher in the >96-192 months age group, HbAS [mean = 671.0, SD 218] and HbAA [mean = 475.0, SD = 88.6], ***P* = 0.009**. Overall, significant positive correlation was observed between the CR1 copy numbers per erythrocyte with age of all individuals in the HbAS and HbAA groups, r (117) = 0.188, ***P* = 0.043**. This was also true for calculated age for HbAS r (47) = 0.296, ***P* = 0.043** and for the cohort, HbAS r (47) = 0.296, ***P* = 0.041**.

5.3.3 Mean CR1 Copy Numbers per Erythrocyte for the various Age Cohorts by Haemoglobin Electrophoresis Results under reduced Oxygen Saturation

The CR1 copy numbers per erythrocyte was observe to vary with age for both HbAS and HbAA groups. The mean CR1 copy numbers per erythrocyte after deoxygenation with sodium dithionite for the various age cohorts are presented. Under deoxygenated conditions, the mean CR1 copy numbers per erythrocyte was again higher for HbAS in the 0-6 month's age group category. Between the age groups >6-48 months; the CR1 copy numbers were almost similar in the HbAA and HbAS. Thereafter the CR1 copy numbers was generally higher in the HbAS than the HbAA category. Under reduced oxygen saturation, there was significant difference between the CR1 copy numbers for the various age cohorts in the HbAS (***P* = 0.048**) but not in the HbAA ($P = 0.295$). There was a positive correlation between CR1 copy numbers and age after deoxygenation $r(117) = 0.218$; ***p* = 0.008**

5.3.4 Relationship between CR1 Copy Numbers per Erythrocyte with Age

The mean CR1 copy numbers per erythrocyte were high in the <6 months age group and was generally low in the >6 to <48 months age group after which there was a general increase. Deoxygenation resulted in a general increase but the same trend was maintained.

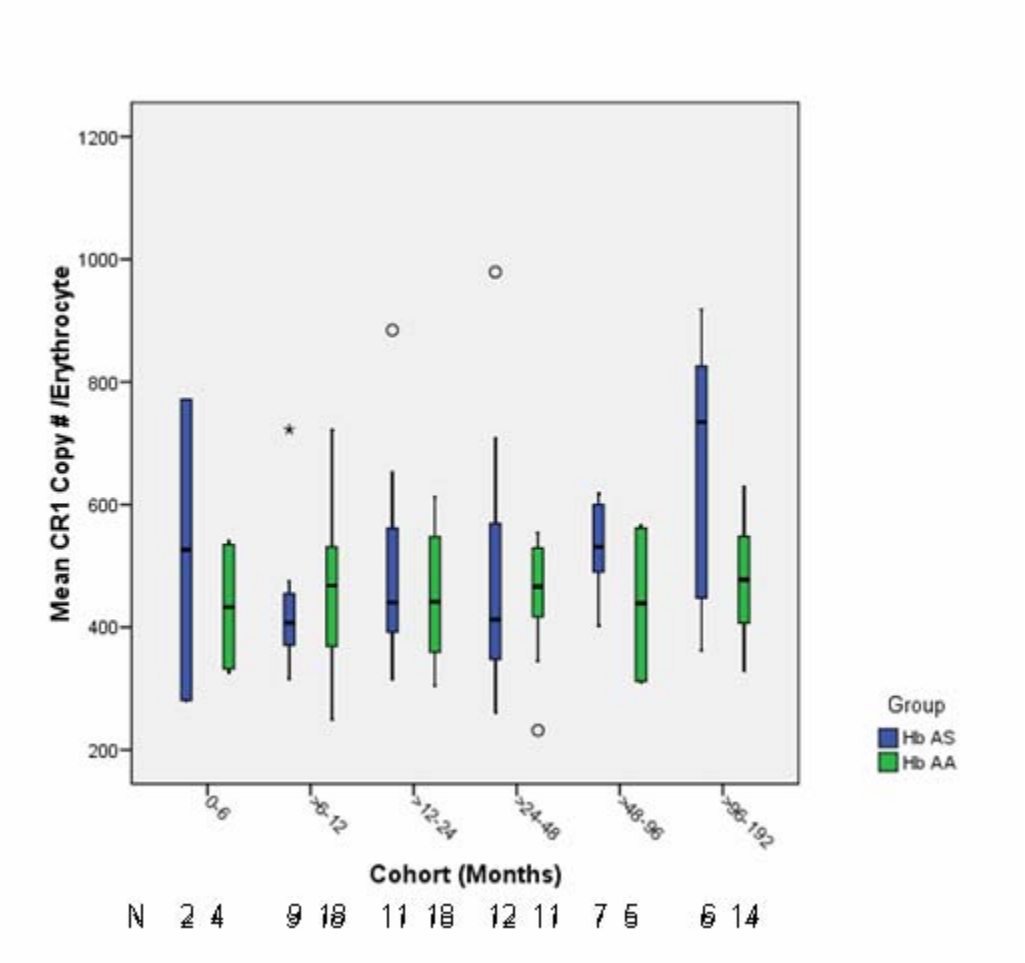

Figure 5.2: Mean CR1 Copy Numbers for HbAS and HbAA Red Cells across the various Cohorts

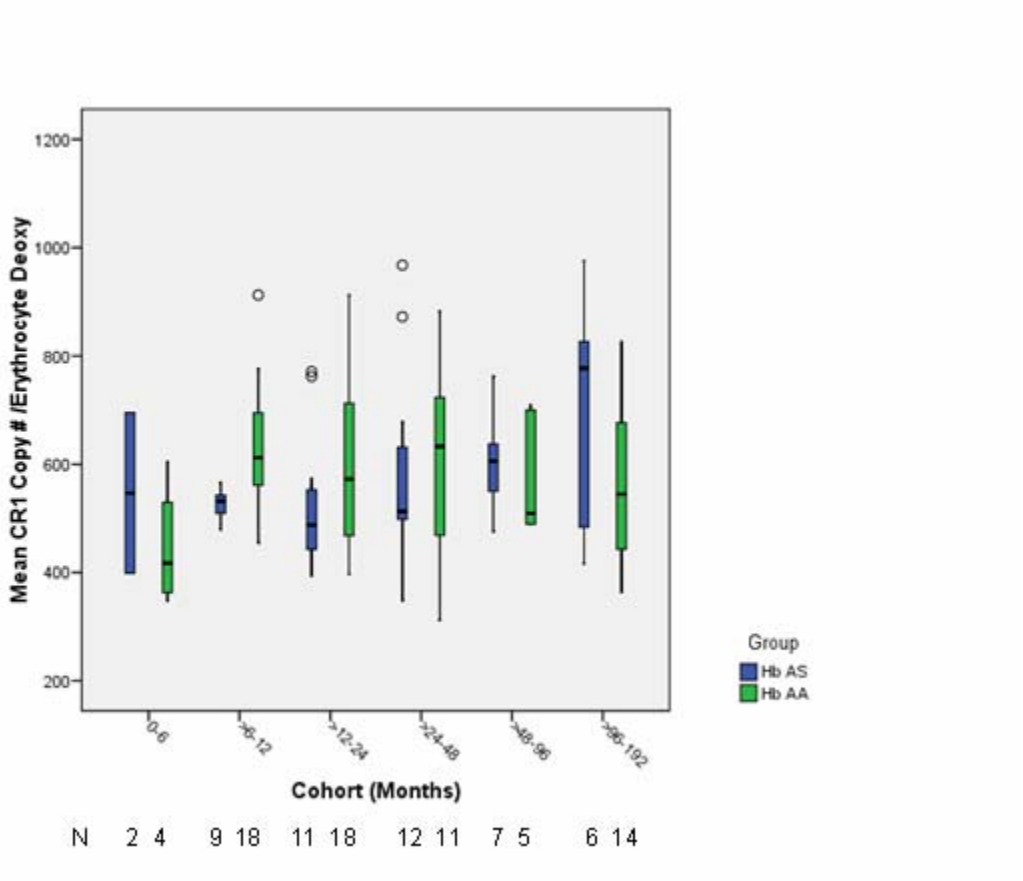

Figure 5.3: Mean CR1 Copy Numbers in HbAS and HbAA Red Cells in each Age Cohort under reduced Oxygen Saturation

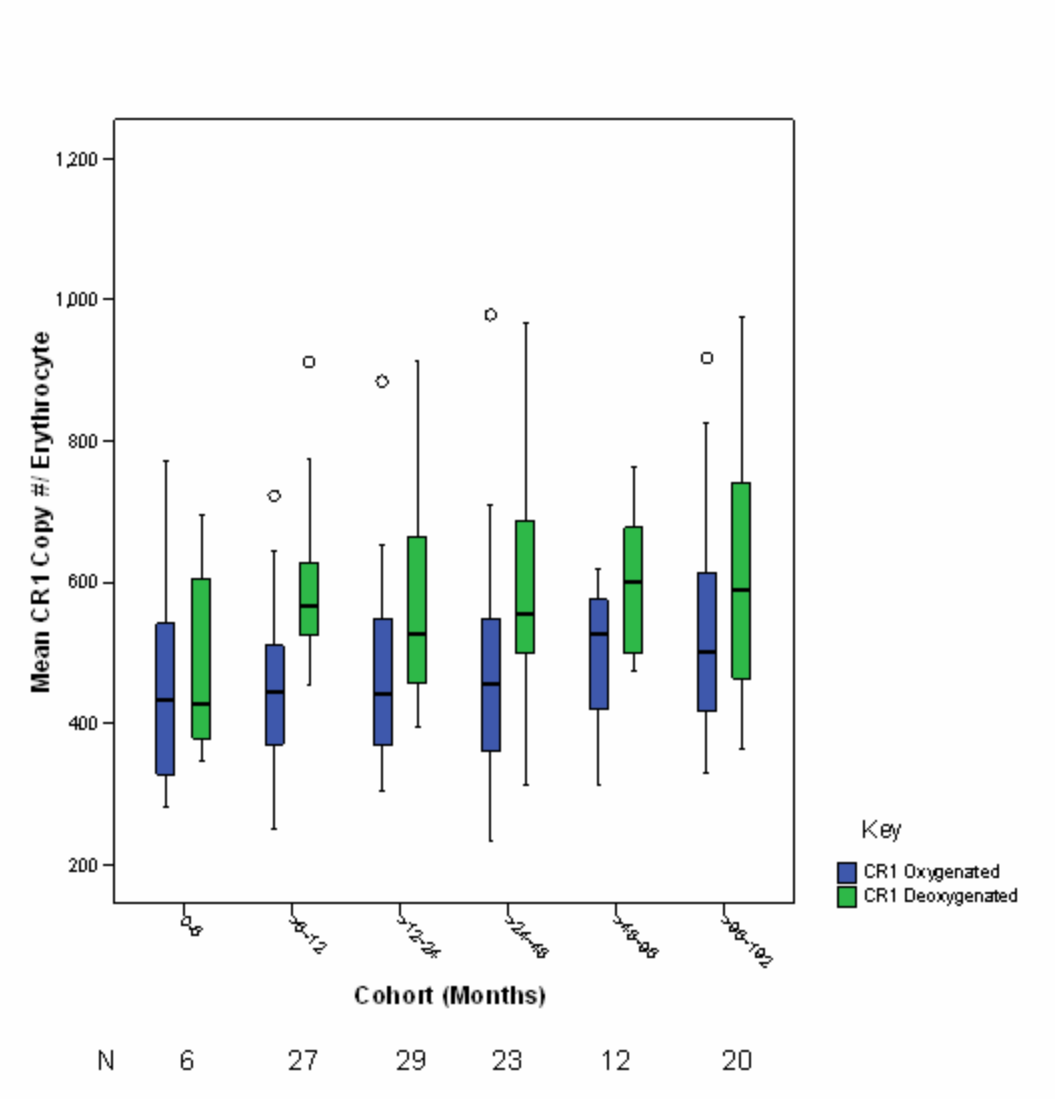

Figure 4.4: Mean CR1 Copy Numbers with Age under normal and reduced Oxygen Saturation

5.3.5 Differences in Mean CD55 Antibody Binding Capacity between HbAS and HbAA Red Cells under normal and reduced Oxygen Saturation

There were no significant differences in the mean CD55 antibody binding capacity between red cells of individuals with HbAS and those with HbAA. The mean antibody binding capacity of HbAS cells (2671, [SD 149]) was higher than HbAA cells (2520, [SD 791]), However, this difference was not statistically significant $P = 0.366$. When the red blood cells were treated with sodium dithionite, the mean antibody binding capacity for

the HbAS (2934, [SD 1107] was higher than for HbAA cell (2571, [SD 914]). This again did not reach statistical significance ($P = 0.058$) (Figure 5.5).

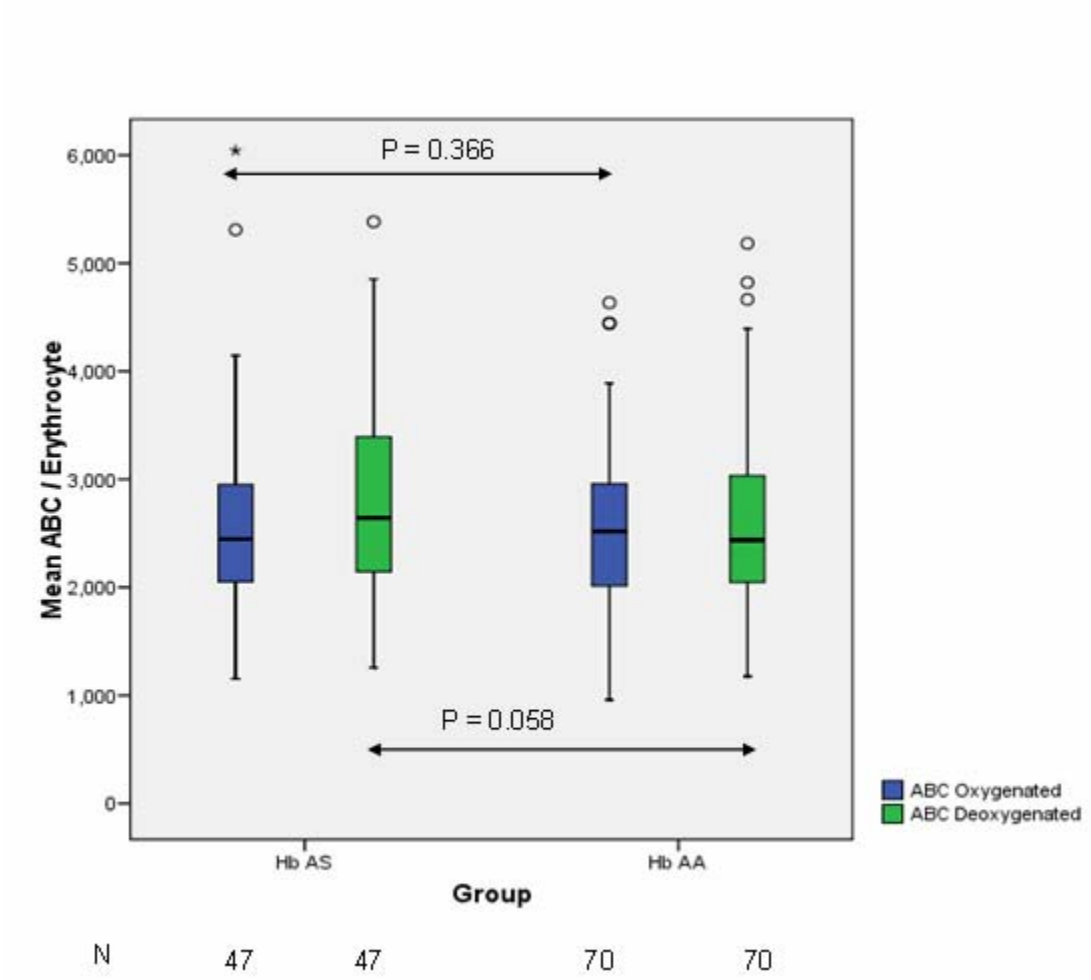

Figure 5.5: Mean CD55 Antibody Binding Capacity of HbAS and HbAA Red Cells under normal and reduced Oxygen Saturation

5.3.6 Differences Mean CD55 Antibody Binding Capacity of HbAS and HbAA Red Cells in each Age Cohort

The mean CD55 antibody binding capacity of erythrocytes under normal and reduced oxygen concentration (Figure 5.6) was observe to vary with age in both groups. Within the HbAS group, mean CD55 antibody binding capacity of erythrocytes was lowest in the 12-24 month age category but increased thereafter. Moreover, the mean CD55 antibody binding capacity of erythrocytes was noted to be generally higher for individuals with

HbAS than those with HbAA both under normal and reduced oxygen concentration (Figure 5.7). For the two groups combined, there was a significant positive correlation between the mean antibody binding capacity and calculated age in months for both groups, r (117) = 0.264; ***P* = 0.004** and more so within the HbAS group r (47) = 0.444; ***P* = 0.002**.

5.3.7 Mean Antibody Binding Capacity for the various Age Cohorts

Reduced oxygen saturation resulted in greater increase the antibody binding capacity for the HbAS erythrocytes after the age of >12-24 months than for the HbAA. For the two groups combined, there was a weak positive correlation between the mean antibody binding capacity and calculated age in months after deoxygenation with sodium dithionite r (117) = 0.264, ***P* = 0.004.**

Similarly, the mean CD55 antibody binding capacity after deoxygenation correlated positively with age of individuals with HbAS r (47) = 0.444, ***P* = 0.002** but not of those with HbAA.

5.3.8 Relationship between Mean CD55 Antibody Binding Capacity with Age

The lowest mean antibody binding capacity was in the >12-24 months age cohort after which there was a general rise. Deoxygenation did not result in significant differences in the mean antibody binding capacity (Figure 5.8). There was a weak negative correlation between the mean CD55 antibody binding capacity was and calculated age with r (117) = -0.285, ***P* = 0.002** this was also true after deoxygenation, r (117) = -0.264, ***P* =0.004**.

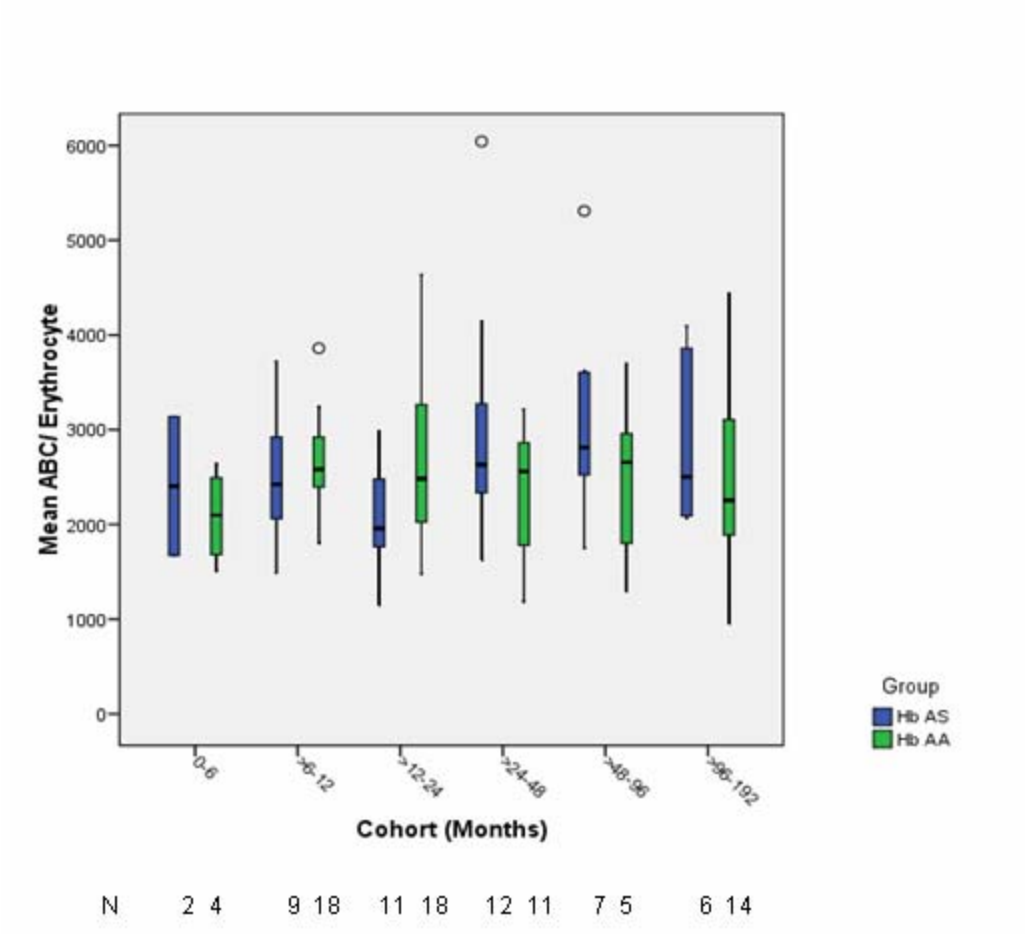

Figure 5.6: Mean CD55 Antibody Binding Capacity for the various Age Cohorts

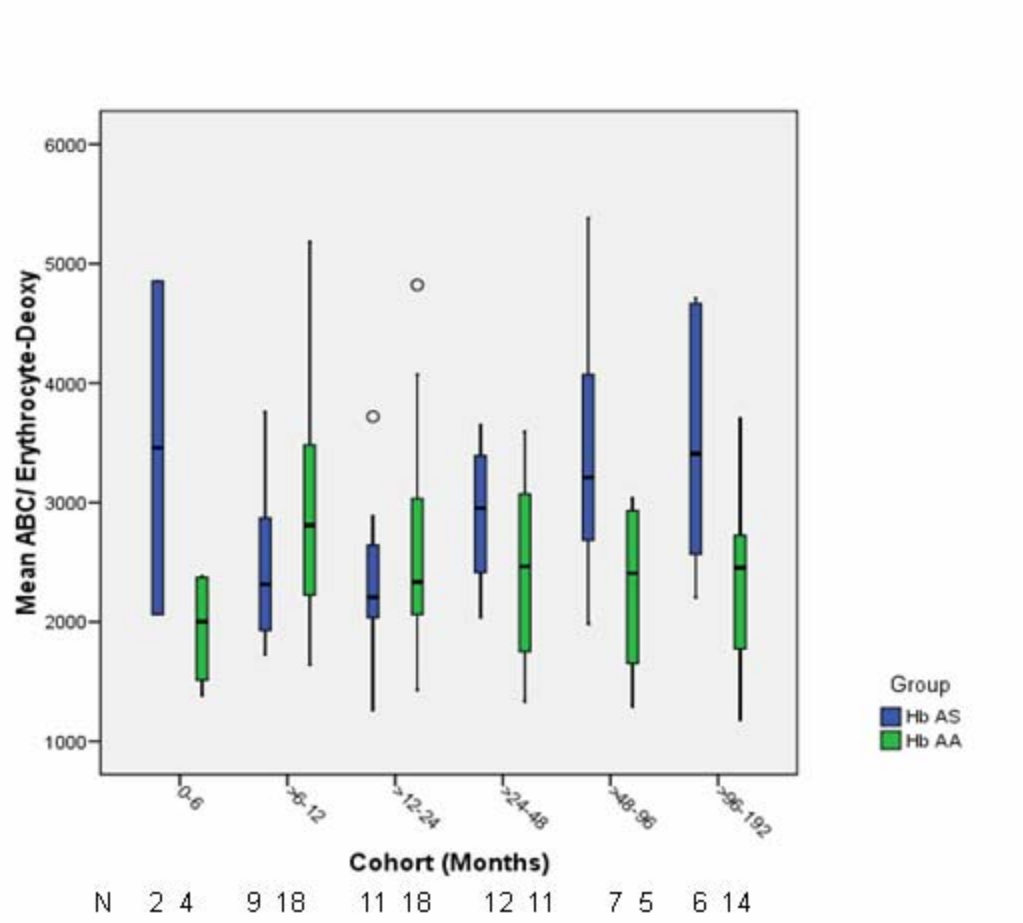

Figure 5.7: Mean CD55 Antibody Binding Capacity for the various Age Cohorts under reduced Oxygen Saturation

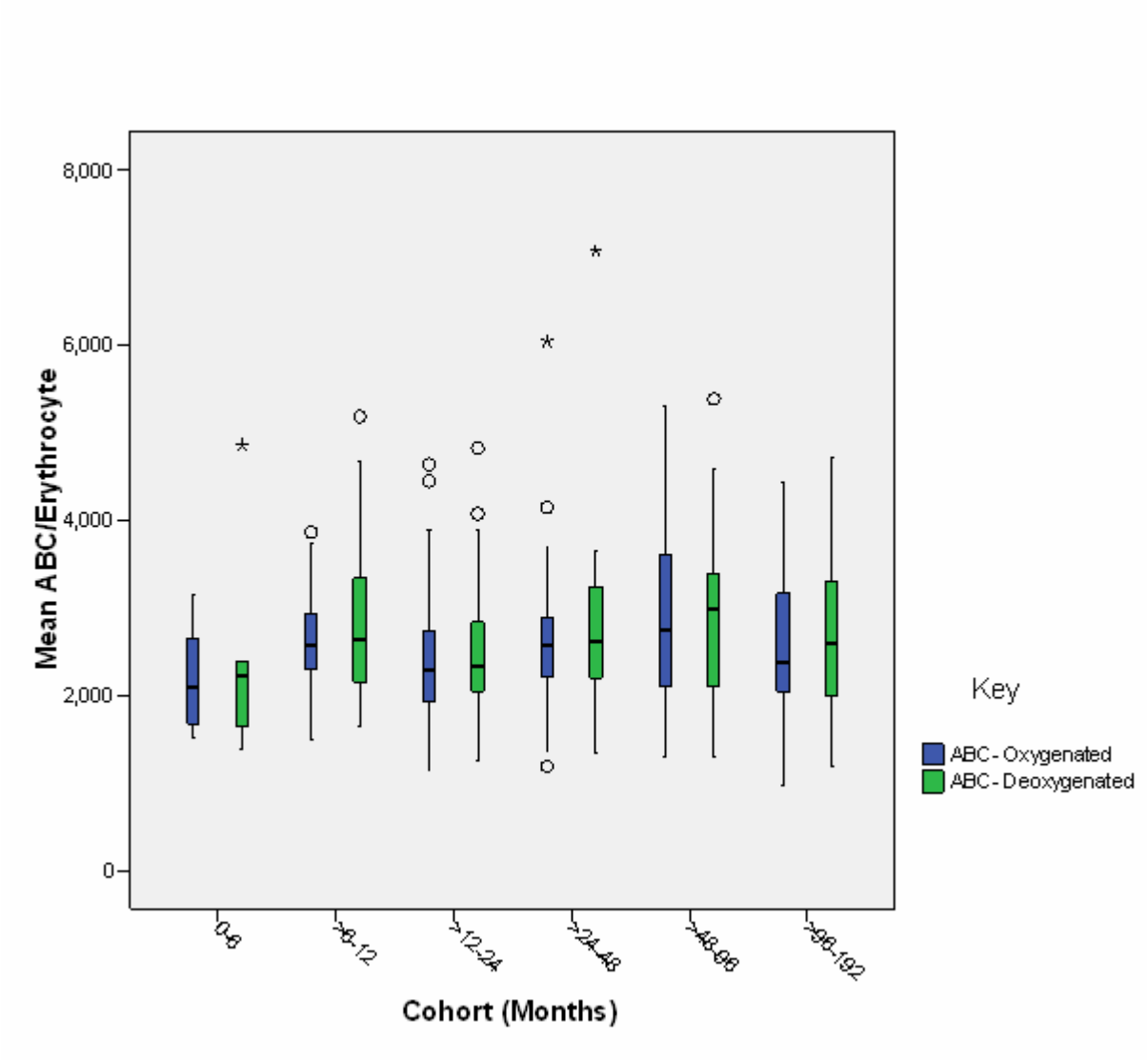

Figure 5.8: Mean CD55 Antibody Binding Capacity across the various Age Cohorts under normal and reduced Oxygenation.

5.4 Discussion

5.4.1 Sickle Cell trait in protection against Malaria

Natural resistance to malaria based on the inheritance of selected resistance genes has been observed in a few individuals living in malaria endemic areas. The relative protection of the sickle cell trait against clinical malaria increases throughout the first 10 years of life, returning thereafter to baseline (Williams *et al.*, 2005a). Transgenic mice expressing sickle cell haemoglobin (HbS) are protected from rodent malaria (Shear *et al.*, 1993) and rodent cerebral malaria (Kaul *et al.*, 1994). In humans HbS provides selective protection to heterozygous sickle cell trait individuals (HbAS) from dying of *Plasmodium falciparum* malaria while homozygous sickle cell trait individuals (HbSS) suffer severe clinical effects (Aluoch, 1995; Aluoch, 1997; Nagel and Fleming, 1992). Studies carried out in The Gambia (Hill *et al.*, 1991)) and Kenya (Marsh, 1992; Williams *et al.*, 2005b) has indicated that HbAS provides more than 90% protection from both cerebral malaria and severe malarial anaemia in children. The mechanism of malaria protection by HbAS is not well understood but has been suggested to be due to enhancement of acquired immunity to the parasite (Verra *et al.*, 2007; Williams *et al.*, 2005a) and innate factors such as impaired parasite growth in erythrocytes containing HbAS attributable to conditions of low oxygen tension (Pasvol, 1980).

This study has shown that the mean CR1 copy numbers per erythrocyte was significantly higher in the HbAS than in the HbAA individuals only in the >96 -192 months age cohort (0.009) under normal oxygenation. It is possible that beyond the age of 96 months, the high mean CR1 copy numbers per erythrocyte could translate to higher immune complex binding capacity and decreased immune complex deposition on the red blood cells and this could protect the red cells from immune complex mediated destruction.

There were no significant differences in the mean antibody binding capacity between individuals with HbAS and HbAA under both normal and deoxygenated conditions.

5.5 Conclusions

The mean CR1 copy numbers per red blood cell was higher in the HbAS group than in the HbAA group, this difference was statistically not significant (P = 0.250). When the red blood cells were treated with sodium dithionite, the mean CR1 copy numbers per erythrocyte were higher in both groups but again, there was no statistically significant difference between the two groups (*P* = 0.400)

A positive correlation was observed between the mean CR1 copy numbers per erythrocyte with age cohort in months of all individuals in HbAS and HbAA groups combined and this was also true for HbAS but not for those with HbAA. Beyond the age of 96 months, the CR1 copy numbers increased with age but more so for the HbAS, there was significant difference in the mean CR1 copy numbers per erythrocyte between HbAS ***P* = 0.009.**

There were no significant differences in the mean CD55 antibody binding capacity between RBCs of individuals with HbAS and those with HbAA. When the red blood cells were treated with sodium dithionite, the mean antibody binding capacity for the HbAS was higher than for HbAA cell but this again did not reach statistical significance (*P* = 0.058).

5.6 Recommendation

It would be interesting to compare expression of CD55 and HbS content for the HbAS erythrocytes to know whether the expression is determined by the S gene content of the cell.

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CHAPTER 6

IMMUNE COMPLEX BINDING CAPACITY OF RED BLOOD CELLS IN CHILDREN WITH SICKLE CELL TRAIT

6.0 Introduction

Severe anaemia is one of the most serious complications of *Plasmodium falciparum* malaria that occurs predominantly in children in the first 3 years of life and is an important cause of childhood morbidity and mortality in sub-Saharan Africa. The pathogenesis of anaemia during malarial infections is not fully understood. Some of the mechanisms that have been implicated include suppression of erythropoiesis (Abdalla, 1988; Chang and Stevenson, 2004; Vernes, 1980), increased destruction of red blood cells (RBCs) as a result of parasite replication (Kai and Roberts, 2008) and immune-mediated accelerated destruction of parasitized as well as non-parasitized erythrocytes (Gyan *et al.*, 2002). Despite all this work, the pathogenesis of severe malarial anaemia is not well understood; recent studies have shown that immune complexes are deposited on red blood cells in children with severe malarial anaemia. These immune complexes have been shown to play an important role in the pathogenesis of severe malarial anaemia (Stoute *et al.*, 2003; Waitumbi *et al.*, 2004; Waitumbi *et al.*, 2000)

Heterozygous sickle cell trait individuals (HbAS) are relatively protected from severe manifestations of malaria such as anaemia (Aidoo, 2002; Dvorak *et al.*, 1975a; Ganczakowski *et al.*, 1995a; Ganczakowski *et al.*, 1995b; Ruwende *et al.*, 1995); The underlying mechanisms of this protection are however not well understood but are thought to be due to several mechanisms (Aluoch, 1997; Carlson *et al.*, 1994; Chippaux *et al.*, 1992a; Chippaux *et al.*, 1992b; Hill *et al.*, 1991; Pasvol *et al.*, 1978; Shear *et al.*, 1993; Weatherall and Clegg, 2001). Given the important role played by immune complex deposition in the pathogenesis of severe malarial anaemia, this study investigated whether there are differences in IC binding capacity between erythrocytes from heterozygous sickle cell trait individuals and normal individuals that could partly explain the protection from severe anaemia in individuals with sickle cell trait.

6.1 Immune Complexes Formation and Role in the Pathogenesis of Severe Malaria

P. falciparum infection leads to severe immunostimulation with generation of several soluble *Plasmodial* antigens (Camus and Hadley, 1985; Jhaveri *et al.*, 1997; Marsh and Howard, 1986; Pichyangkul *et al.*, 1997). The formation of circulating immune complexes (CICs) is the physiological consequence of the binding of antibodies that are produced in response to the different antigens (Jhaveri *et al.*, 1997) and under normal circumstances; mechanisms for their efficient and rapid clearance exist in mammals (Schifferli, 1996). However, in some abnormal circumstances, their accumulation in tissue or circulation induces pathological consequences as seen in disorders such as rheumatoid arthritis or systemic lupus erythematosus (Birmingham and Hebert, 2001; Kohro-Kawata *et al.*, 2002). Under normal circumstances ICs activate complement either through the classical or the alternative pathways. In humans, the subsequent covalent binding of C3b or C4b to the complexes allows them to bind to complement receptor 1 (CR1, CD35) expressed on erythrocytes. Once bound to erythrocytes via CR1, the C3b-containing ICs are carried to the liver and spleen, where they are removed by macrophages in a process known as the transfer reaction and the erythrocytes return to circulation (Craig *et al.*, 2005; Reinagel *et al.*, 1997; Schifferli, 1996). Thus, low CR1 expression levels on erythrocytes may result in poor IC clearance capacity, which then predisposes one to IC-mediated complications.

The ICs that are not bound to erythrocytes can deposit in tissues and on endothelium where they continue to activate complement and stimulate leukocyte production of nitric oxide (NO) and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) by leukocytes (Waxman *et al.*, 1984); in turn contributing to development of severe anaemia. Children with severe malaria-associated anaemia and cerebral malaria have significant higher circulating immune complexes than their respective controls and this predisposes their red blood cells to increased erythrophagocytosis due to a relative

deficiency of CR1 and CD55. This deficiency is thought to result in impaired clearance of immune complexes from the circulation (Mibei *et al.*, 2005; Waitumbi *et al.*, 2000).

6.1.1 Immune Complex Binding Capacity of E-Complement Receptor -1

Erythrocyte CR1 binds immune complexes and acts as inert shuttle transporting these bound ICs through circulation to the liver and the spleen where the bound immune complexes are removed by macrophages in a process known as the transfer reaction and the erythrocytes return to circulation (Ahearn and Fearon, 1989; Birmingham, 2001). This multi-valent binding is favored by the known clustering of CR1 and by the multiple binding sites on each CR1. Almost 50% of CR1 are distributed in clusters of greater than or equal to three units compared to less than 15% on the surface of polymorphonuclear leukocytes (Paccaud *et al.*, 1990). Although the numbers of CR1 clusters vary from cell to cell, the mean number of clusters correlates significantly with the mean number of CR1 per cell. This cluster could explain the high binding efficiency of C3b coated IC to erythrocytes (Paccaud *et al.*, 1988). The clustering of E CR1 makes CR1 a privileged site on erythrocytes and prevents phagocytosis of erythrocytes opsonized via CR1 (Reinagel *et al.*, 1997). The number of CR1 molecules is known to decrease with aging of the erythrocytes in normal individuals and also in certain disease states such as systemic lupus erythematosus, HIV and some hemolytic anaemias. In these conditions, the erythrocyte clearance mechanism for the ICs is interfered with and the ICs deposit on tissues such as the skin, lungs and kidney resulting in disease (Birmingham, 2001).

Recent studies in our laboratory have associated increased levels of circulating immune complexes (CICs) with SMA (Mibei *et al.*, 2005; Stoute *et al.*, 2003). Patients with SMA have been shown to have low CR1 (Stoute *et al.*, 2003; Waitumbi *et al.*, 2000) and this may result in poor ICs clearance capacity through phagocytosis in the spleen and liver (Stoute *et al.*, 2003). Owuor and colleagues (Owuor *et al.*, 2008) have further demonstrated that deficiencies in red cell CR1 in children with SMA were accompanied by a marked decline in IC binding capacity. Collectively, these data indicate that IC binding capacity by erythrocytes influences the ICs clearance capacity in *P. falciparum*

malaria patients and may determine an individuals' susceptibility to severe malarial malaria.

The ICs binding capacity by erythrocytes of sickle cell trait individuals has not been examined for association with resistance to SMA.

6.2 Materials and Methods

6.2.1 Preparation of Immune Complexes

50 µl of 49 mg/ml rabbit anti-BSA (Sigma-Adrich, St.Louis, MO) and 3 µl of 5 mg/ml BSA-FITC (Accurate Chemical and Scientific Corp., Westbury, NY) were added to 950 µl of RPMI1640 (Sigma-Aldrich). This combination was noted to be the point of equivalence in preliminary experiments. The mixture was incubated at 37°C for 1 hr and overnight at 4°C. The next day, the IC preparation of soluble and insoluble IC was aliquoted and stored at -20°C.

6.2.2 Measurement of IC Binding Capacity of Erythrocytes

For immune complex opsonization, 5µl of stock IC or RPMI 1640 (unstained control) was incubated in a total volume of 100 µl containing 30% AB+ serum in wells of a 96-well plate. For a negative control, a separate set of wells contained IC plus 10mM EDTA. Following incubation at 37ºC for 30 minutes with constant rocking motion, 100µl 1% hematocrit suspension of freshly thawed red cells from each study participants or from a standard aparasitemic control in RPMI 1640 was added to each of the wells of the above 96-well plate. This was followed by further incubation for 30 minutes at 37ºC. The erythrocytes were then washed twice in 200µl of ice cold RPMI, resuspended in PBS containing 1% paraformaldehyde, and stored at 4 ºC until acquisition. After gating, the erythrocyte FITC fluorescence was measured using logarithmic amplification and the positive cutoff was set using unstained cells. The percent of the positive cells (IC Binding) was calculated based on this cutoff. To control for day to day variation, the IC binding capacity was normalized to the mean IC binding capacity of the red cell standard used throughout using a formula similar to the one used for the correction of CR1 and CD55 (above)

6.3 Results

6.3.1 Differences in IC Binding Capacity between HbAS and HbAA Red Cells under normal and reduced Oxygen Saturation

In order to determine if the high CR1 levels and high CD55 levels would translate into increased immune complex binding capacity, we measured the mean immune complex binding capacity for these cells under both normal and reduced oxygenation saturation. The mean immune complex binding capacity before and after deoxygenation with sodium dithionite for HbAS and HbAA are presented (Figure 6.1). The data are presented as box-and-whisker plots. For each group, the horizontal line in the middle of the box marks the median of the sample. The box represents the interquartile range and the central 50% of the data falls within the range of the box. The whiskers are the vertical lines extending up and down from each box and they represent the upper and the lower 25% of the data. The mean immune complex binding capacity for the HbAS cells (52.5[SD=6.4]) was higher than HbAA cells (48.1[SD=11.6]). This difference was statistically significant with ***P* = 0.017**. When the cells were deoxygenated, the mean immune complex binding capacity for the HbAS cells (52.6=7[SD=7.8]) was higher than HbAA cells (48.1[SD=11.6]). This difference was statistically significant with ***P* =0.003** (Figure 6.1).

When UNIANOVA with matching variable was done, the immune complex binding capacity was again significantly higher for the HbAS than HbAA (***P* = 0.025**). This was also true under deoxygenated conditions (***P* = 0.003**).

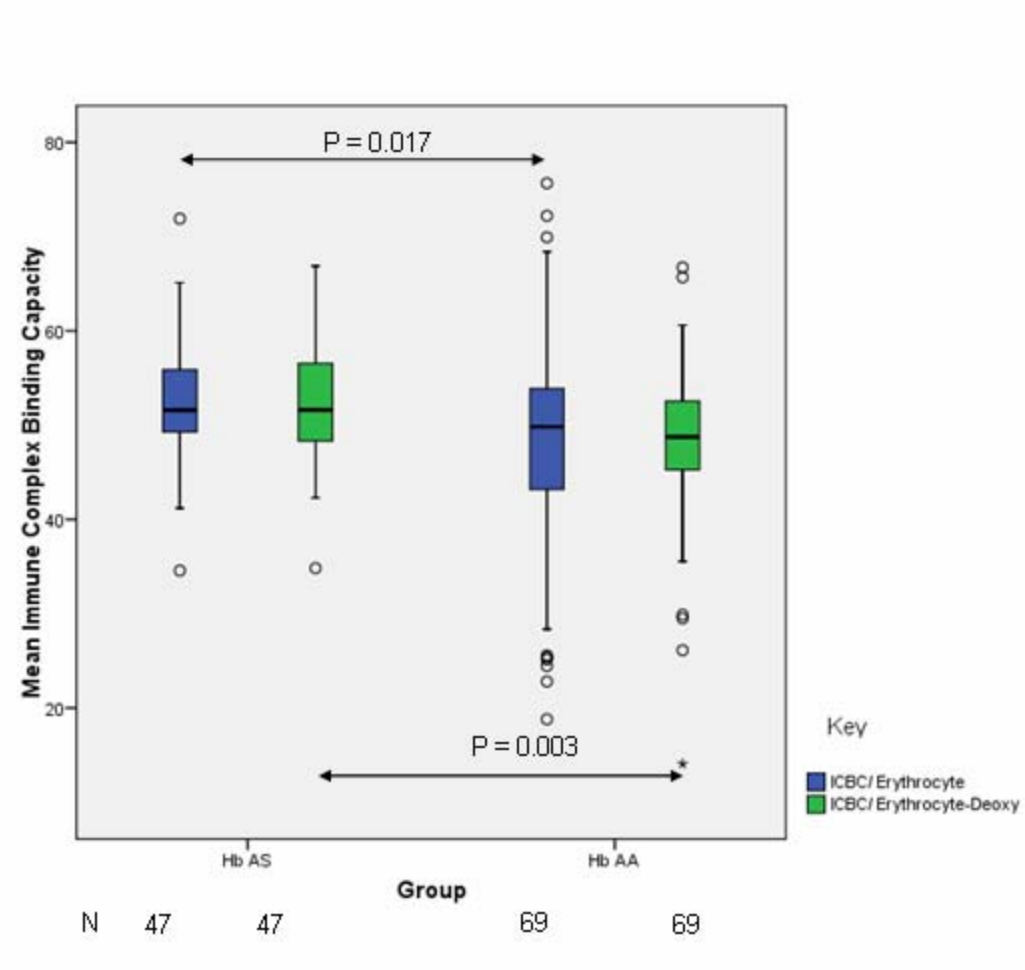

Figure 6.1: Immune Complex Binding Capacity by Haemoglobin Type

6.3.2 Differences in Mean IC Binding Capacity in HbAS and HbAA Red Cells in each Age Cohort

In order to see whether the differences in red cell complement regulatory protein had any effects on immune complex binding capacity and whether the red cells IC binding capacity varied with age, we measured the immune complex binding capacity in the various age groups. The mean IC binding capacity per erythrocyte under normal conditions (Figure 6.2) was observed to be generally higher in HbAS than HbAA individuals in all the age cohorts. Moreover, the mean immune complex binding capacity for red cells was lowest in the >6-12 months age group categories for both HbAS and HbAA individuals after which there was arise up-to age 48-96 months and thereafter, no much difference.

There was a weak positive correlation between the immune complex binding capacity and the age cohort r (117) = 0.198, ***P* = 0.034**.

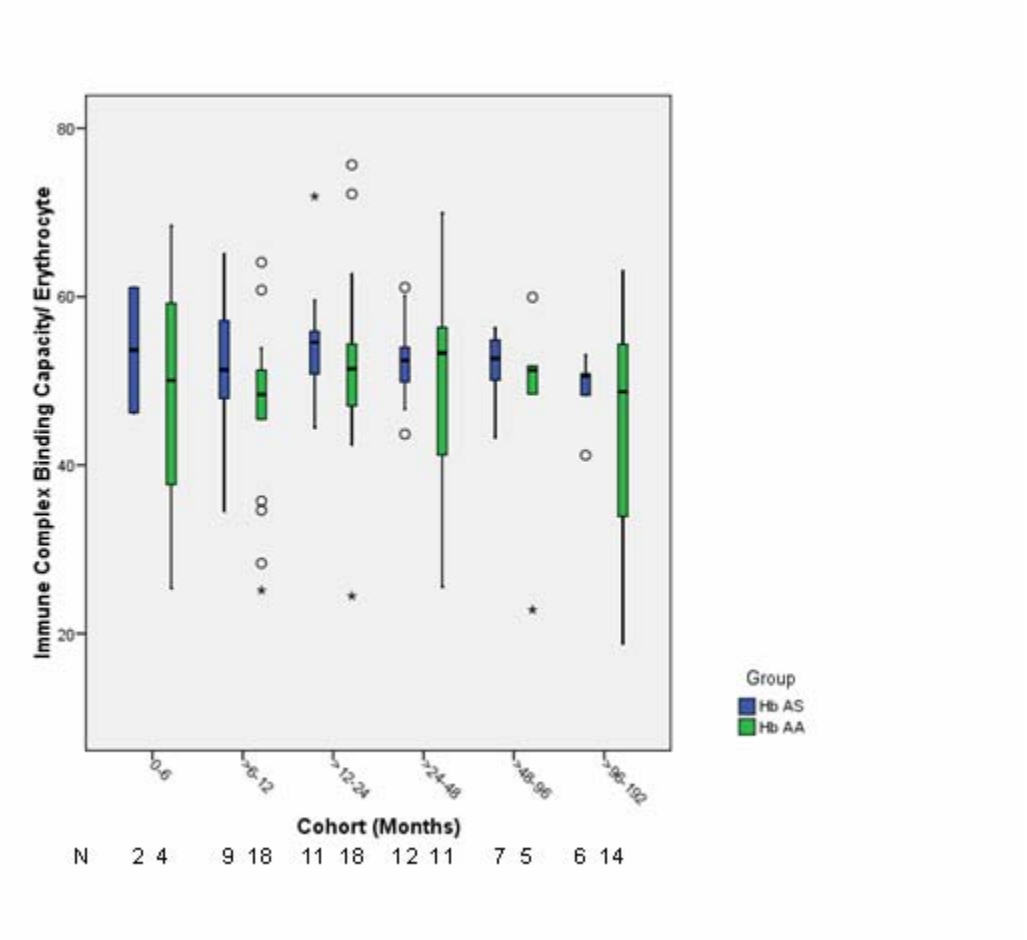

Figure 6.2: Mean IC Binding Capacity in HbAS and HbAA Red Cells in each Age Cohort under reduced Oxygen Saturation

6.3.3 Differences in Mean IC Binding Capacity in HbAS and HbAA Red Cells in each Age Cohort under reduced Oxygen Saturation.

The mean immune complex binding capacity under reduced oxygen saturation was lowest in 12-24 months age groups for HbAS and 24-48 months age groups for HbAA. An increase was noted in subsequent age groups for both HbAS and HbAA individuals.

In general, the immune complex binding capacity under reduced oxygen saturation was higher in the HbAS than HbAA across the various age cohorts (Figure 6.3).

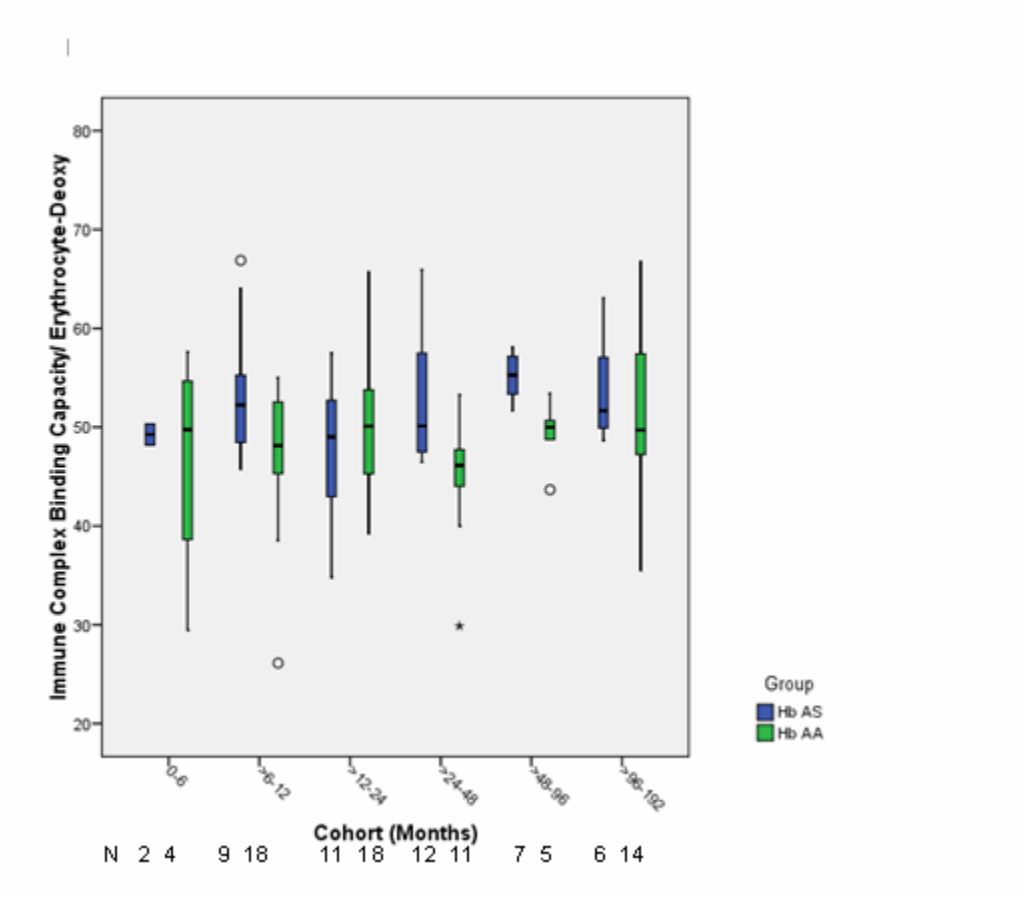

Figure 6.3: Mean IC Binding Capacity in HbAS and HbAA Red Cells in each Age Cohort under reduced Oxygen Saturation

6.4 Discussion

In malaria, a number of immune complexes are generated. These circulating immune complexes may lead to end organ damage by depositing on erythrocytes and other organs like the kidney (Mohammed, 1982). Studies from our laboratory have shown that ICs are elevated in severe malarial anaemia at the time of diagnosis (Mibei *et al.*, 2005) these ICs can stimulate pro-inflammatory cytokines that may play a role in the pathogenesis of severe malaria for example severe malarial anaemia (Waxman *et al.*, 1984)

The ability to bind circulating immune complex is critical for mopping up of the circulating ICs. High ability to mop up immune complexes lead to less chances of getting severe manifestations of malaria for example severe malaria anaemia. The immune complex binding capacity for HbAS cells were much higher than for the HbAA cells (***P* = 0.017**). This was also true after deoxygenation (***P* = 0.003**). It is apparent that the HbAS cells bind more immune complexes than the HbAA cells and this may lead to increased mopping up of the immune complexes generated during malaria infections and therefore prevent severe manifestations of malaria. After deoxygenation, the apparent increase in the immune complex binding capacity of the HbAS erythrocytes could be due to increased exposure of the binding sites. This may be due to conformational changes in the CR1 molecules resulting in the exposure of more binding sites.

Like in other studies, age correlated with immune complex binding capacity with high levels in the 0 - <6 months age cohort, thereafter decreasing to the lowest levels between <6-24 age cohort (Waitumbi *et al.*, 2004). It was also noted that, the lowest level of immune complex binding capacity corresponded to the lowest levels of CR1 in this population (Waitumbi *et al.*, 2004) and the age where the majority of children get severe malarial anaemia (Snow *et al.*, 1999; Stoute *et al.*, 2003)

6.5 Conclusions

1) The mean immune complex binding capacity for the HbAS cells was higher than HbAA cells both before and after deoxygenation (***P* = 0.017** and **0.003** respectively). When a matching variable was factored in, this was again true (***P* = 0.025**)
2) The mean immune complex binding capacity was lowest in the >6-12 months age group for both HbAS and HbAA; however, the overall picture showed that HbAS individuals had higher immune complex binding capacity than HbAA in all the age cohorts
3) In general, there was a weak positive correlation between the immune complex binding capacity and the age cohort r (117) = 0.198, ***P* = 0.034**.

6.6 Recommendation

A study to relate the immune complex binding capacity, the HbS percent content and the various complement regulatory proteins would be appropriate.

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CHAPTER 7

COMPLEMENT DEPOSITION ON RED BLOOD CELLS IN CHILDREN WITH SICKLE CELL TRAIT

7.0 Introduction

Malaria infection is a significant cause of anaemia, particularly in children living in areas of high transmission (Murphy and Breman, 2001). The pathogenesis of *P. falciparum* malarial anaemia is complex and cannot be explained solely on the basis of haemolysis of parasitized red blood cells as often the anaemia is disproportionate to the level of parasitemia. In addition to the destruction of parasitized erythrocytes by rupture of cells after completion of the parasite's intra-erythrocytic life cycle as well as opsonization and clearance of intact infected RBCs (Kai and Roberts, 2008), increased clearance of uninfected erythrocytes, has been proposed to contribute significantly to the development of malarial anaemia (Jakeman *et al.*, 1999; Kai and Roberts, 2008). The mechanisms responsible for clearance of uninfected erythrocytes in malaria have not been clearly defined. Several factors including immune-mediated mechanism, such as deposition of complement (C3b) on erythrocytes leading to enhanced receptor-mediated uptake by macrophages has been proposed to contribute partly to the destruction of non-infected cells leading to anaemia (Kai and Roberts, 2008). Therefore, the extent of complement (C3b) deposition on red cells may influence an individuals' resistance or susceptibility to severe malarial anaemia.

In this study, cytofluorometry was used to determine susceptibility of red cells from individuals with normal haemoglobin and those with heterozygous sickle cell trait to complement (C3b) deposition. The same was done under deoxygenated conditions to see if deoxygenation had any effects on the C3b deposition.

7.1 Immune Complex Deposition on Erythrocytes

Severe anaemia is one of the most lethal complications in children infected with *P. falciparum*. The pathogenesis of this anaemia is not completely understood. It has been

indicated that the degree of red blood cell (RBC) loss in malaria cannot be explained entirely by the direct destruction of RBCs by the parasite (Arese *et al.*, 2005; Salmon *et al.*, 1997). However, the destruction of uninfected red cells which also takes place contributes significantly to the anaemia (Jakeman *et al.*, 1999). Indeed, several investigations have reported decrease in life span of uninfected red cells in malaria animal models (Arese *et al.*, 2005; Salmon *et al.*, 1997) and patients with *P. falciparum* (Kai and Roberts, 2008). Moreover, a mathematical model of severe malarial anaemia has revealed that with each lysed infected erythrocyte ; a further 8.5 uninfected erythrocytes are destroyed (Jakeman *et al.*, 1999).

Complement is activated during malaria infection (Jhaveri *et al.*, 1997)) and C3d which is one of the molecules implicated in the removal of senescent red cells via erythrophagocytosis have been detected on red cells of children with severe malaria (Facer, 1980a; Facer, 1980b). Recently, Odhiambo and colleagues (Odhiambo *et al.*, 2008) demonstrated deposition of the opsonin C3b on red cells of patients with malaria, thus suggesting further a role for complement-mediated damage of RBCs.

The factors that contribute to reduced susceptibility to anaemia in heterozygous sickle cell traits are unclear. This study aimed to determine if there are differences in susceptibility to C3b deposition between red cells of individuals with normal haemoglobin and those with sickle cell trait.

7.2 Methods

7.2 1 Measurement of C3b Deposition on Red Cells

All centrifugation steps were at x500g for 5 min. Rabbit polyclonal anti-C3a [negative control antibody, (Nordic immunological laboratories, Tilburg, The Netherlands)] and anti-C3b (Accurate) were pre-adsorbed x3 by adding a 1:50 dilution of antibody in phosphate buffered saline (PBS) pH 7.4 to an equal volume of packed pre-washed erythrocytes from the normal standard control. The cells were incubated for 1 hr at 37ºC with constant rocking followed by centrifugation. The pre-adsorbed antibody was frozen at -20ºC in single use aliquots. 100µl of pre-washed freshly thawed erythrocytes at 1%

hematocrit in Alsever's buffer was added to wells of a 96-well plate and resuspended in 50µl of pre-adsorbed rabbit anti-C3b, anti-C3a or in PBS (unstained control), and incubated for 10 minutes at 37ºC. After two washes in PBS, the cells were resuspended in 1:50 anti-rabbit PE (Sigma Aldrich) for 30 minutes at room temperature, washed twice, and resuspended again in PBS then acquisition was carried out. The %C3b –positive cells were calculated by Overton subtraction of the baseline C3a histogram from the baseline C3b histogram (Overton, 1988).

7.3 Results

7.3.1 Differences in % C3b Deposition between HbAS and HbAA Red Cells under both normal and reduced Oxygen Saturation

The % C3b deposition on RBCs before and after deoxygenation with sodium dithionite for HbAS and HbAA cells are presented. The data are presented as box-and-whisker plots. For each group, the horizontal line in the middle of the box marks the median of the sample. The box represents the interquartile range and the central 50% of the data falls within the range of the box. The whiskers are the vertical lines extending up and down from each box and they represent the upper and the lower 25% of the data (Figure 7.1).

The mean percentage C3b-positive cells for the HbAS cells (18.5[SD=12.6]) was lower than HbAA cells (18.7[SD=12.2]). This difference was not statistically significant P = 0.937. When the cells were deoxygenated, the mean percentage C3b-positive cells for the HbAS cells (13.8[SD=11.0]) was again lower than HbAA cells (14.7[SD=9.3]). This difference was again not statistically significant P = 0.601.

Pearson's correlation using haemoglobin electrophoresis results as the grouping variable revealed no difference between the HbAS and HbAA cells, r (117) = 0.043, P = 0.643. When this was done under reduced oxygenation, there was again no statistical significance r (117) = 0.007, P = 0.938.

There was no significant correlation between calculated age and C3b deposition r (117) = 0.076, *P* = 0.415. This was again true when the assay was done under deoxygenated conditions, r (117) = 0.113, *P* = 0.227

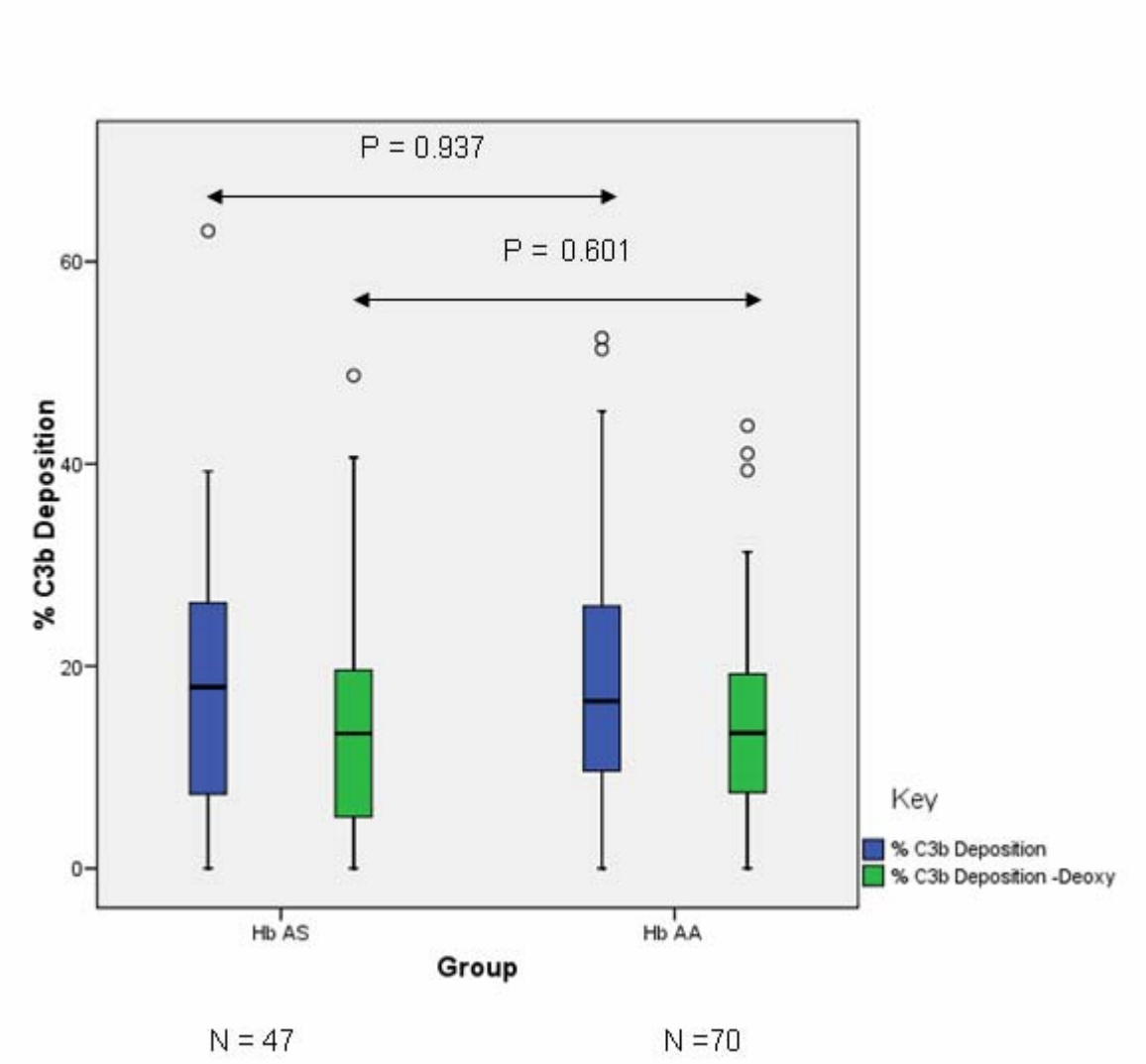

Figure 7.1: Percent C3b Deposition on Erythrocytes of HbAS and HbAA under normal and reduced Oxygen Saturation

7.3.2 Differences in % C3b Deposition between HbAS and HbAA Red Cells in each Age Cohort

Under normal oxygen conditions, the percentage C3b deposition was observed to be highest in both HbAS and HbAA in the >6-24 months age cohort and lowest in the >24-48 months age cohort for both HbAS and HbAA. Thereafter there was a minimal rise in percent C3b deposition for both age cohorts under normal and deoxygenated (Figure 7.2) conditions. The difference between the %C3b deposition and the age cohort in months was however not statistically significant p = 0.526.

There was no correlation between the percentage C3b deposition and the age cohort in months r (117) = 0.012, $P = 0.899$. This was again true using the calculated age, r (117) = 0.076, $P = 0.415$

There were no significant differences in percent C3b deposition between erythrocytes of HbAS and HbAA individuals in all the age cohorts under reduced oxygen saturation (Figure 7.3).

There was a weak negative correlation between previous malaria infection and the C3b deposition r (117) = - 0.198, $P = 0.033$.

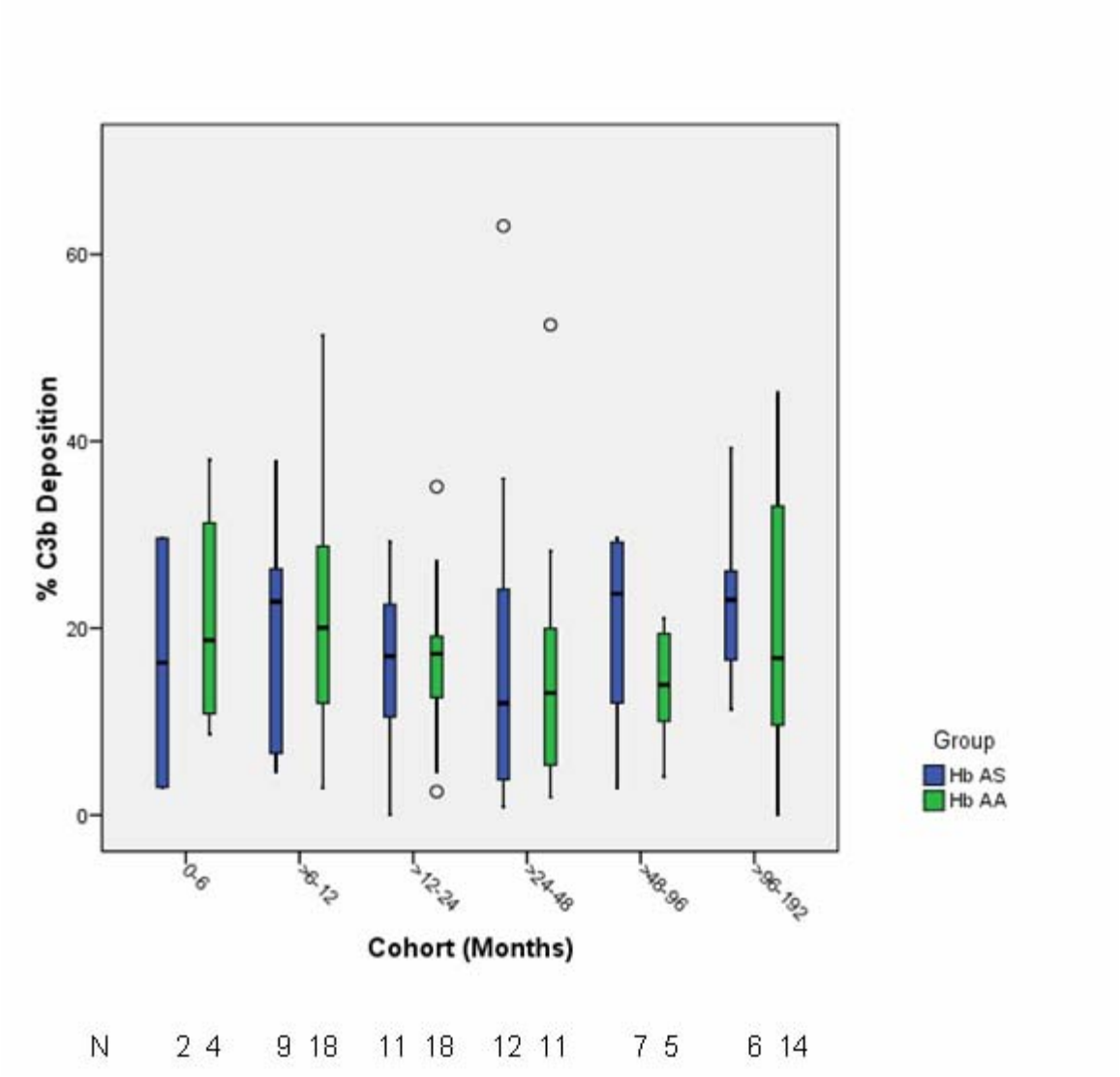

Figure 7.2: Percentage C3b Deposition for the various Age Cohorts by Haemoglobin Type

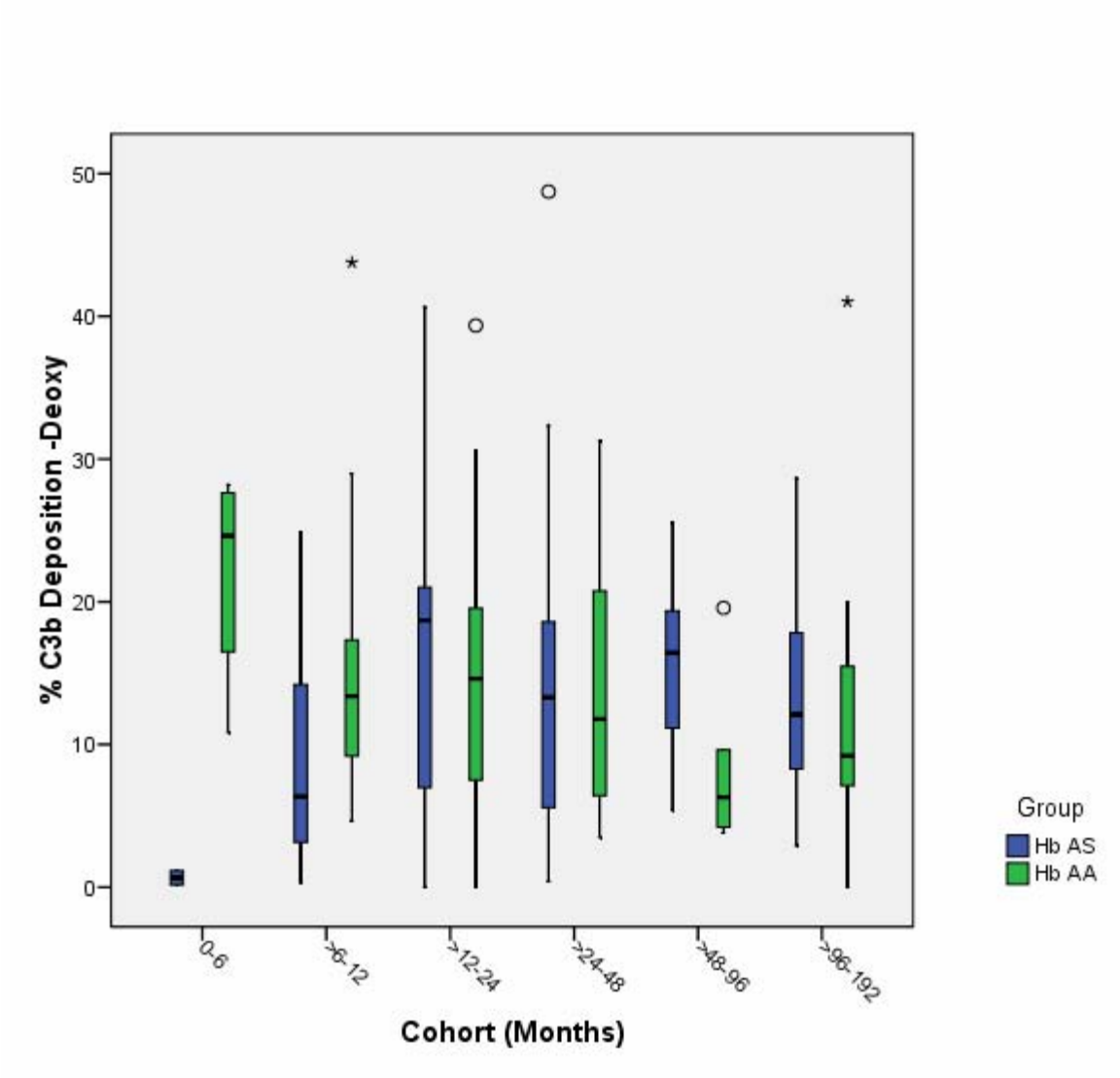

Figure 7.3: C3b Deposition on Erythrocyte of Individuals with HbAS and HbAA in each Age Cohort under reduced Oxygen Saturation

7.4 Discussion

Malaria infection leads to complement activation and C3b deposition on red cells (Arese *et al.*, 2005; Owuor *et al.*, 2008). Red cells of individuals with low complement regulatory proteins were found to be more susceptible to C3b deposition compared to

controls (Odhiambo *et al.*, 2008). Moreover, the pattern of C3b deposition on red blood cells has been shown to be opposite that of CR1 levels.

In the present study, C3b deposition was lowest in the 0-6 month age cohort probably due to the corresponding high levels of complement regulatory proteins in this age cohort. This high level of complement regulatory proteins in the neonates may make these children more equipped to handle IC formation. This can explain their reduced susceptibility to complement deposition and therefore protection against severe manifestations of malaria for example severe malarial anaemia. This is in agreement with earlier studies which found similar results (Odhiambo *et al.*, 2008) and this can explain their reduced susceptibility to complement deposition. This is in agreement with earlier studies which found similar results (Odhiambo *et al.*, 2008).

Although a similar pattern was observed between deposition of C3b, CR1 and CD 55 levels, no significant differences in susceptibility to C3b deposition were noted between red cells of HbAS and HbAA individuals. These data suggests that differences in susceptibility to C3b deposition between HbAS and HbAA erythrocytes may not be an important factor that determines protection from severe malarial anaemia in individuals with sickle cell traits.

In this study, deoxygenation did not result in any significant difference in % C3b deposition between the HbAS and HbAA cells.

7.5 Conclusion

1) The % C3b deposition was similar in both HbAS and HbAA cells under normal and reduced oxygen saturation, the highest incidence of % C3b deposition was in the 6 to < 24 and lowest in the 0-6 month's age cohort for both the HbAS and HbAA.

2) There was significant negative correlation between %C3b deposition and previous malaria infection in the children r (117) = -0.198, ***P* = 0.033**.

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CHAPTER 8

COMPLEMENT REGULATORY PROTEINS, IMMUNE COMPLEX BINDING CAPACITY AND C3b DEPOSITION

8.0 Introduction

Epidemiological studies by Allison (Allison, 1954) demonstrated correlation between geographical distribution of the sickle-cell mutation in the beta haemoglobin gene (*HBB*) and malaria endemicity. The HbS provides selective protection to heterozygous sickle cell trait individuals (HbAS) against *Plasmodium falciparum* malaria mortality and its severe clinical effects (Nagel and Fleming, 1992) and heterozygous sickle-cell trait carriers have approximately 15 percent selective advantage as a result of their malaria resistance (Allison, 1954). Indeed, children with HbAS have been shown to have significant lower parasite densities than those with HbAA during both mild and severe malaria episodes (Adams, 1992; May *et al.*, 2007; Williams *et al.*, 2005b). Moreover, parasitaemic children with HbAS are 50–90% less likely to progress to mild or severe malaria (Adams, 1992; May *et al.*, 2007; Williams *et al.*, 2005b) and HbAS children who do develop cerebral malaria are less likely to die from the disease compared to those with HbAA (Olumese *et al.*, 1997). A recent study at the Kenyan coastal town of Kilifi showed that HbAS had no effect on symptomless parasitemia but was 50% protective against mild clinical malaria, 75 % protective against admission to hospital for malaria and almost 90% protection against severe or complicated malaria (Williams *et al.*, 2005a; Williams *et al.*, 2005b). Thus, the current prevalence of HbS in many tropical populations may reflect survival advantage for the sickle cell carriers against life-threatening *P. falciparum* malaria (Aluoch, 1997; Hill *et al.*, 1991). The lower risk of chronic malnutrition in early childhood, mediated by protection against mild malaria episodes, may contribute to the survival advantage of HbAS carriers in areas of high malaria transmission (Kreuels *et al.*, 2009).

Despite the abundant epidemiological evidence for the protection of HbAS against severe malaria, the exact mechanism by which it exerts protection remains unclear. A number of studies carried out to explain this protection suggests involvement of factors including inhibition of invasion, growth, and development of *Plasmodium falciparum* parasites in red cells containing HbAS due to low oxygen tension (Pasvol, 1980; Pasvol *et al.*, 1978) and increased removal of parasitized erythrocytes containing HbAS by the spleen due to their enhanced sickling (Luzzatto, 1979; Roth *et al.*, 1978; Shear *et al.*, 1993). Recent studies suggest that malaria protection by HbAS also involves enhancement of acquired immunity to the parasite (Verra *et al.*, 2007; Williams *et al.*, 2005a; Williams *et al.*, 2005b). However, none of these studies can fully explain why people with sickle cell trait are protected from severe manifestations of malaria especially severe malarial anaemia.

Complement activation and immune complex (IC) formation are important features of malaria infection and both have been implicated to play a role in the development of severe malarial anaemia (Mibei *et al.*, 2005; Odhiambo *et al.*, 2008; Stoute *et al.*, 2003). The complement regulatory proteins CR1 and CD55, protects RBCs from complement activation and IC formation that occur during malaria infection (Jhaveri *et al.*, 1997). Studies have indicated that low levels of these complement regulatory proteins on erythrocytes surfaces may increase their susceptibility to phagocytosis or complement mediated lysis (Stoute *et al.*, 2003; Waitumbi *et al.*, 2000).

Although the role played by the complement regulatory proteins in protection of red cells from complement mediated destruction and immune complexes clearance is acknowledged and their acquired deficiencies associated with severe anaemia (Stoute *et al.*, 2003; Waitumbi *et al.*, 2004; Waitumbi *et al.*, 2000), no work has been done to evaluate their role in protection of sickle cell trait individuals against severe malarial anaemia.

The mechanism of protection afforded by sickle cell trait from severe manifestations of malaria is relevant especially in clinical research for example in malaria vaccine trials. It is important to document the occurrence of some of these red blood cell polymorphisms including sickle cell trait and to know the mechanisms by which they offer protection so

that we may know whether the protection is from malaria vaccine or from some of these polymorphisms. Knowing exactly how some of these protections occur could also hold the key to future novel therapeutic interventions and prophylactic measures against severe malaria.

Therefore, in this study, the involvement of complement regulatory proteins in protection of sickle cell traits against severe malarial anaemia was evaluated by determination of erythrocyte levels of CR1 and CD55, immune complex binding capacity and susceptibility to complement deposition in heterozygous carriers of the sickle cell gene and compared to those of individuals with normal haemoglobin under both normal oxygenation and reduced oxygenation.

8.1 Methods

8.1.1 Study Design and Population

This was as detailed elsewhere (Chapter 1-5). Briefly, 47 heterozygous sickle cell trait individuals aged 0-16 years were matched to 70 individuals with normal haemoglobin of similar age (± 2 months or ± 2 years for those below or more than 8 years, respectively) at a ratio of 1:1 or 1:2. As many conditions including malaria (Roestenberg *et al.*, 2007; Stoute, 2005; Stoute *et al.*, 2003; Waitumbi *et al.*, 2000), HIV infection (Aries *et al.*, 1997) and others (Spendlove *et al.*, 2006) can alter the level of red cell complement regulatory proteins, the exclusion criteria were evidence on clinical grounds of malnutrition manifested by marasmus or kwashiorkor; immunocompromised status manifested by weight loss, thrush, or diffuse adenopathy; severe anaemia (Haemoglobin ≤ 5.0 g/dl); bacterial infection such as pneumonia; malignancy; and blood transfusion within 3 months preceding the study.

8.1.2 Blood Samples and Smears

This was as outlined earlier (Chapter 3 to 7)

8.1.3 Statistical Analysis

Statistical analyses were performed as outlined earlier (see chapters 1 – 4).

8.2 Results

8.2.1 Demographic, Clinical and Laboratory Characteristics of Study Participants

A total of 117 children (age, ≤192 months.) of which 47 had HbAS and 70 had HbAA were included in the nested cohort study as described earlier were included in the study. Demographic, Clinical and Laboratory characteristics of study participants are presented in Table 8.1. There were no significant differences in gender, median age in months, median haemoglobin level, mean CR1 copy numbers per erythrocyte, mean erythrocyte copy numbers per erythrocyte after deoxygenation, mean CD55 antibody binding capacity, mean CD55 antibody binding capacity after deoxygenation, percentage C3b deposition, percentage C3b deposition after deoxygenation and the presence of malaria parasitemia between the HbAS and HbAA groups ($P = 0.339$, $P = 0.349$, $P = 0.468$, $P = 0.336$, $P = 0.377$, $P = 0.716$, $P = 0.136$, $P = 0.914$ and $P = 0.480$ respectively). The mean immune complex binding capacity before and after deoxygenation were significantly higher in the HbAS than in the HbAA group [($P = 0.037$ and $P = 0.005$ respectively), Table 8.1. Overall, these results illustrate that the protection of HbAS cells from severe manifestations of malaria like severe malaria anaemia may be related to the differences in the ability of the HbAS cells to bind more immune complexes (Figure 6.1) generated during infection with *P. falciparum* malaria through yet unidentified mechanisms.

Table 8.1: Demographic, Clinical and Laboratory Characteristics of the Study Population

Characteristic	**HbAA (n = 70)**	**HbAS (n = 47)**	***P***
Gender			
Male n (%)	28 (54.9)	23 (45.1)	0.339[a]
Female n (%)	42 (63.6)	24 (36.4)	
Age (Median; 25th -75th percentiles)	21.67 (11.28-73.62)	24.63 (11.47-94.40)	0.347[b]
Haemoglobin levels (Median; 25th -75th percentiles)	10.60 (9.30-12.00)	11.10 (10.20-11.60)	0.468[b]
Mean CR1 copy #/Erythrocyte (95% CI)	470.72 (431.49-509.95)	509.43 (457.43-561.44)	0.336[b]
Mean CR1 copy #/Erythrocyte Deoxygenated (95% CI)	602.79 (558.84-646.74)	575.92 (532.14-619.70)	0.377[b]
Mean ABC/Erythrocyte (95% CI)	2520.02 (2331.36-2708.68)	2671.44 (2388.00-2954.88)	0.716[b]
Mean ABC/Erythrocyte Deoxygenated (95% CI)	2570.96 (2352.92-2789.01)	2934.16 (2609.07-3259.24)	0.136[b]
Mean IC binding capacity (95% CI)	48.14 (45.36-50.93)	52.48 (50.60-54.35)	**0.037**[b]
Mean IC binding capacity Deoxygenated (95% CI)	48.00 (45.97-50.02)	52.74 (50.44-55.04)	**0.005**[b]
% C3b deposition(Median; 25th -75th percentiles)	16.90 (9.84-26.38)	17.92 (6.76-26.34)	0.914[b]
% C3b deposition deoxygenated (Median; 25th -75th percentiles)	13.88 (7.89-19.38)	13.34 (4.79-19.98)	0.480[b]
Numeric *P. falciparum* read			
Negative n (%)	39 (61.9)	24 (38.1)	0.621
Positive n (%)	31 (57.4)	23 (42.6)	

Data are presented as median (25th – 75th percentiles) or means (95% CI). Children (n=117) were categorized according to the haemoglobin types into either HbAA (n=70) or HbAS (n=47). [a]Statistical significance determined by the Chi-square analysis. [b]Statistical significance determined by Mann-Whitney U test.

8.2.2 Relationship between Mean CRI Copy Numbers for HbAS and HBAS and Haemoglobin Type

The mean CR1 copy numbers per red blood cell was statistically not significantly different between HbAA and HbAS groups before and after deoxygenation [(*P* = 0.250, *P* value = 0.400 respectively), Figure 5.1]. The CR1 copy numbers per erythrocyte was observed to vary with age for both HbAS and HbAA groups, being low in the >6-48 months age category and increasing thereafter. The mean CR1 copy numbers per erythrocyte were noted to be generally higher for individuals with HbAS than those with HbAA between the ages of >48-96 months. Beyond the age of 96 months, the CR1 copy numbers increased with age but more so for the HbAS, there was significant difference in the mean CR1 copy numbers per erythrocyte between HbAS [(mean = 671.0, SD 218) and HbAA (mean = 475.0, SD = 88.6), ***P* = 0.009**; see Figure 5.2]. In addition, a bivariate regression analysis demonstrated no association between the mean CR1 copy numbers per erythrocyte and haemoglobin type before and after deoxygenation (OR = 1.001, 95% CI 0.999-1.004, *P* = 0.239 and OR = 0.999, 95% CI 0.997-1.001, *P* = 0.407 respectively) (Table 8.2).

8.2.3 Relationship between CD55 Antibody Binding Capacity and Haemoglobin Type.

Analysis of the mean CD55 Antibody Binding Capacity showed no difference between the HbAS and HbAA groups (Figure 5.5). Interestingly, treatment of red blood cells with sodium dithionite (deoxygenation) resulted in greater increase in the antibody binding capacity for the HbAS erythrocytes after the age of >12-24 months than for the HbAA. Similarly, the mean CD55 antibody binding capacity after deoxygenation correlated positively with age of individuals with HbAS r (47) = 0.444, *P* = 0.002 but not of those with HbAA. In a bivariate logistic regression analysis assessing the relationship between Antibody Binding Capacity and haemoglobin type (HbAA and HbAS), the results showed no association between mean CD55 Antibody Binding Capacity and haemoglobin type (OR = 1.000, 95% CI 1.000-1.001, *P* = 0.354), but demonstrated a borderline association when the red blood cells were treated with sodium dithionite (OR

= 1.000, 95% CI 1.000-1.001, *P* = 0.062, Table 8.2), implying that children with HbAA had a much lower antibody binding capacity when compared to those with HbAS.

8.2.4 Relationship between Mean Immune Complex Binding Capacity and Haemoglobin Type

The mean immune complex binding capacity for the HbAS cells was significantly higher than HbAA cells both before and after deoxygenation (***P* = 0.017** and ***P* = 0.005** respectively). When the matching variable (± 2 months or ± 2 years for those below or more than 8 years, respectively) was factored in, this was again true (***P* = 0.025**). The mean immune complex binding capacity was lowest in the >6-12 months age group for both HbAS and HbAA (Figure 6.1), after which there was arise up-to age 48-96 months and thereafter a decrease with age. A bivariate logistic regression analysis demonstrated that children with HbAS bind significantly more immune complex than those with HbAA (OR = 1.046, 95% CI 1.003-1.091, ***P* = 0.034**) (Table 8.2).

8.2.5 Relationship between Percentage C3b Deposition and Haemoglobin Type

Percentage C3b deposition per erythrocyte did not differ between haemoglobin types (Figure 7.1). However, the percentage C3b deposition was low in the 0-6months age cohort, but thereafter remained similar in both HbAS and HbAA cells under normal and reduced oxygenation. The highest incidence of percentage C3b deposition was in the < 24 months age group in both the HbAS and HbAA (Figure 7.1). A bivariate logistic regression analysis failed to show any significant association between C3b deposition and either HbAA or HbAS before (OR = 0.999, 95% CI 0.969-1.029, P = 0.937) and after treatment of the cells with sodium dithionite (OR = 0.991, 95% CI = 0.955-1.029, P=0.642) (Table 8.2).

8.2.6 Multivariate Regression Analyses Model to Investigate the Relationship between Complement Regulatory Protein Levels and Haemoglobin Type

Complement regulatory protein levels were modeled in a multivariate logistic regression analyses to assess associations between the complement regulatory protein levels and

haemoglobin types. In the model, the complementary regulatory proteins that demonstrated a difference, at $P < 0.25$, between HbAA and HbAS in the bivariate logistic regression analyses were included. As such, the mean CR1 copy number per erythrocyte, mean CD55 antibody binding capacity per erythrocyte following treatment of the cells with sodium dithionite and the mean immune complex binding capacity per erythrocyte were considered (Table 8.3). A multivariate regression analyses demonstrated that children with HbAS had a significantly higher mean immune complex binding capacity than children in the HbAA group (OR = 1.046, 95% 1.0032-1.092, P = 0.040) (Table 8.3), implying the protective effect of HbAS erythrocytes against severe manifestations of malaria could be related to the ability of HbAS cells binding more immune complexes generated during malaria infection.

Table 8.2: Determination of Odds Ratio (OD) and the 95% Confidence Interval (CI) for the various Laboratory Parameters

Variable	**HbAA**		
	Odd Ratio (OR)	95% CI	***P***
Haemoglobin levels	1.065	0.861-1.317	0.564
Mean CR1 copy #/Erythrocyte	1.001	0.999-1.004	0.239
Mean CR1 copy #/Erythrocyte Deoxygenated	0.999	0.997-1.001	0.407
Mean ABC/Erythrocyte	1.000	1.000-1.001	0.354
Mean ABC/Erythrocyte Deoxygenated	1.000	1.000-1.001	0.062
Mean Immune complex binding capacity	1.046	1.003-1.091	**0.034**
Mean Immune complex binding capacity Deoxygenated	0.991	0.995-1.029	0.642
Percentage C3b deposition	0.999	0.969-1.029	0.937
Percentage C3b deposition Deoxygenated	0.991	0.955-1.029	0.642
Numeric *P. falciparum* read	0.829	0.395-1.741	0.621

Children (age, ≤192 months; n = 117) were stratified on the basis of haemoglobin type into two groups; HbAA (n =70) and HbAS (n = 47). For each individual sample, mean Haemoglobin levels, complement regulatory protein levels, mean immune complex binding capacity , percentage C3b deposition and presence or absence of *P. falciparum* malaria parasites were determined. A bivariate logistic regression analyses were performed to determine the Odds Ratio (OR) and 95% CI. In order to determine the impact of HbAS on the above parameters, individuals with HbAA were used as the reference group in the bivariate analyses.

Table 8.3: Relationship between Complement Regulatory Protein Levels, Mean IC Binding Capacity and Haemoglobin Type.

Variable	**HbAA (n=70)**		
	Odd Ratio (OR)	95% CI	***P***
Mean CR1 copy #/Erythrocyte	1.001	0.998-1.003	0.660
Mean ABC/Erythrocyte Deoxygenated	1.000	1.000-1.001	0.160
Mean IC binding capacity	1.046	1.0032-1.092	**0.040**

Children (age, ≤192 months; n = 117) were stratified on the basis of haemoglobin type into two groups; HbAS (n = 47) and HbAA (n = 70). For each individual sample, means of CRI copy numbers per erythrocyte, CD55 Antibody Binding Capacity per erythrocyte under deoxygenated conditions (ABC/Erythrocyte deoxygenated) and Immune Complex (IC) binding capacity were determined. The Odds Ratio (OR) and 95% CI were determined using multivariate logistic regression. In order to determine the impact of HbAS on mean CR1 copy numbers per erythrocyte, mean ABC/Erythrocyte deoxygenated and mean IC binding capacity, individuals with HbAA were used as the reference group in the multivariate analyses.

8.3 Discussion

8.3.1 Sickle Cell trait in Protection against Malaria

The relative protection of the sickle cell trait against clinical malaria has been reported to increase throughout the first 10 years of life, returning thereafter to baseline (Williams *et al.*, 2005a). The results of this study suggests that the age at which mean CR1 copy numbers increase in HbAS is lower (4yrs) than that for HbAA (8yrs). Although not statistically significant, individuals with sickle cell trait were noted to exhibit higher CR1 levels compared to those with HbAA. Beyond the age of 96 months, the CR1 copy numbers per erythrocyte was significantly higher for the HbAS than HbAA. HbAS [mean = 671.0, SD 218] and HbAA [mean = 475.0, SD = 88.6], p value = 0.009. This may be a contributing factor to the protective effects of HbAS from an early age when they are most vulnerable to getting severe manifestations of malaria.

The higher levels of CD55, immune complex binding capacity for HbAS compared to HbAA red blood cells may be additional factors that protect them from increased risk of complement attack and therefore the development of severe malarial anaemia.

It was notable that the HbAS cells had significantly higher immune complex binding capacity than the HbAA cells. This observation was true across all the various age groups. The ability to bind up and mop immune complexes generated especially during malaria infection is critical to prevent destruction of erythrocytes during malaria infection (Odhiambo *et al.*, 2008; Owuor *et al.*, 2008).

Natural resistance to malaria based on the inheritance of selected resistance genes has been observed in individuals living in malaria endemic areas. Transgenic mice expressing sickle cell haemoglobin (HbS) are protected from rodent malaria (Shear *et al.*, 1993) and rodent cerebral malaria (Kaul *et al.*, 1994). In humans HbS provides selective protection to heterozygous sickle cell trait individuals (HbAS) from dying of *Plasmodium falciparum* malaria while homozygous sickle cell trait individuals (HbSS) suffer severe clinical effects (Aluoch, 1995; Aluoch, 1997). Studies carried out in The Gambia (Hill *et*

al., 1991)and Kenya (Marsh, 1992; Williams, 2006) has indicated that HbAS provides more than 90% protection from both cerebral malaria and severe malarial anaemia in children. The mechanism of malaria protection by HbAS is not well understood but has been suggested to be due to enhancement of acquired immunity to the parasite (Verra *et al.*, 2007; Williams, 2006) and innate factors such as impaired parasite growth in erythrocytes containing HbAS attributable to conditions of low oxygen tension (Pasvol, 1980).

The Complement Receptor 1 (CR1/CD35) and Decay Accelerating Factor (DAF/CD55) are complement regulatory proteins (CRPs) that play an important role in the protection of red blood cells (RBCs) from complement mediated destruction and clearance of immune complexes (ICs) that occur during malaria and their acquired deficiencies have been associated with severe *P. falciparum* malaria.

The lowest level of immune complex binding capacity corresponds to the lowest levels of CR1 in this population (Waitumbi *et al.*, 2004).This also corresponds to the age where the majority of children get severe malarial anaemia (Snow *et al.*, 1999; Stoute *et al.*, 2003) The lowest levels of CR1 correspond to the lowest immune complex binding capacity predisposing these children to immune complex deposition and therefore increased risk of red cell destruction leading to anaemia.

In malaria, a number of immune complexes are generated. These circulating immune complexes may lead to end organ damage by depositing on erythrocytes and other organs like the kidney. Studies from our laboratory have shown that ICs are elevated in severe malarial anaemia at the time of diagnosis (Mibei *et al.*, 2005) these ICs can stimulate pro-inflammatory cytokines that may play a role in the pathogenesis of severe malaria especially CM

The ability to bind circulating immune complex is critical for mopping up of the circulating ICs. The high ability of red blood cells to mop up immune complexes results in decreased chance of getting severe manifestations of malaria (Odhiambo *et al.*, 2008; Owuor *et al.*, 2008). The immune complex binding capacity for HbAS cells were much

higher than for the HbAA cells ($P = 0.037$). This was also true after deoxygenation ($P = 0.003$). It is apparent that the HbAS cells bind more immune complexes than the HbAA cells and this may lead to increased mopping up of the immune complexes generated during malaria infections and therefore prevent severe manifestations of malaria. After deoxygenation, there was apparent increase in the binding sites may be due to conformational changes in the CR1 molecules resulting in the exposure of more binding sites.

Like in other studies, age correlated with immune complex binding capacity with high levels in the 0 - < 6 months age cohort, thereafter decreasing to < 6 – 24 months age cohort. This is in agreement with other studies (Stoute *et al.*, 2003; Waitumbi *et al.*, 2000)

C3b is an important opsonin and promotes phagocytosis of target membranes such as bacteria, viruses and immune complexes (Craig *et al.*, 2005). C3b participates in stimulating immune response by a variety of mechanisms and promotes the localization of foreign antigens to lymphoid tissues, stimulates B cell growth and participates in stimulating the cytokine production (Marie *et al.*, 2002). Malaria infection leads to complement activation and C3b deposition on red cells. Red cells with low complement regulatory proteins are more susceptible (Odhiambo *et al.*, 2008). The pattern of C3b deposition on red blood cells has been shown to be opposite that of CR1. In our study although the pattern of deposition of C3b was opposite the CR1 and CD 55 levels with the mean percentage C3b-positive cells for the HbAS cells being lower than HbAA cells, this difference was not statistically, $P = 0.914$. After deoxygenation, the mean percentage C3b-positive cells for the HbAS cells was again lower than HbAA cells but this difference was again not statistically significant $P = 0.480$.
The peak % C3b deposition for the various age cohorts was between the ages of >6-12months after which it generally decreased to the lowest level at age >24-48 months. With the general higher CR1 levels in the age cohort >96 months for the HbAS, the general higher immune complex binding capacity for the HbAS and the low C3b deposition, it is possible that this is protective in the HbAS in the older age group.

8.4 Conclusion

This study has shown that the age at which mean CR1 copy numbers increase in HbAS was lower (4yrs) than that for HbAA (8yrs). Although not statistically significant, individuals with sickle cell trait were noted to exhibit higher CR1 levels compared to those with HbAA. Beyond the age of 96 months, the CR1 copy numbers increased with age but more so for the HbAS, there was significant difference in the mean CR1 copy numbers per erythrocyte between HbAS, p value = 0.009. This may be a contributing factor to the protective effects of HbAS from getting severe manifestations of malaria especially in the older age group.

Although not statistically significant, the HbAS erythrocytes had higher mean CD55 antibody binding capacity than the HbAA cells.

The mean immune complex binding capacity for the HbAS cells was significantly higher than HbAA cells both before and after deoxygenation (***P*** = 0.017 and 0.005 respectively). This was again true when a matching variable was factored in, (P = 0.025).

Despite the high mean CR1copy numbers per erythrocyte, the high mean CD55 antibody binding capacity and the high immune complex binding capacity of the HbAS compared to HbAA, there was no statistically significant difference in the percentage C3b deposition between the HbAS and the HbAA red cells.

8.5 Recommendations

1) It would be interesting to do high performance liquid chromatography to know the percentage of HbAS for the participants to know whether the protection afforded by sickle cell trait carriers is HbS content dependent or not.

2) It is recommended that a large cross section survey be done for the HbAS and HbAA to look at CR1 expression with a view to knowing whether they are high, medium or low expressors.

3) It would be interesting to do a western blot to know the CD55 percentage in HbAS as opposed to HbAA.

4) The protection of sickle cell gene from malaria has been shown to be HbS content related (Aluoch, 1997). In this study we did not look at the percentage of HbS content which has been shown to vary from 20% to 45% with a mean value of 38 ± 5% (Al-Shakour, 2000). It would be important to relate the complement regulatory proteins, the immune complex binding capacity and the % C3b deposition with HbS content of the cells to see if this changes the scenario.

5) It would be important to look at the complement regulatory protein machinery between birth and the age of 8 years in a bid to bring out these differences. This study has shown that there is distinct difference in the level of CR1 between HbAS and HbAA from the age of 4 years to 8 years. Before this and after this, the levels mirror each other and there is not much difference.

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APPENDICES

Appendix 1: Informed Consent

Kenya Medical Research Institute US Army Medical Research Unit, Kenya Study Specific Procedure	SSP NO.: SM-01 Supercedes: N/A Effective Date: 15-SEP-04
Study Name: Changes in Erythrocyte Immune Complex Binding Capacity and Complement Sensitivity with Age in Populations with Different Malaria Risks	
Principal Investigator: Jose A. Stoute, M.D.	
Title: Recruitment of Study Subjects and Administration of Informed Consent	
For: Investigators, Co-Investigators, Clinical Research Coordinators, Senior Field Supervisor, Field	
Purpose: To describe the procedure to be followed in recruiting study subjects and administering Informed	
Prepared by:	Date:
Approved by:	Date:

Procedure: Informed Consent will be obtained from the subject in accordance with applicable FDA, OHRP, and ICH Guidelines for GCP. Records will be obtained of the entire informed consent process.

1. Fieldworkers will use recruitment scripts (see sample recruitment script, Appendix A) to interview and schedule appointments for individuals interested in attending briefing sessions at the WRP Kombewa Clinic. Upto 10 participants may be scheduled per briefing session.

2. The recruitment script will include a brief introduction to the study and general questions designed to determine whether the individual is potentially eligible for the study. The information collected may include:

Date of the interview

Name of the subject (in case of a child, the name of the subjects' father, mother and/or guardian).

Subjects' Date of Birth and age
Primary language of the respondent
Willingness to attend a briefing session at the WRP Kombewa clinic
Briefing appointment day and time
Interviewer name and signature
All completed recruitment scripts will be filed with the regulatory documents.
Interested individuals will be invited to attend a briefing session at the WRP Kombewa clinic. Participants (and/or parents or guardians of participants) will be asked to register in the Informed Consent Briefing Sign-in Log (see sample Log, Appendix B). The log will contain the following information:

Name of the study and Principal Investigator
Name of the individual conducting the session (Provider)
Provider's role (Designation)
Start and Stop Times
Date
Name of the participant
Date of Birth
Compound Number
Village
Signature (or thumbprint)
Whether consent form was signed

A Physician Assistant/Clinical Officer stationed at the study site will conduct each briefing. The briefing sessions will consist of an oral presentation, and the information contained in the informed consent document will be presented in the participants' native language. At the end of each presentation, participants will be invited to ask questions of the individual conducting the briefing.

The Informed Consent form that is given to the participants will be a certified translation, of their native language, of the IRB approved consent form.

The participant will be allowed time to read the Informed Consent Explanation and Consent Form.

In the event that illiterate participants are in attendance, an impartial witness will be present to ensure that the briefing was conducted appropriately, and that all issues and concerns raised were addressed to the participants' satisfaction. The witness will sign the informed consent forms of any illiterate participants, indicating that (s)he witnessed the entire consent process. Another potential participant may serve as an impartial witness.

Instructions on the proper completion of the consent document will be given to those participants who wish to sign after the briefing session. An impartial witness will be available to witness the voluntary application of the participants' signature or thumbprint.

Participants who sign the consent form will be given a copy of the signed consent document.

Individuals who are unwilling to consent on the day of briefing will be given a copy of the consent document to take home for their further review and consideration. They will be advised to return to the clinic if they decide at a later date that they would like to give consent. They will be encouraged to return if they have questions at any time.

The original Informed Consent Form will be placed in a manila folder and the subject will then begin the screening process.

The Informed Consent Briefing Sign-in Log will be completed to indicate which of the individuals who attended the briefing have signed the consent form.

Attachments: Appendix A: Sample Recruitment Script

Sample Informed Consent Briefing Sign-In Log

RBC X-Section

RECRUITMENT SCRIPT

We are from the Walter Reed Project Kombewa Clinic based in Kombam village. We are about to begin a study in children and adults to determine how red blood cells vary with age and to determine how this affects the chances of someone getting malaria. There will be only 1 clinic visit if the participant has no malaria and a maximum of 3 visits if the participant has malaria.

Parents will sign the consent form for children below 18 years of age. We will need to verify the age of each participant, both the children and the adults, from any record you have, for example a record from Ministry of Health, baptismal card or national identity card.

Which Language do you best understand?

☐ Luo

☐ English

☐ Kiswahili

Date_____ Cpd No:_____ Village_____

(Check as appropriate)

Child < 18yrs ☐ Adult 18 yrs to 45 ☐

(If child)

Is there a document to verify DOB? ☐ Yes ☐ No

Name of child_____ Sex _____

DOB _____/_____/_____ Age_____(days/weeks/months/years)

dd *mmm* *yy*

Name of father _____

Name of mother_____

Name of guardian_____

Other_____

(*If adult)*

Is there a document to verify DOB? ☐ Yes ☐ No

Name of adult_____Sex_____

DOB _____/_____/_____Age_____(years) *dd*

mm……yy……

Which language do you best understand_____

(Please check appropriately)

YES NO

☐ ☐

Are you willing to attend a briefing session at Kombewa clinic?

(Briefing appointment days. Briefing time is 9am on all days)

Day	*Date*
Monday	
Tuesday	
Wednesday	
Thursday	
Friday	

Interviewer's Printed Name_____

Interviewer's Signature_____

Date_____

RBC x-section

INFORMED CONSENT BRIEFING SIGN IN LOG

Dr. José A. Stoute

PROVIDER _____

TITLE/ROLE_____

DATE OF BRIEFING: _____ START TIME_____

STOP TIME_____

The following individuals attended the Informed Consent Briefing, were given the opportunity to have their questions answered, and have been provided with a copy of the consent form to take home

NAME	DOB dd/mm/yy	VILLAGE	CPD. NO.	PARENT SIGNATURE (OR RIGHT THUMB PRINT) SELF	WAS CONSENT SIGNED YES NO

INFORMED CONSENT EXPLANATION

(To be read and questions answered in a language in which the volunteer is fluent.)

TITLE OF STUDY: Changes in Erythrocyte Immune Complex Binding Capacity and Complement Sensitivity with Age in Populations with Different Malaria Risks.

INSTITUTIONS: The Kenya Medical Research Institute and the US Army Medical Research Unit, Kenya.

PRINCIPAL INVESTIGATOR: Dr José A. Stoute, Walter Reed Army Institute of Research, Dept. of Cellular Injury, 503 Robert Grant Ave., Silver Spring, MD 20910, Tel 1-301-319-9652.

PARTICIPATION INFORMATION: We would like you or your child to participate in a medical research study of malaria. It is very important that you understand the following general principles that apply to all participants in our studies: 1) participation is entirely voluntary. 2) you or your child may withdraw from participation in this study or any part of the study at any time. Refusal to participate will involve no penalty or loss of benefits to which you or your child are otherwise entitled. 3) After you read the explanation, please feel free to ask any question that will allow you to understand clearly the nature of the study.

INTRODUCTION: Malaria is a disease that affects many people throughout the world. It is caused by parasites that are normally transmitted by mosquito bites. Malaria may be mild but can also sometimes be severe. Malaria attacks the red cells of the body and can kill by causing anaemia or coma. As part of the study we would like to see how red cells vary with age to determine how that affects the chances of someone having severe malaria. We hope this study will help us figure out how anaemia and coma come about in malaria and how to prevent it.

PROCEDURES TO BE FOLLOWED: If you agree for yourself or your child to participate in this study, first, a physical examination will be done by a clinician working for the study. A blood smear will be done by finger prick to diagnose malaria. If the smear is negative, 2.5 ml (1/2 teaspoonful) of blood will be obtained from a vein in the arm or by finger or heel prick. If the smear is positive, treatment for malaria will be given and you will be asked to return in two weeks. If at that time the smear is negative, then the blood sample will be obtained and enrollment completed. If the smear is negative a second time a different malaria medicine will be given and you will be asked to return in another two weeks. If the smear is positive the third time you will be treated and excluded from the study. In addition to the test for malaria, a variety of other tests will be done to measure red cell function. Additional procedures may be required that normally would be performed in any hospital in order to properly treat your child's condition. These may include obtaining a stool sample to look for worms or obtaining urine to exclude the possibility of kidney infection.

RISKS: The risks from participation in this study are minimal. There is the possibility of mild discomfort, bruising, and very rarely infection, at the site from where blood is obtained. The medicines used in this study are used routinely in many other hospitals. In rare occasions, medicines can cause allergic reactions which can be mild, severe, or even fatal.

DURATION OF VOLUNTEER'S PARTICIPATION: I day but may last 3 weeks.

BENEFITS: All medications will be provided free of charge. Transportation will be provided whenever possible.

DISCOMFORTS: There is the possibility of mild discomfort at the site from where blood is obtained.

PERFORMANCE SITES AND NUMBER OF VOLUNTEERS IN THE STUDY: Four sites will be chosen from the following districts: Kisumu, Kakamega, Kericho, Kisii, Nandi, Nyamira, Baringo, Nakuru, Nyeri, Kirinyaga, Nairobi, and Kilifi. 330 participants per site, making a total 1320.

ASSURANCE OF CONFIDENTIALITY OF VOLUNTEER'S IDENTITY: Records relating to participation as a research subject will remain confidential. Representatives of the Kenya Medical Research Institute, the U.S. Army Medical Research and Materiel Command (USAMRMC), and the National Institutes of Health and designees may review the study records as part of their responsibility to oversee research. The name of the participants will not be used in any report resulting from this study. You will receive a copy of this consent form.

USE OF BLOOD SAMPLES: The blood samples obtained in this study will not be used for any other purpose, other than the ones stated in the protocol and consent form, without prior approval of the KEMRI Ethical Review Committee.

MEDICAL CARE FOR INJURY OR ILLNESS: Should an injury occur as a direct result of participating in this research project, medical care will be provided at no cost for that injury. No other compensation will be given, only medical care. You should also understand that this is not a waiver or release of your legal rights. You should discuss this issue thoroughly with one of the investigators before you enroll in this study.

PERSONS AND PLACES FOR ANSWERS IN THE EVENT OF RESEARCH RELATED INJURY: If you think you or your child has a problem as a result of participation in this study, call or come the Walter Reed Project Office, to see Dr. Walter Otieno, Walter Reed Project, P.O. Box 54, Kisumu, Kenya Tel. 057-22942.

FOR INFORMATION OR ANSWERS TO QUESTIONS CONCERNING YOUR RIGHTS AS A RESEARCH SUBJECT YOU MAY CONTACT:

The Chairman of the Kenya National Ethical Review Committee, C/O Kenya Medical Research Institute, P.O. Box 54840, Nairobi, Kenya Tel. 020-2722541.

IF THERE IS ANY PORTION OF THIS CONSENT EXPLANATION SHEET THAT YOU DO NOT UNDERSTAND, ASK THE INVESTIGATOR BEFORE SIGNING.

TITLE OF STUDY: Changes in Erythrocyte Immune Complex Binding Capacity and Complement Sensitivity with Age in Populations with Different Malaria Risks.

Participant's Name: _____Participant's Age: _____

The research study has been explained to me by _____and I understand and agree to the following:

It is up to me to decide whether I or my child can participate in this study and I can withdraw at any time without fear of any penalty.

The duration of the study is 1 day but may last 3 weeks.

About ½ teaspoon of blood will be obtained.

All outpatient medications will be provided free of charge.

Transportation to and from the clinic will be provided.

I have read and received a copy of the informed consent explanation and I have been given the opportunity to ask questions to my satisfaction.

If I have any further questions, I may contact Dr. Walter Otieno at 057-22942.

_____ _____

Parent's / Subjects signature Date

Parent's/ Subjects Printed Name: _____

_____ _____

Signature of Person Giving Explanation Date

Printed Name: _____

Thumbprint of Parent/ subject if unable to sign:

Address: _____

Locality: _____

Village: _____

House Number: _____

PO Box:_____

Witness to Consent Interview

On the date given next to my signature, I witnessed the "Consent Interview" for the Research Study named above in this document. I attest that the information in this consent form and the written summary was explained to the subject or the subject's legally authorized representative, and the subject or subject's representative indicated that his/her questions and concerns were adequately addressed.

Name of Witness _____

Signature of Witness _____ Date _____

Witness to Subject's Signature

On the date given next to my signature, I witnessed the subject or the subject's legally authorized representative sign his/her name or imprint his impress his thumbprint on this consent form.

Name of Witness _____

Signature of Witness _____ Date _____

Appendix 2: Volunteer Flow on the Day of Briefing.

Kenya Medical Research Institute US Army Medical Research Unit, Kenya Study Specific Procedure	SSP NO.: SM-02 Supercedes: N/A Effective Date:15-SEP-04
Study Name: Changes in Erythrocyte Immune Complex Binding Capacity and Complement Sensitivity with Age in Populations with Different Malaria Risks	
Principal Investigator: José A. Stoute	
Title: Briefing and Enrollment Day Procedures	
For: Investigators, Associate Investigators, Clinical Research Coordinators, Ancillary Staff	
Purpose: To establish the volunteer flow and personnel responsibilities on the day of Briefing and	
Prepared by:	Date:
Approved by:	Date:

Procedure:

Station 1 (Meeting Room) – Reception and Registration

Designated staff will greet the volunteers as they arrive and direct them to the registration desk.

Participants will be registered in the Informed Consent Briefing Sign-in Log (see attached).

Participants (and/or parents/guardians of participants) will be required to provide proof of age.

Illiterate subjects will be advised to identify a literate individual from among the participants who will act as a witness during the signing of the informed consent (refer to SSP: SM-01)

Participants will be given a copy of the Informed Consent Form at the registration desk to refer to during the course of the briefing. This informed consent will be useful in ensuring that the individual has been logged in at the registration desk.

Designated personnel will ensure that all participants have a copy of the Informed Consent Form before the presentation begins. Persons found not to have a copy of the consent form will be advised to report to the registration desk to collect a copy of the consent form and to register their attendance.

Participants will be directed to the area where the briefing will be carried out.

The Physician Assistant/Clinical Officer conducting the briefing will be introduced to the participants.

Participants will be informed of the program of events for this session.

Station 2 (Conference Room) – Informed Consent Explanation

The Physician Assistant/Clinical Officer will conduct the Informed Consent Briefing in a language that all participants are familiar with.

After the conclusion of the briefing, the Physician Assistant/Clinical Officer conducting the session, as well as other investigators will be available to answer questions and address issues as a group, or individually in private. A translator will be present if required.

Once it has been determined that all questions and concerns have been adequately addressed, participants willing to sign the informed consent form will be directed to station three.

Those who are unwilling to consent on the day of briefing will be directed back to the registration desk where this action will be recorded. They will be given a copy of the consent document to take with them for their further review and consideration, and

advised to return to the clinic if they decide at a later date that they would like to give consent. They will be encouraged to return if they have questions at any time.

Station 3 (Consenting Booths) – Signing of Informed Consent

There are 4 consenting booths and these will be manned by designated personnel. These personnel will be responsible for ensuring that the Informed Consent form is signed appropriately. Participants will be channeled to this station in a manner that ensures both order and efficiency. Guides stationed in the main clinic building will coordinate to ensure that individuals are directed to the Consenting booths only when there are free booths.

Personnel manning each booth will greet the participants (and/or parents/guardians of the participants)

Personnel will inquire if the participants (and/or parent(s)/guardians of participants) need to ask any further questions. If they do, they will be directed to a designated room, where one of the Investigators will be stationed. After their concerns have been addressed, they will be directed back to the Consenting Booths (if they still wish to sign) or to the Reception Desk (if they do not wish to sign at that time).

Personnel will explain the role of the witness to the illiterate parent(s) or guardians.

Personnel will show the parent(s)/guardian (and witness, if applicable) where to sign and direct them to station 4.

Station 4 (Office area) – Copying of Informed Consent

Subjects who have signed the informed consent will be directed to the Reception Desk.

Personnel will receive the signed Informed Consent from the parent/guardian. Personnel will check that the consent has been signed as required.

The Informed Consent Document will be signed by the Physician Assistant/Clinical Officer who conducted the briefing, before a copy is made.

The Informed Consent Form will then be copied. Designated personnel will then place the original consent form in a manila folder. Participants will then begin the screening process.

Station 5 (Office Area) –Registration

The Informed Consent document will be placed in a manila folder. This folder will have been pre-prepared with the study forms.

Subject IDs will be allocated in order of appearance (e.g. the first subject will have ID number. 001, the second subject will be 002, etc).

Subjects will be asked to sign the Screening Volunteer Registry Record (Appendix A). There will be a total of 8 different Screening Volunteer Registry Records stratified into the following age cohorts:

0-6 months

7-12 months

13 - 24 months

25 - 48 months

49 - 96 months

9 -16 years

17-32 years

33-45 years

The number of subjects enrolled into the cohorts will be as indicated below:

0-6 months (30 subjects)

7-12 months (60 subjects)

13 - 24 months (60 subjects)

25 - 48 months (60 subjects)

49 - 96 months (30 subjects)

9 -16 years (30 subjects)

17-32 years (30 subjects)

33-45 years (30 subjects)

Personnel at this station will complete the Registration section of the Screening Day Checklist. Designated personnel will then escort the subject to the next station in the screening process, Physical Examination.

Station 6 (Examination Rooms) – Physical Examination

The participant will undergo a standardized physical examination.

The subject will then be sent to the lab for a malaria smear. If the smear is negative, the subject will be directed back to the clinicians' office, enrolled and sent back to the lab where a blood sample will be obtained.

If the smear is positive, the subject will be treated for malaria and asked to return two weeks later for re-evaluation and to give the blood sample. If an individual who was positive for malaria at the initial visit is still positive at the time of re-evaluation, they will be treated again with the second line agent (Artemether/Lumefantrine (Coartem/Riatem.) and asked to return in two weeks. if at the time of the third visit this individual is still positive for malaria, they will be treated with quinine, 10mg/Kg twice daily for 7 days and excluded from participation.

Minor illnesses such as Upper Respiratory and Urinary Tract Infections may be treated if identified. In such cases, the standard of care will be identified.

Individuals found not eligible to continue with the screening process will be informed of the reasons for their exclusion, and directed to the dining area.

If the individual is found to be smear negative, they will be enrolled and directed to the next station in the screening process, sample collection.

Subject flow will be as in Appendix A.

Station 7 (Phlebotomy Area) – Sample Collection

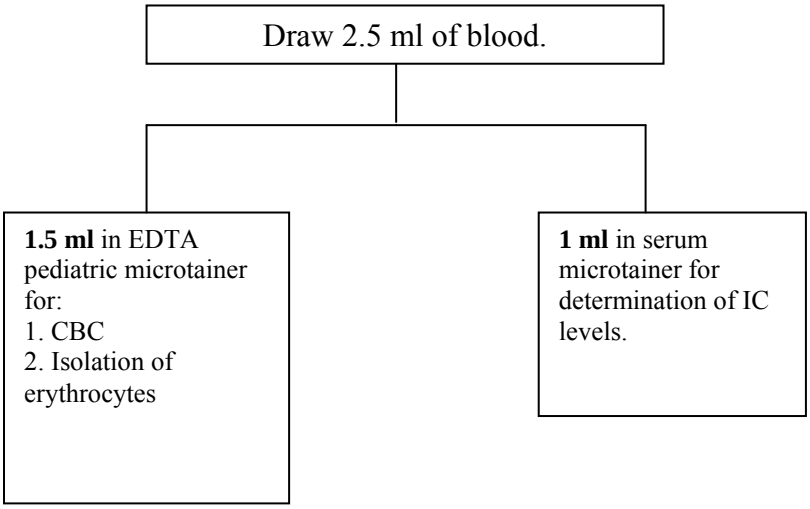
Blood will be collected only when the participant is well and free of malaria by microscopic examination of Giemsa-stained blood smears. Trained clinical personnel will collect all the blood samples using sterile techniques and disposable needles and syringes or lancets.

Specimens will be collected as indicated on the Laboratory Request Form. Blood will be collected for:

CBC

Plasma/Serum storage

Hgb Electrophoresis



Laboratory specimen collection devices will be labeled using the pre-prepared laboratory labels.

Laboratory personnel will complete the laboratory section of the Screening Day Checklist.

Laboratory personnel will retain the subjects' charts and complete laboratory results. After completion of laboratory results, charts will be turned over to the CRC for QA and filing.

Subjects will be directed to Station 8, Clearance and Exit.

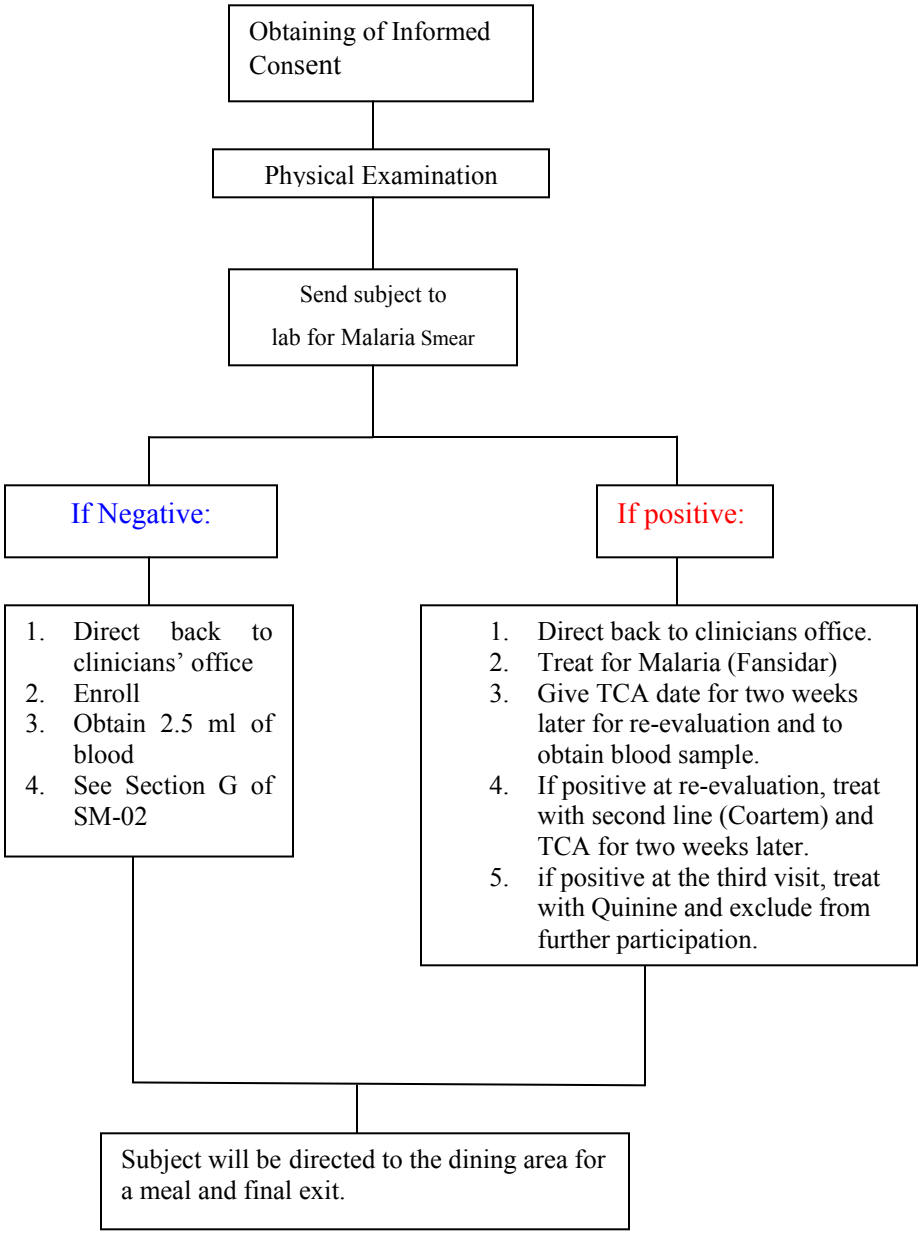
Station 8 (Dining Area) – Clearance and Exit

1. Designated personnel acting as guides stationed in the corridor area will thank subjects for their interest and participation.

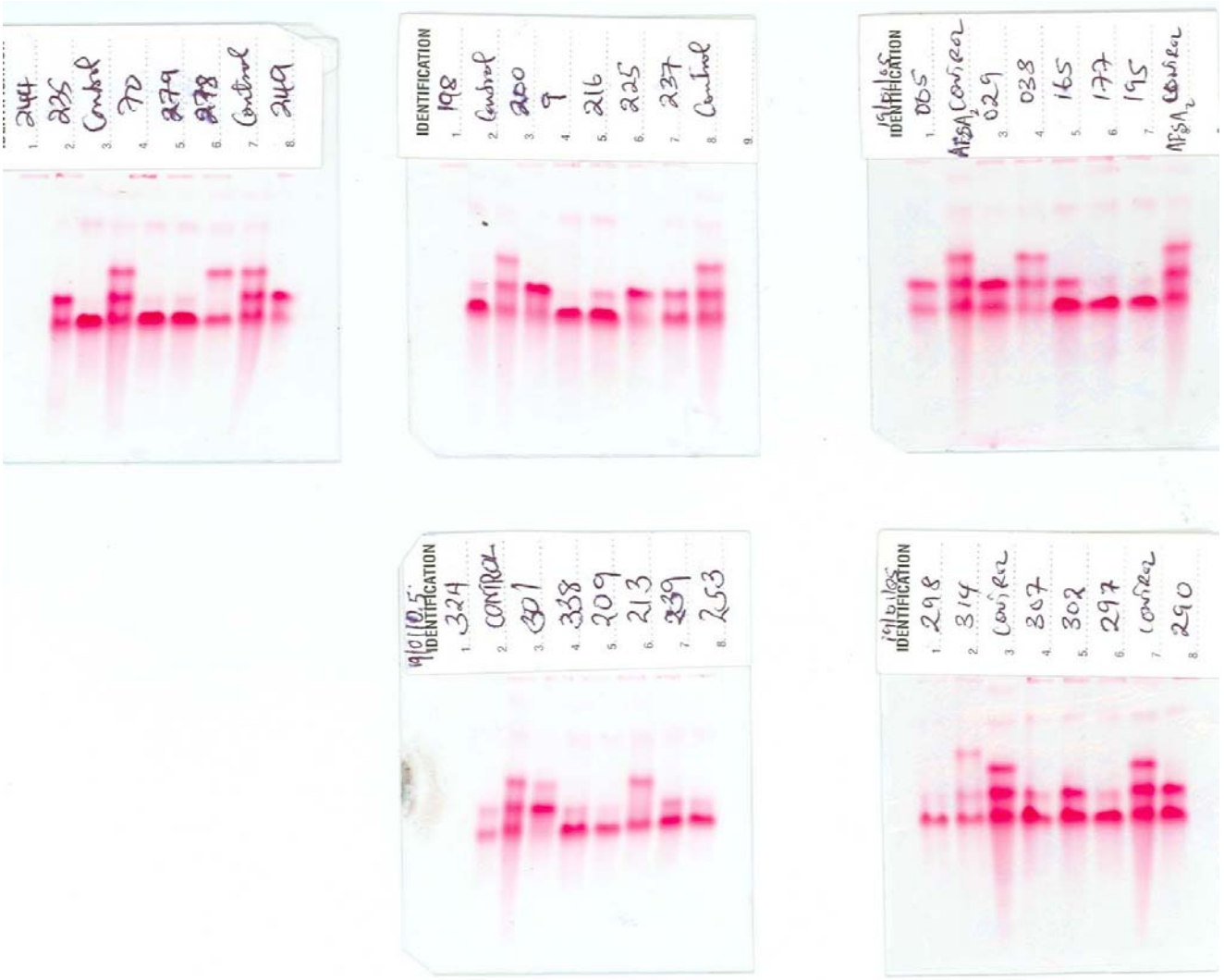
2. Designated personnel acting as guides will direct the subjects to the dining area.

3. Subjects will be served and will be given transportation back to their homes.

Subject Flow on the Day of Screening



Appendix 3: Scanned Photos of Haemoglobin Electrophoresis



Appendix 4: Abstracts for Conferences/Workshops/Seminars

1. American Association of Tropical Medicine and Health Washington D.C December 2005.

Expression of Erythrocyte Complement Regulatory Proteins in Individuals with Sickle Cell Trait and Normal Haemoglobin in a Malaria Endemic Area of Western Kenya.

Walter Otieno,*[1,2] Joash R. Aluoch,[2] Benson Estambale,[2] and José A. Stoute.[3,4]

1 The US Army Medical Research Unit and the Kenya Medical Research Institute, Nairobi, Kenya.

2 The University of Nairobi, College of Health Sciences, Nairobi, Kenya.

3 The Dept. of Cellular Injury, Walter Reed Army Institute of Research, Silver Spring, MD, USA.

4 The Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD, USA.

*Presentor

Abstract:

Sickle cell trait (HbAS or SCT) has similar distribution pattern to malaria in the malaria holoendemic areas of the tropics. This is attributed to the relative protection offered by the SCT from severe malaria. The mechanism of protection is not well understood. Erythrocyte Complement Regulatory protein-1 (E-CR1) and Decay Accelerating Factor (DAF or CD55) are important in protecting RBCs from complement mediated damage and in controlling the complement activation cascade. CR1 and CD55 levels are low in children with severe malarial anaemia (SMA). This increases the susceptibility of their red blood cells to phagocytosis or complement mediated lysis. We think that E-CR1 and

CD55 may have a role in protecting HbAS children from SMA. We propose to investigate the level of CR1 and CD55 expression, the immune complex binding capacity and the susceptibility to complement activation of children with HbAS versus HbAA in a cohort of children from a malaria endemic region of Western Kenya. We hypothesize that sickle cell trait could be associated with increased CR1\Erythrocyte ratio and over expression of DAF. This may lead to increased rate of clearance of immune complexes from the circulation and may offer an explanation as to why children with HbAS are protected from SMA. To achieve our objective, we have done a cross section study in Kombewa division of Kisumu district, a malaria holoendemic region……..

Complement Regulatory Protein Levels, Immune Complex Binding Capacity, and Complement Susceptibility of Red Cells from Children with Sickle Cell Trait or Normal Adult Haemoglobin in a Malaria Endemic Area of Western Kenya

2) Fifth MIM Pan-African Malaria Conference, 2-6 November 2009, Nairobi, Kenya Registration ID: 16729946

Walter Otieno[1,2], Joash R. Aluoch[4], Benson Estambale[2] & José A. Stoute[3]

1. The US Army Medical Research Unit and the Kenya Medical Research Institute, Nairobi, Kenya.

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Introduction

Severe anaemia is one of the most serious complications of *Plasmodium falciparum* malaria that occurs predominantly in children in the first 3 years of life and is an important cause of childhood morbidity and mortality in sub-Saharan Africa. Malaria infection leads to the formation of immune complexes that can activate complement and lead to the production of pro-inflammatory cytokines by interaction with macrophages.

Heterozygous sickle cell trait individuals (HbAS) are relatively protected from severe manifestations of malaria such as anaemia. The underlying mechanisms of this protection are however not well understood but are thought to be due to several mechanisms. Given the important role played by immune complex deposition in the pathogenesis of severe malarial anaemia, this study investigated whether there are differences in IC binding capacity between erythrocytes from heterozygous sickle cell trait individuals and normal individuals that could partly explain the protection from severe anaemia in individuals with sickle cell trait.

Materials and Methods

We used flow cytometry to measure red cell complement regulatory protein levels, C3b deposition, and immune complex binding capacity of children with AS haemoglobin or AA haemoglobin.

Results

The analysis of these results is in progress and will be presented at the conference.

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