PREVALENCE OF FETAL HEMOGLOBIN AND ANTIBODY RESPONSES TO *Plasmodium Falciparum* ANTIGENS IN SICKLE CELL DISEASE PATIENTS IN WESTERN KENYA

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DECLARATION

I hereby declare that this thesis is my original work and has not been presented for degree or other awards in any other University

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DEDICATION

To my family members especially my mother Mary Webala and brothers Oscar Ochieng' and the Late Derrick Odhiambo for their dedicated support and patience during the time of my study

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ABBREVIATIONS AND ACRONYMS

AMA Apical membrane antigen

CSP Circumsporozoite protein

DNA Deoxyribose nucleic Acid

EBA Erythrocyte binding antigen

EBP Erythrocyte binding protein

GLURP Glutamate rich protein

HbAS Heterozygous sickle cell

HbF Fetal Hemoglobin

HbS Sickle hemoglobin

HbSS Homozygous Sickle cell

ICSH International Council for Standardization in Hematology

KEMRI Kenya Medical Research Institute

LSA Liver stage antigen

MEA Malaria endemic area

MFI Median Fluorescence Intensity

MSP Merozoite surface protein

MVEC Micro-vascular endothelial cells

NACOSTI National Commission for Science, Technology and Innovation

PCR Polymerase Chain Reaction

RBC Red blood cell

SAT Suspension Array Technology

SCA Sickle Cell Anemia

SCD Sickle Cell Disease

WHO World Health Organization

DEFINITION OF TERMS

Prevalence of fetal hemoglobin:	The proportion of individuals with fetal hemoglobin level above 1% in the SCD patients' population sampled
Levels of fetal hemoglobin:	The amount of fetal hemoglobin in the SCD individuals as determined by Betke method
High fetal hemoglobin:	Amount above 10%
Low fetal hemoglobin:	Amount below 10%
Betke method:	A technique used for quantification of fetal hemoglobin by the principle of alkali denaturation

ABSTRACT

Malaria is a major public health problem worldwide with increasing cases and deaths in sub-Saharan Africa. Sickle cell disease conditions relate geographically with malaria endemic areas. Fetal hemoglobin (HbF) moderates the clinical severity of sickle cell disease (SCD) and also provides protection against malaria. Consequently, it provides survival advantage but the data is limited. Designing a study linking HbF with protection against malaria infection has been a challenge due to potential confounders on the exposure outcome. This study therefore investigated the prevalence and levels of HbF and the IgG responses to *Plasmodium falciparum* antigens in 100 SCD patients aged 5-30 years living in a malaria-endemic area in Western Kenya. A cross-sectional study was conducted to determine the prevalence and levels of HbF and the IgG responses to a panel of eleven recombinant *P. falciparum* antigens in SCD patients. The levels of HbF and the IgG responses to each of the 11 antigens were determined using the alkali denaturation (Betke) method and the cytometric bead assay in a Luminex-suspension array technology respectively. The study reports a prevalence of up to 77 % of the SCD patients with high fetal hemoglobin (>10%) with a mean and range of 19.09% (1.44-56.25%) respectively. Generally the levels of HbF increased with age ($r = 0.17$, $P < 0.05$) indicating that fetal hemoglobin provides survival advantage in SCD, in males there was an increase in HbF with age ($r=0.31$; $P<0.05$) while in females it was not significant ($r = 0.02$; $P>0.05$). The IgG responses to the multiple *P. falciparum* antigens were differently expressed in the SCD patients, pre-erythrocytic antigens showed a statistical difference when the mean IgG levels were compared using unpaired T test between the seropositive SCD patients and non-SCD individuals with the later having high IgG levels ($P<0.05$). In contrast LSA-NRC had high IgG levels in SCD patients ($P<0.05$). The IgG responses to blood stage antigens on the other hand were not statistically different between the SCD patients and non- SCD individuals ($P>0.05$). The IgG responses to MSP-1-42-FVO were high both in the seropositive SCD and non-SCD individuals. However, when compared with the non-SCD individuals using unpaired T-test, the non- SCD individuals had significantly high levels of IgG responses to both the pre-erythrocytic and the blood stage antigens than the SCD patients ($P<0.05$). Using Spearmans' rank correlation analysis, HbF positively correlated with the IgG responses to LSA-NRC ($r= 0.26$; $P<0.05$), other antigens showed no correlation. This implies that HbF can provide protection against malaria in SCD patients living in malaria endemic areas and thus increase their life expectancy. The findings also reinforce the previous findings that antibody cooperates with fetal hemoglobin to provide protection against malaria. Nonetheless, further rigorous study design approach should be used for investigations on the role of HbF on pathogenesis and chemotherapy of malaria in SCD patients.

CHAPTER 1: INTRODUCTION

1.1 Background information

Malaria is a prevalent protozoan infectious disease, which is a public health concern in the sub Saharan Africa (Aneni *et al.*, 2013). The World Health Organization (WHO) estimated 207 million cases of malaria in 2012 and 627 000 deaths, 90% of which were in the sub-Saharan Africa. About 482,000 children under 5 years of age die annually, translating to 1,300 children every day or one child every minute (WHO, 2013). Most of these deaths are due to *Plasmodium falciparum* (WHO, 2010; Cullen and Arguin, 2013). The common symptoms include: exhibiting 'uncomplicated' malaria characterized by fever and other symptoms including headache, body aches and malaise, as well as acute and chronic anemia (Allen *et al.*, 1992).

Sickle cell disease (SCD) is a chronic debilitating blood disorder, which is prevalent in sub-Saharan Africa especially in malaria-endemic areas (Makani *et al .,* 2007). Malaria and SCD pose major health problems in Kenya (Aluoch, 1997). The relationship between SCD and malaria is complex (Aluoch, 1997; Newton *et al.,* 1997). Malaria is widely believed to be a major environmental determinant and cause of morbidity and mortality among individuals with SCD in Africa (Konotey-Ahulu and Ringelhann, 1969; Molineaux *et al.*, 1979). Piel *et al*. (2010) indicated that there is clear evidence that malaria and the hemoglobin S gene are intimately connected by the fact that they have similar geographic distributions. Sickle hemoglobin (HbS) in the sickle cell trait (HbAS) is considered protective against malaria (Williams *et al.,* 2005) thus explaining the high frequency of HbS gene

and prevalence in malaria endemic areas (Makani *et al.,* 2007). Sickle cell trait is estimated to reduce malaria admission rates by 70% and is 90% protective against severe complicated malaria (Williams *et al.,* 2005). Similarly, sickle cell trait reduces severe malarial anemia by 60% (Aidoo *et al.,* 2002). The mechanism by which sickle cell trait imparts resistance to malaria is unknown. The homozygous SCD state (HbSS) on the other side is associated with increased susceptibility to malaria (Aluoch, 1997; Newton *et al.,* 1997). The relationship between HbAS or HbSS and malaria is complex, and there are wide research gaps still being investigated.

Fetal hemoglobin (HbF) is the main hemoglobin component throughout fetal life and at birth it accounts for approximately 80% of total hemoglobin in newborns. Fetal hemoglobin is produced starting from the sixth week of gestation and during the rest of fetal life replacing the embryonic hemoglobin Gower I (HBG1) hemoglobin Gower II (HBG) and Portland (Akinsheye *et al.,* 2012). Fetal hemoglobin (α2γ2) is formed by (two α- and two γ-globin chains), consisting of 141 and 146 amino acid residues respectively. The fetal hemoglobin switch begins to take place from the HBG1 and HBG2 (γ-globin) genes to the adult HBB (β-globin) gene (Stamatoyannopoulos, 2005). This switch is normally completed during infancy and typically lasts until approximately 6 months of age. In normal adults, HbF is heterogeneously distributed in a small percentage of erythrocytes referred to as F cells (Boyer *et al.,* 1984; Thein and Menzel, 2009). Fetal hemoglobin makes up to between 60% and 90% of red cell hemoglobin at birth.

This usually decreases to the adult range of 0-2% at 6 months of age, but may also vary (Akinsheye *et al*., 2011). Elevated levels of HbF, usually between 2-5%, have been reported in some cases of hereditary spherocytosis, leukemia, aplastic anemia, megaloblastic anemia, and carcinoma, neoplasia and refractory anemias (Akinsheye *et al.,* 2011). Patients with homozygous α-thalassemia and SCD always have markedly increased levels of HbF ranging from 15-100%.

Fetal hemoglobin gene has been defined as a powerful modulator of the clinical and hematologic features of SCD (Akinsheye *et al*., 2011), where different concentrations of HbF have been shown to be required to protect against various disease complications. Platt *et al.* (1994) describes the ability to live longer given that one has a condition that is debilitating as survival advantage and he further explains that it determines the life expectancy and thereby gives a clear picture in counseling and care given to the SCD patients. Low fetal hemoglobin levels has been described to be one of the risk factors enhancing the complications in SCD patients (Aidoo *et al.*, 2002). Patients with high levels of fetal hemoglobin have been shown to have better survival as compared to those with low HbF levels, since the fetal hemoglobin ameliorates the clinical severity of SCD with major reduction of acute pain crisis, leg ulcers and vasocclusion (Aidoo *et al.,* 2002). This is important to the policy makers and SCD care takers to emphasize on the use of therapeutics that boost the levels of fetal hemoglobin and hence survival advantage. Management of SCD by use of therapeutics that boost the levels of

fetal hemoglobin enhances the life expectancy of the patients up to around 50 years of age (Schnog *et al.*, 2004).

Fetal hemoglobin (HbF) has been described to provide protection against malaria, but this remains unclear (Makani *et al.*, 2007). In SCD patients, high HbF is associated with generally milder but not asymptomatic clinical malaria (Mpalampa *et al.*, 2012). Fetal hemoglobin has been shown to work co-operatively with maternal IgG antibodies to protect infants against severe malaria (Amaratunga *et al*., 2011). Fetal hemoglobin has been shown to impair the cytoadherence of infected red blood cells to micro vascular endothelial cells (MVECs) thereby reducing the sequestration of the parasites and thus reduced parasitemia and severity of malaria manifestations (Amaratunga *et al*., 2011).

Fairhurst *et al.,* (2005) described the role of HbF in malaria resistance: it reduces the expression of *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP-1); this is the major virulence factor i.e. used for cytoadherence in MVECS and iRBCS, by causing an abnormal *Pf*EMP-1 knob display.

The immune response induced in humans by infection caused by malarial parasites is complex and varies depending on the level of endemicity, epidemiological factors, genetic makeup, host age, parasite stage and parasite species. Repeated infection and continuous exposure are required to achieve clinical immunity and later anti-parasitic immunity (Mohan and Stevenson, 1998). Resistance involves genetic-based resistance mechanisms, cell-mediated immunological mechanisms

and specific antibodies, which are able to reduce the severity of the symptoms and mortality.

The baseline data on HbF levels are known in several populations around the world; Nigeria, Saudia Uganda, but there are no studies that have examined the HbF levels in SCD patients in the western region of Kenya or how this may correlate with antibodies to *P. falciparum* malaria antigens and also the chances of survival of the SCD patients in malaria endemic areas. Antibodies have also been demonstrated to act co-operatively with HbF to provide protection against malaria but there is limited information to support these finding. The level of the IgG responses to a panel of up to eleven *P. falciparum* antigens in SCD patients has not been studied in western Kenya. The purpose of the present study was therefore to determine the prevalence and the levels of HbF in SCD patients aged 5-30 years old in a malaria endemic area and also to find out if fetal hemoglobin has any relationship with the IgG responses to multiple *P. falciparum* antigens.

Therefore to answer the above questions the study determined the levels of fetal hemoglobin by alkali denaturation method (Betke method). The classical method for the determination of fetal hemoglobin which is based on the alkali denaturation (Betke *et al.,* 1959). This method relies on the resistance to denaturation by alkali of HbF compared to adult hemoglobin (HbA), the denaturation being activated by the ionization of buried, weakly acidic side chains (one tyrosine and two cysteines) present in HbA and not in HbF (Perutz, 1974). Alkali denaturation is a kinetic test performed only under well standardized conditions in order to obtain reproducible

results; it is fast and also easy to perform (Ducrocq *et al.,* 1998). Using the Multiplex Suspension Array Technology (SAT) referred by the commercial name Bio-plex or Luminex based upon the traditional flow cytometry technology the study established the magnitudes of IgG responses in the study participants.

1.2 Statement of the problem

Fetal hemoglobin (HbF) has been described to provide protection against malaria. However, how it provides protection against malaria infection is has not been described (Makani *et al.*, 2007). The prevalence of HbF in the general population is very low and those with high level of HbF are infants considered to have the trans-placental transferred maternal immunity (Akinsheye *et al*., 2011). A recent study concluded that HbF prevents the growth of *P. falciparum* by impairing the cytoadherence of the infected red blood cells (iRBCs) to micro-vascular endothelial cells (MVECs) and non parasitized RBCs, thereby reducing parasitemia (Amaratunga *et al.,* 2011) but this is yet to be clarified. Antibodies have also been demonstrated to act co-operatively with HbF to provide protection against malaria (Amaratunga *et al.,* 2011), but there is limited information to support these findings. Data on the prevalence and the levels of fetal hemoglobin and IgG responses to sickle cell disease patients in Western Kenya is lacking and therefore, designing a study to test how HbF may be associated with antibodies in providing protection against malaria remains a big challenge.

1.3 Justification of the study

Studies have indicated that SCD patients have a life expectancy of between 40-52 years however most of them die early before the age of five years (Konotey-Ahulu and Ringelhann, 1969). Sickle cell disease patients have HbF persisting in their body system and studies indicate that the high HbF provides survival advantage in the SCD patients (Platt *et al.,* 1994). Malaria is the environmental determinant of SCD (Konotey-Ahulu and Ringelhann, 1969). An association between HbF and antibody responses to protection against malaria has only been demonstrated in infants (Amaratunga *et al.*, 2011). The present study provides an avenue to test the association of HbF with antibody responses to malaria in a malaria endemic area. The approach attempts to overcome the study design challenges of this kind of study by targeting SCD patients who have survived beyond 5 years and are likely to have high level of HbF and limited or no effect of maternal immunity. The design of this study therefore presents the chance to demonstrate the effect of acquired immunity and fetal hemoglobin on the outcome of malaria infections. Considering the potential challenges, the study is designed to relate the levels of HbF and IgG antibody responses to multiple pre-erythrocytic and blood stage *P. falciparum* antigens. Testing the relationship of HbF to the IgG responses to *P. falciparum* antigens will provide evidence that informs both the intervention strategy and malaria vaccine development targeting the SCD population.

1.4 Research questions

i. What is the prevalence and levels of fetal hemoglobin in SCD patients aged 5-30 years in a malaria endemic area in Western Kenya?

ii. What is the effect of SCD on the IgG responses to multiple *P. falciparum* vaccine candidate antigens?

iii. What is the relationship between levels of fetal hemoglobin and the IgG responses to *P. falciparum* vaccine candidate antigens?

1.5 Hypotheses

i. There is no difference in the IgG responses to multiple *P. falciparum* vaccine candidate antigens between the SCD patients and the non-SCD individuals living in a malaria endemic area.

ii. There is no relationship between the levels of HbF and IgG responses to *P. falciparum* vaccine candidate antigens in SCD patients in malaria endemic area.

1.6 Objectives

1.6.1 General objective

To determine the prevalence and the levels of fetal hemoglobin (HbF) and the IgG responses to *Plasmodium falciparum* antigens among sickle cell disease (SCD) patients.

1.6.2 Specific objectives

i. To determine the prevalence and levels of HbF in SCD patients aged 5-30 years in a malaria endemic area in western Kenya.

ii. To determine the IgG responses to multiple *P. falciparum* vaccine candidate antigens in SCD patients in a malaria endemic area.

iii. To determine the relationship between levels of fetal hemoglobin and the IgG responses to multiple *P. falciparum* vaccine candidate antigens in SCD patients.

1.7 Significance of the study

The study sought to establish the prevalence and the levels of fetal hemoglobin in sickle cell disease patients in a malaria endemic area in western Kenya. This will to be used as a guide in designing other studies on SCD in malaria endemic areas. The results of the relationship between antibodies and fetal hemoglobin from the study will provide more insight on the management of both malaria and sickle cell disease thus reduce morbidity and mortality in these patients and also give critical information for therapeutic and vaccine development.

CHAPTER 2: LITERATURE REVIEW

2.1 Historical background

The epidemiology of sickle cell disease serves as a remarkable feature of the distribution of *P. falciparum* malaria. Haldane (1949) in terms of the Darwinian evolution theories, hypothesized that the genetic make-up of an individual is the major determinant of the clinical severity of *P. falciparum* malaria infection. He further demonstrated that both the HbS and the HbAS are frequent in areas of high malaria transmission and that the HbAS individuals are more protected against malaria than HbAA individuals. Malaria and SCD pose a major challenge to both the infectious disease medicine and hematology and is also a public health problem in the sub-Saharan Africa.

2.2 Epidemiology, Transmission and Cause of Malaria

Malaria is a life threatening disease caused by parasites that are entirely transmitted through bites of the female *Anopheles* mosquitoes. The latest estimates are 198 million cases of malaria in the year 2013 and 584,000 deaths (Cullen and Arguin, 2013). The epidemiology of clinical malaria is of great importance as there is need for better clinical definitions of malaria in the evaluation of the control measures such as insect treated bed nets and malaria vaccine. Malaria endemicity historically has been defined in terms of rates of parasitemia or palpable-spleen rates in children 2 to 9 years of age as hypoendemic (< 10%), mesoendemic (11 to 50%), hyperendemic (51 to 75%), and holoendemic (> 75%) (Breman, 2009). The entomologic inoculation rate is the number of infectious

(sporozoite carrying) female anopheline bites per year (EIR), a term used to indicate transmission intensity of a defined area (Breman, 2009).

Malaria in humans is caused by four *Plasmodium* spp and these include *P. falciparum*, *P. ovale*, *P. malariae* and *P. vivax* (Breman, 2009). Among the causative agents of malaria *P. falciparum* is the most pathogenic (Hay *et al.*, 2004). The infection with malaria parasites may result in a wide variety of symptoms, ranging from absent or very mild symptoms to severe disease and even death. Diagnosis of malaria depends on the demonstration of the parasite in the blood by microscopy (Perlmann and Troye-Blomberg, 2002).

2.3 *Plasmodium falciparum* life cycle

Plasmodium falciparum parasite is exclusively transmitted by female anopheles mosquitoes, these are mainly from the members of the anopheles *gambiae* complex (Breman, 2009). The asexual cycle is in the vertebrate host and the sexual cycle is completed in the female anopheles mosquito (CDC, 2012). In humans, the infections are initiated by the injection of sporozoites into the host during a blood meal. The sporozoites are released into the bloodstream and invade the hepatocytes. The Co-receptor on sporozoites that mediate invasion involves in part thrombospondin domain on the circumsporozoite protein (CSP) and on Thrombospondin-related adhesion protein (TRAP), this domain binds specifically to heparin sulphate proteoglycans on hepatocytes. Asexual multiplication during the exo-erythrocytic schizogony leads to the production of merozoites that are

released into the bloodstream to invade the erythrocytes (Figure 2.1). The subsequent stages after schizogony involve distinct morphological phases, these are: the ring stage, which is metabolically inactive for approximately 24 hours, and the trophozoite stage, which is very active and consumes most of the red blood cells. After 4-5 rounds of binary divisions 8-36 new merozoites are produced which burst from the host cell to invade new erythrocytes starting another round of infection (Figure 2.1). The asexual stage where the merozoites are released in the blood stream is responsible for malaria pathogenesis.

The sexual stage occurs when some of the merozoites differentiate into male (microgametes) and female (macrogametes) and are ingested by the mosquito. The flagellated microgametes fertilize the macrogametes to form a zygote that develops into a motile ookinete that penetrates the gut epithelial cells and develops into an oocyst. Sporozoites are then produced after the oocyst undergoes multiple rounds of asexual replication. The rupturing of the mature oocyst releases the sporozoites into the hemocoel of the mosquito where they migrate and invade the salivary gland (Figure 2.1).

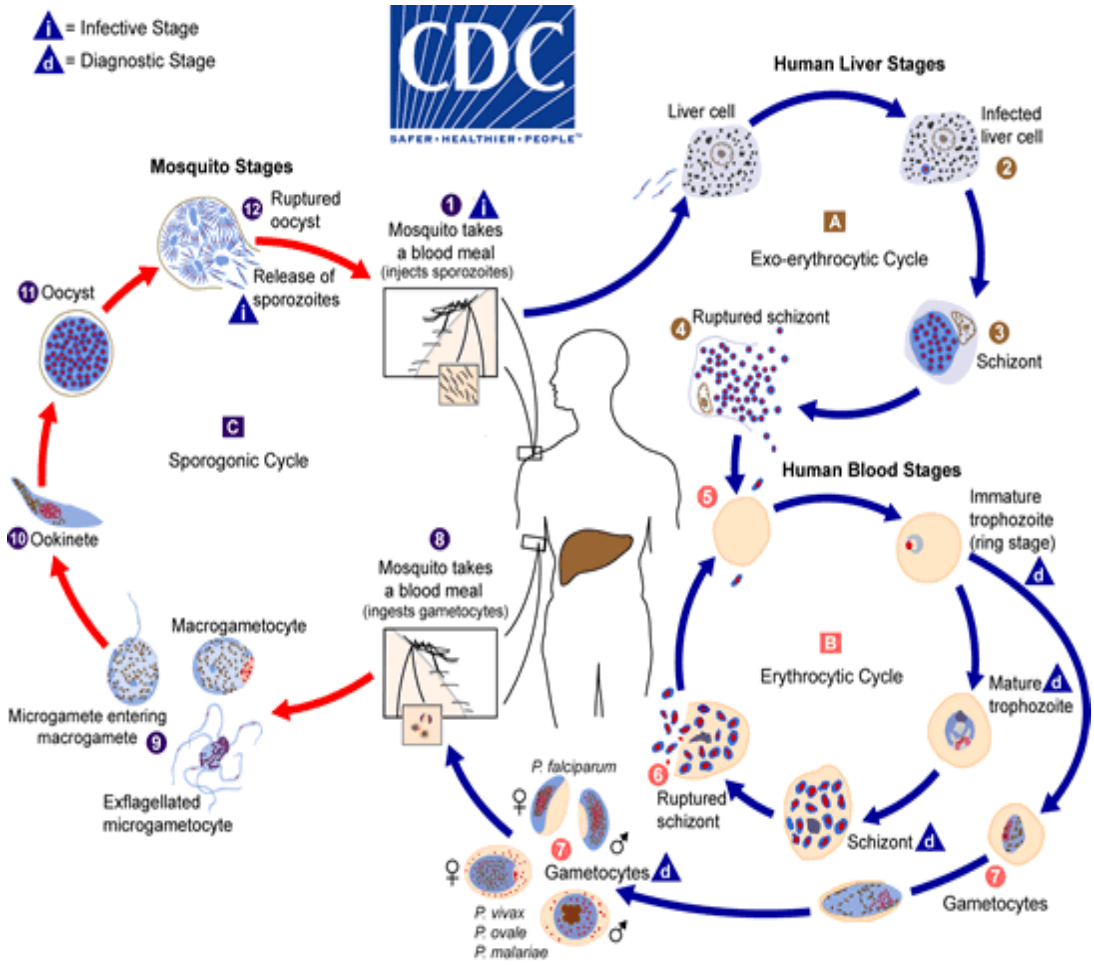

Fig 2.1: Lifecycle of *Plasmodium falciparum* with the vertebrate stages (1-4-Pre-erythrocytic stage and 5-6 as blood stages, 6-7 sexual stage and the mosquito stages 8-12).

Adapted from (CDC, 2012).

2.4 *Plasmodium falciparum* antigens in malaria vaccine development

2.4.1 Pre-erythrocytic stage antigens

Vaccine development targeting this stage of antigens aims to prevent the invasion of hepatocytes by sporozoites and/or prevent liver-stage parasite from developing to maturity (Taylor-Robinson, 2002). The vaccine induces production of

antibodies that block invasion of the sporozoites and induce effector cells that destroy infected hepatocytes directly or via their mediators such as cytokines (Hollingdale and Krzych, 2002) and this reduces the morbidity and mortality by eliminating the parasite load (Nardin *et al.,* 1999).

2.4.1.1 Circumsporozoite protein (CSP)

Circumsporozoite protein (CSP), is the predominant surface antigen on sporozoites (Sinnis and Nardin, 2002). The CSP is composed of three regions; an N terminus that binds heparin sulfate proteoglycans, a four amino acid repeat region N- Acetyl neuraminic Acid Phosphatase (NANP), and a C terminus that contains a thrombospondin-like type I repeat (TSR) domain (Plassmeyer *et al.*, 2009). Vaccine development has majorly focused on the central repeat region that contains the immunodominant B cell epitope. Vaccine constructs have evolved and they incorporate both the central repeat region that contains both the B cell epitopes and the C terminus which has the TSR domain, T cell epitopes, and B cell epitopes (Nardin *et al.,* 2000). The CSP RTS, S/AS01 or mosquirix is the current most effective malaria vaccine and is composed of a portion of the central repeat and the C-terminal regions linked to the hepatitis B surface antigen (Bejon *et al.,* 2008).

2.4.1.2 Liver stage antigen 1 (LSA-1)

Liver-stage antigen 1 (LSA-1) gene encodes a 230-kDa protein which is characterized by a large central repeat region varying in length containing 86

repeats of the 17-amino-acid sequence flanked by two highly conserved N- and C-terminal regions (Fidock *et al.*, 1994). LSA-NRC is a recombinant LSA-1 construct incorporating the N and C terminal regions of the protein and two of the centrally placed 17 amino acid repeats. LSA-1 is exclusively expressed in hepatocytes immediately after the sporozoite has invaded the liver hepatocytes. Hollingdale and Krzych (2002), suggested that LSA-1 is localized within the parasitophorous vacuole as a flocculent material and is separate from the developing parasites; this therefore, suggests its involvement in liver schizogony and merozoite release. Merozoites released from ruptured hepatic schizonts are encased in LSA-1 vacuoles as they traverse through the liver sinusoid into the bloodstream therefore protecting the merozoite (Hollingdale and Krzych, 2002). The non repeat regions in LSA have been shown to have both B- and T- cell epitopes and is also highly conserved and this makes it a target for vaccine design at both B and T cell level (Fidock *et al.,* 1994). Proteins and peptides to LSA-1 have been associated with protection and reduced parasitemia in individuals exposed to either natural or experimental malaria infection (John *et al.,* 2008).

2.4.1.3 Apical membrane antigen-1 (AMA-1)

Apical membrane antigen-1 (AMA-1) is an 83-kDa polymorphic membrane protein expressed in both sporozoites and merozoites. This protein is located in the micronemes, rhoptry organelles of merozoites and is involved in the reorientation and formation of tight-junction that is necessary for invasion of red blood cells by merozoites (Crewther *et al.,* 1990). Other studies have indicated that AMA-1 is a

potential vaccine candidate. This is based on observations in mice and monkeys which upon vaccination with recombinant AMA-1 are protected from parasitemia due to *P. chibaudi* and *P. knowlesi* (Anders *et al*., 1998). Protection is also acquired in the rodents after immunization with recombinant AMA-1 against *P. chabaudi* and *P. yoelii* infections. Further demonstrations indicate that polyclonal anti-AMA-1 antibodies inhibit *in-vitro* merozoite invasion and in this way interfere with the processing of the antigen (Narum *et al.,* 2002). Studies in humans using antibodies to the full- length ectodomain of AMA-1 (FVO and 3D7) have demonstrated the role of AMA-1 in natural immunity against *P. falciparum* malaria (Polley *et al.,* 2004).

2.4.2 Blood stage antigens

A number of proteins have been identified on the merozoite surface and the apical organelles that play a role in RBC invasion and are thought to be targets of immunity. Since clinical symptoms of malaria manifest only during the blood stage, therefore, a vaccine against this stage of the parasite life will prevent or reduce severity and complication of the disease.

2.4.2.1 Merozoite surface protein -1 (MSP-1)

Merozoite Surface Protein-1 (MSP-1) is the most abundant surface component of the merozoite stage of the parasite life cycle, totaling up to 40% of the GPI-anchored merozoite surface protein coat (Sanders *et al*., 2005). MSP-1 is synthesized as a high molecular weight precursor (195-kDa). It is membrane

anchored that undergoes proteolytic processing to yield fragments of several sizes 83, 42, 36, 28 - 30 and 19-kDa (Wipasa *et al*., 2002). MSP-1 plays a role in the binding to and invasion of erythrocytes by merozoites (Cowman *et al.,* 2000). Immune responses to the 83-kDa and 42-kDa fragments have been associated with protection against natural infections in West African children (Riley *et al.*, 1992).

2.4.2.2 Merozoite surface protein-3 (MSP-3)

Merozoite surface protein-3 (MSP-3) is a 48-kDa protein (Oeuvray *et al.,* 1994). It is a polymorphic parasite antigen that may have a role in parasite invasion, which is evidenced by the finding that truncation of the MSP-3 gene reduces parasite invasion of erythrocytes (Mills *et al.,* 2002). Antibodies to the conserved portion of MSP-3 have been shown to mediate antibody dependent cellular inhibition (ADCI) of parasite growth in cooperation with monocytes *in vitro* (Oeuvray *et al.,* 1994).

2.4.2.3 Glutamate rich protein (GLURP)

The *P. falciparum* glutamate-rich protein (GLURP) is a 220-kDa protein expressed both in the pre-erythrocytic and blood stages of the parasite lifecycle, including on the surface of newly released merozoites (Borre *et al.,* 1991). GLURP contains an N-terminal non-repeat region (R0), a central repeat region (R1) and an immunodominant C-terminal repeat region (R2). GLURP is associated with clinical protection against malaria where high GLURP-specific antibody levels are associated with low parasite densities. Additionally, a recent

study of controlled experimental *P. falciparum* infections demonstrated that non-immune individuals acquire GLURP antibodies following a single, brief low-density *P. falciparum* infection. Moreover, it was recently shown that the antibodies produced in response to GLURP in naturally exposed individuals can inhibit the *in vitro* growth of *P. falciparum* with or without co-operation from monocytes, suggesting that GLURP could play an important role in controlling parasitemia.

2.4.2.4 Erythrocyte binding antigen (EBA-175)

Erythrocyte-binding antigen (EBA) is a 175-kDa merozoite expressed protein located in the micronemes; it mediates sialic acid-dependent invasion of red blood cells (RBC) (Sim *et al.,* 1999). It has also been shown to elicit potentially protective antibody responses (Okenu *et al.*, 2000). EBA-175 was the first member of the erythrocyte-binding ligand family characterized and shown to bind to the major glycoprotein found on human erythrocytes; glycophorin A (Narum *et al.*, 2002). Recombinant fragments of EBA-175 are recognized by human sera from malaria endemic areas (Daugherty *et al.,* 1997; McCarra *et al.,* 2011). Additionally, IgG1 antibodies to EBA-175 peptide 4 are associated with protection against clinical malaria (Toure *et al.,* 2006).

2.5 Humoral immunity to malaria

Malaria infection gives rise to host responses that are regulated by both the innate and adaptive immune system as well environmental factors (Langhorne *et al.,* 2008). Acquired immunity is both species and stage-specific, this is non sterile; immunity that wanes from the system i.e. short lived and is associated with low-grade parasitemia and episodes of clinical disease throughout life (Perlmann and Troye-Blomberg, 2002). In malaria endemic areas, the children born to immune mothers are protected against the disease during their first half year of life by maternal IgG (Baird, 1995). The acquisition of active immunity to malaria is slow and requires repeated parasite exposure to be maintained (Doolan *et al.,* 2009). Malaria infection in endemic areas induces strong humoral responses that involves the production of IgM and IgG and other immunoglobulin isotypes. Cohen *et al.*, (1961) suggested that the passive transfer of IgG antibodies from immune individuals to non-immune individuals is protective and thus reduces parasitemia and clinical disease.

Malaria induces both polyclonal and specific IgG production. IgG has been shown to be the most protective with cytophilic antibodies (IgG 1 and IgG3 isotypes) being the most prevalent in protected individuals (Aucan *et al.*, 2001). Antibodies protect against malaria by first inhibiting merozoite invasion of erythrocytes and intra-erythrocytic growth. The antibodies enhance clearance of infected erythrocytes from the circulation by binding to their surface thereby preventing sequestration in small vessels and promoting clearance by the spleen (Cohen *et al*.,

1961). Opsonization of the infected erythrocytes increases their susceptibility to phagocytosis, cytotoxicity and parasite inhibition by various effector cells like neutrophils and monocytes or macrophages which later on induce the production of TNF α which is toxic to the parasite (Greenwood, 2008). The predominant antigens involved in production of IgG are members of highly variant families and this variability enables the parasites to evade immune responses and therefore, constitutes an important virulent factor (Guerin *et al.,* 1987). The variant surface antigens on *P. falciparum* infected erythrocytes are encoded by the multigene variants (Breman, 2009). The gene products called *Pf*EMP-1 are highly variant polypeptides of 200-350-kDa equipped with several binding sites mediating adhesion of infected erythrocytes to the vascular endothelium of capillaries and post capillary venules.

2.6 Sickle Cell Disease

Sickle cell disease (SCD) affects millions of individuals throughout the world, especially in sub-Saharan Africa (Makani *et al.,* 2007). It is the commonest genetic disease worldwide with a prevalence of approximately 5% (WHO, 2011). Sickle cell disease (SCD) is an autosomal recessive genetic condition due to a mutation in the beta-globin gene resulting in the replacement of glutamic acid in position 6 of the beta-globin chain by valine (Ekulu *et al.,* 2013). The mutation results in an abnormal hemoglobin molecule called the sickle hemoglobin (HbS). Sickle cell disease occurs when an individual inherits abnormal hemoglobin genes from both parents. These defective genes are responsible for abnormal red blood cell or red

blood cells with the sickle like structure (Aneni *et al.,* 2013).

Healthy red blood cells usually are smooth and donut-shaped, whereas sickled red blood cells cannot squeeze through small blood vessels but instead they pile up and cause blockages that deprive organs and tissues of oxygen-carrying blood (Makani *et al.,* 2007). This process produces periodic episodes of pain and can damage tissues and vital organs leading to other serious medical problems (Mpalampa *et al.,* 2012). Normal red blood cells live about 120 days in the bloodstream, but sickled red cells die after about 10 to 20 days (Ekulu *et al.,* 2013). Replacement usually is not fast enough resulting to the blood that is chronically short of red blood cells; a condition commonly referred to as anemia. Sickle cell disease patients have been shown to die at an early age especially before they attain the age of five years; however, those who survive have a life expectancy of up to 50 years (Konotey-Ahulu and Ringelhann, 1969).

2.7 Clinical events of SCD

Sickle cell disease is a multisystem disorder affecting multiple organ systems of the body. These clinical events are divided into groups that include; anemia and hematological complications. Chronic hemolytic anemia develops with the synthesis of adult hemoglobin. Anemia also results from the acute splenic sequesteration due to the rapid trapping of the red blood cell in the spleen, which is the major cause of mortality in SCD.

Vasoocclussion is the major cause of painful crises, acute splenic sequesteration and priapism that affects arms, legs, back, abdomen, chest and head. Sickle cell individuals are susceptible to infections with most encapsulated organisms for example *Streptococcus pneumonia* (Boyer *et al.,* 1975) and research has suggested that the SCD individuals be vaccinated with the anti pneumococcal vaccine and be given penicillin as a control to reduce bacteremia (Magnus *et al.,* 1999). Other infections are transfusion transmissible for example hepatitis B.

2.8 Fetal hemoglobin

Fetal hemoglobin (HbF) is produced only in humans, apes and Old World monkeys. Fetal hemoglobin has a stronger affinity for oxygen than adult hemoglobin (HbA), this facilitates oxygen transfer across the placenta from the mother to the fetus during gestation (Sankaran *et al.,* 2010). Hemoglobin production in the red blood cells largely switches from HbF to HbA at the beginning of the final trimester of pregnancy with the fraction of the F cells; the RBCs containing HbF also declining. In most adults, only about 1% of RBCs contain HbF. However, those with Hereditary Persistence of Fetal Hemoglobin (HPFH); defined as a benign condition in which mutations due to deletions in the beta (β-) or gamma (γ-) globin genes or regulatory regions alter normal hemoglobin switching and their red blood cells contain about 10-100% of HbF (Billig *et al.,* 2012).

Fetal hemoglobin is composed of 2 alpha (α-) globin polypeptide chains and 2 γ-globin chains. The γ-globin chains are encoded by 2 nearly identical genes (*HBG2* and *HBG1*) within the β-globin gene-like cluster on chromosome 11p that differ by a glycine or alanine residue at amino acid position γ136 (Akinsheye *et al.*, 2011). In normal adults, HbF is less than 1% of the total hemoglobin and is distributed unevenly among the erythrocytes (Boyer *et al.*, 1975). Increased HbF levels have been reported in adults with the hereditary pathological and SCD conditions (Akinsheye *et al.,* 2011). Fetal hemoglobin is the major genetic modulator of the hematologic and clinical features of SCD (Akinsheye *et al.,* 2011). The genes are genetically regulated and the level of HbF and its distribution among sickle erythrocytes is highly variable.

2.8.1 Fetal hemoglobin determination and quantification

Fetal hemoglobin measurement is clinically useful in the study and diagnosis of some important globin gene disorders where the concentration of HbF varies. Several methods have been developed for the estimation of fetal hemoglobin levels by alkali denaturation (Singer *et al.,* 1951; Betke *et al.,* 1959). Since then there have been increasing demands for a simpler method which is sensitive and accurate enough to measure reproducibly small elevations of fetal hemoglobin in both inherited and acquired hematological disorders. The method described by Betke *et al.* (1959) met the requirements to be the standard technique for fetal hemoglobin quantification. Pembrey *et al.* (1972) described a modification of this method that is simple and gives highly reproducible results in the lower range of

hemoglobin F values. They further reported that it was capable of giving excellent reproducibility, sensitive and accurate readings at 415 nm. However, like other alkali denaturation techniques, it still gives falsely low values of HbF when it rises above 50%.

The alkali denaturation technique of Betke *et al.* (1959) was thus recommended for fetal hemoglobin determination and quantitation with concentrations of at least between 2% and 40%. Radioimmunoassay is used to quantify HbF concentrations below 2% (Garver *et al,* 1976), while chromatographic procedures shown to be more accurate are used when HbF is above 40% (Dozy *et al.,* 1968). However, these methods have been found not to be applicable in smaller hematological laboratories and in developing countries. Moreover, comparison of results of HbF quantitation between different laboratories is difficult because of the variety of modifications of alkali denaturation tests employed.

Since quantitative HbF determination is important for the detection of abnormal hemoglobins and thalassaemia syndromes, International Council for Standardization in Hematology (ICSH) agreed to develop HbF reference preparations to be used for quality control purposes for HbF determination by alkali denaturation methods and to make recommendations for their preparation and use. For quantitative HbF determination, the alkali denaturation method of Betke *et al.* (I959) was recommended as it has the advantage of prior conversion of Hb to hemoglobincyanide (HiCN). However, methods using oxyhemoglobin have been shown not to be suitable for routine use, especially in developing

countries. This is because of the likelihood of spontaneous methemoglobin formation, as methemoglobin-F is alkali labile, in contrast to the alkali stable HiCN-F. The Betke method is known to be accurate in the range of HbF levels between 1% and 12 %, but being difficult to perform for large series of samples, it may thus be efficiently replaced by HPLC which is more convenient to practice (Ducrocq *et al.,* 1998).

2.8.2 Fetal hemoglobin levels

Fetal hemoglobin (α2 γ2) is the main hemoglobin component in the fetus, with about 65-90% at birth and usually drops to less than 2% by 6 to 12 months of age **(**Schwartz *et al.,* 1990). After birth the HbF- gamma-gene switches down and the HbA beta-gene switches on so that adults mainly produce HbA (α2 β2). Low levels of HbF are produced henceforth and distributed heterogeneously within the F cells (Boyer *et al.,* 1975).

Patients with severe inherited disorders of the beta globin chain structure or synthesis, in particular sickle cell anemia and β-thalassemia (β-thal) have unusually high levels of fetal hemoglobin (Wood, 1993). The beta globin chain structure disorder comprises a heterogeneous group of conditions, in which HbF production persists throughout adult life in the absence of hematological abnormalities called hereditary persistence of fetal hemoglobin (HPFH) (Herman and Conley, 1960). The persistent production of variable levels of HbF into childhood and adult life is a characteristic finding in sickle cell anemia and more

severe forms of β-thalassemia. Some patients with SCD have been reported to have high levels of fetal hemoglobin that have been associated with the Senegal and Saudi-Indian haplotype of HBB-like gene cluster (Akinsheye *et al.,* 2011).

Fetal hemoglobin levels are also useful for predicting the clinical severity of sickle cell disease (Charache *et al.,* 1992) where both the laboratory and the clinical features are influenced (Platt, 1994). Patients with higher HbF concentrations have been described to have fewer pain episodes and this has been associated with patients of the African origin (Rogers *et al.,* 1978). Sickle cell disease patients with high HbF levels have less severe clinical course and mild clinical complications because of the increased hemoglobin F that inhibit the polymerization of the sickle hemoglobin (Herman and Conley, 1960). Low levels of HbF have been associated with dactylitis, acute splenic sequestrations, painful crises and chest syndrome (Pembrey *et al.*, 1978; Bailey *et al.,* 1992). The varying levels of fetal hemoglobin in erythrocytes account for a larger part of clinical heterogeneity observed in patients with SCD (Bailey *et al.,* 1992). It is also a major prognostic factor for several clinical complications (Molineaux *et al.,*1979).

The variations in the HbF levels in HbSS patients and others from different localities could be due to common single nucleotide polymorphisms (SNPs) at the BCL11A and HBS1L-MYB loci, which have been implicated previously in HbF level variation in non-anemic European populations (Akinsheye *et al.,* 2012). An association between a BCL11A SNP and HbF levels in a SCD cohort study in the USA has also recently been demonstrated. (Sedgewick *et al.,* 2008) reports that

BCL11A gene is a potential regulator of HbF expression. The prevalence of fetal hemoglobin is of greater importance as described in various studies done in Africa and it has been suggested that the levels of HbF must be part of the initial steps in studying patients with SCD (Mpalampa *et al.,* 2012). The prevalence of SCD in western Kenya Asembo was reported to be 1.6% (Suchdev *et al.,* 2014). However, the prevalence as well as the levels of fetal hemoglobin in these individuals is yet to known.

2.9 Fetal hemoglobin and malaria

Recent studies indicate that there is evidence suggesting that HbF retards the expansion of *P. falciparum* population; the parasites do not survive well in HbF containing red blood cells (Amaratunga *et al*., 2011), but the details and specific mechanism(s) remain unclear. Some of the reasons suggested for this retardation include; HbF containing red blood cell serves as "dead end" where the parasite can only invade but not replicate and neither can it escape and therefore dies within the red blood cell. The growth process is suppressed in HbF-containing RBCs, such that fewer merozoites are released per infected RBC, or they take longer to develop to the point of bursting (Billig *et al.,* 2012). In addition, *P. falciparum* may have a higher affinity for HbF-containing RBCs than HbA-containing RBCs and also have a higher affinity for reticulocytes, present in higher proportions in growing infants. This affects the infection dynamics suggesting that the proportion of HbF-containing RBCs may be a factor in the protection of infants from severe clinical disease and high parasitemia. Thereby slowing

parasite population growth thus providing extra time for an effective response by the infant's developing immune system or maternal antibodies (Billig *et al.,* 2012).

Amaratunga *et al.* (2011) suggested that HbF and maternal IgG act co-operatively to impair the cytoadherence of parasitized RBCs in the first few months of life. Antibodies against MSP-3 and GLURP have been shown to associate with HbF to provide protection against febrile malaria in children aged 0-2 years (Kangoye *et al.,* 2014). In malaria endemic areas an infant's contemporaneous expression of HbC or HbS and development of an immune IgG repertoire may effectively reconstitute the waning protective effects of HbF and maternal immune IgG, thereby extending the malaria resistance of infancy into early childhood. *Plasmodium falciparum* expresses PfEMP-1 cytoadherence proteins and concentrates them in knob-like protrusions on the RBC surface, where they mediate binding to a variety of host cells thus enhancing development of high parasite densities (Fairhurst *et al.,* 2012). Infant susceptibility to *P. falciparum* malaria increases substantially as fetal hemoglobin (HbF) and maternal immune IgG disappear from circulation (Amaratunga *et al.,* 2011). Antibody responses to malaria parasites have been the major focus in most of the recent studies done in areas of both low and high transmission. These studies all focus on vaccine development to eradicate the most deadly disease in the sub-Saharan Africa. Sickle cell disease patients being the most vulnerable population in malaria endemic areas, it serves as the rationale where more research has to be involved (Mpalampa *et al.,* 2012).

CHAPTER 3: MATERIALS AND METHODS

3.1 Study site

The study was conducted at Lwak health center in Asembo, Siaya County, western Kenya. The site lies between Latitudes 0° 10' 60" South and Longitude 34° 22' 60" East (Appendix 1). Asembo covers an area of approximately 200 Km^2 inhabited by a population of about 100,000 people per Km^2 this is according to the Kenya bureau of statistics (Volume 2-Population and Household Distribution by Socio-Economic Characteristics, 2009). The prevalence of SCD in this population is 1.6% as reported by Suchdev *et al*. (2014). However, the prevalence and levels of HbF in the population are unknown. The Luo ethnic group predominantly occupies the area with subsistence farming and fishing as their main source of livelihood. The housing types included both the semi-permanent and temporary structures consisting mainly of grass thatched and mud walled structures. Intense malaria transmission occurs in this region throughout the year, peaking in the rainy seasons, these are May to June (Ototo *et al.,* 2015).

3.2 Study participants and enrolment

Sickle cell disease patients aged 5-30 years attending the clinic during the study period were eligible. The homozygous sickle cell disease (HbSS) patients already registered at the health center were confirmed by PCR. Other patients referred from the peripheral health facilities were also tested and their HbSS status confirmed before being included in the study. The HbSS status of non-SCD

individuals aged 5-30 years from the same population was also confirmed by PCR. Each study participant was given a unique study identification number.

3.3 Sample size

The study recruited 100 SCD patients, 20% of the proposed sample size was expected to have a high prevalence of HbF. The detectable effect sizes or odd ratio of 1.2 (a two-tailed test) were obtained at 80% power and α (false-positive rate) of 0.05. The proposed sample size gave a detectable effect size of 1.2 for HbF to antibody responses in the context of prevalence of antibodies. Seventy (70) individuals without SCD from the same location were also randomly selected for the control group (Singh and Masuku, 2014).

3.4 Inclusion and exclusion criteria

Sickle cell disease patients and non-SCD individuals aged 5-30 years from Luo ethnic group and those who consented were included. However those who had blood transfusion within a period of four months, those with chronic infections, the severely anemic and also those on hydroxyurea were excluded from the study.

3.5 Study design

This was a cross-sectional study using 5-30 years old randomly recruited 100 SCD patients and 70 non-SCD individuals from the same study area. Blood samples were obtained from both the SCD patients and the non-SCD individuals. Approximately, 200 μL of blood was used for the PCR confirmatory test. For

those confirmed to be SCD positive, 6 ml of blood was withdrawn. The collected blood samples were then used to determine both the levels of fetal hemoglobin and for the testing and the quantification of IgG responses to a panel of 11 recombinant *P. falciparum* antigens. Approximately 500 µL of the blood from the SCD patients was used for determining the HbF levels and 1 ml of plasma used for antibody testing and quantification. Approximately 3 ml of blood was withdrawn from the study participants who were SCD negative and used for antibody testing and quantification.

3.6 Laboratory procedures

3.6.1 Microscopy for malaria testing

Thick and thin blood films were made for malaria microscopy. The blood smears were stained using 5% Giemsa solution at pH 7.2. Parasites in the thick smears were counted against 200 white blood cells (WBCs) and the counts recorded for *Plasmodium* spp asexual forms and gametocyte. Individuals diagnosed with malaria were referred for treatment at the local health centers.

3.6.2 Determination of fetal hemoglobin levels

Two independent tests were carried out to determine the levels of fetal hemoglobin in the SCD patients and a third test was carried our when the difference between test 1 and 2 was greater than five. The test relies on the resistance to denaturation of the fetal hemoglobin by alkali being activated by the buried, weakly acidic side chains of the hemoglobin molecule (Betke *et al.,* 1959). Blood samples in EDTA

vacutainers was centrifuged at 2500X g for ten minutes to obtain the hemolysate. The hemoglobin contained in the hemolysate was first transformed into a more stable form of cyanmethemoglobin by adding 1.3 ml of Drabkins solution to the hemolysate, this was left to stand for 5 minutes after which, 50 µL of 1.2 N Sodium hydroxide was added to 2.8 ml of cyanmethemoglobin. The reaction was then stopped immediately after 2 minutes by addition of 0.5 ml of saturated ammonium sulphate and then filtered. The optical density for the filtrate and the control solution were measured at 540 nm on a spectrophotometer ELISA reader, (Molecular Devices Sunnyvale, CA). The % of alkali resistant hemoglobin was derived from the following formula:

$$\% \text{ HbF level} = \frac{\text{OD 540 nm of test solution}}{\text{OD 540 nm of control solution} \times 20} \times 100$$

Fetal hemoglobin levels greater than ten percent (>10%) are defined as high level of fetal hemoglobin, while those with less than ten percent (<10%) are defined as low levels of fetal hemoglobin (Mpalampa *et al.,* 2012).

3.6.3 Antibody testing and quantification

Antibody testing and quantification was carried out in the University of Minnesota/KEMRI Laboratories, Kisumu. The volume of working solution (50 µL / well) was determined together with the number of beads that would result in 1,000 beads/region/well or 5,000 beads/region/well. Coupled bead stocks, were

then combined in a 15 ml amber conical tube and diluted with PBNT to result in 100 microspheres/ µL, the working bead solution. Millipore microtiter 96-well plates (MABVN 1250, Millipore Corporation, Billerica, MA) were pre-wetted with 100µL of PBNT/well and aspirated using a millipore vacuum manifold. Fifty µL of the working bead solution was transferred to it. Fifty µL of diluted plasma was added to each of the well of the microtiter plate. The plasma was mixed with the beads three times by pipetting up and down. The plates were incubated in the dark on a shaking microplate shaker (IKA® MTS, Wilmington, NC) at 600 rpm for 30 sec, followed by 300 rpm for 30 min. Plates were aspirated using a millipore vacuum manifold and washed twice with 100 µL /well of PBNT before the beads were resuspended in 50 µL PBNT by pipetting. Fifty µL of diluted (1: 100) goat antihuman IgG gamma-chain specific, F (ab`) 2 fragment-R-phycoerythrin (Sigma, P-8047 St. Louis, MO) in PBNT was added to each well, and incubated in the dark on a shaking microplate shaker at 600 rpm for 30 sec, followed by 300 rpm for 30 min. Plates were then aspirated using a millipore vacuum manifold and washed twice with 100 µL/well PBNT. The beads were finally resuspended in 100 µL PBNT by pipetting and analyzed on bioplex^{200} machine (Hercules, CA).

The reader was set to read a minimum of 100 beads with a unique fluorescent signature/region and the results expressed as median fluorescence intensity (MFI) on bioplex manager software Version 5.0 (Bio-rad). For each plasma sample, the threshold value (mean+3 standard deviations) was determined using MFI values

from 18 malaria-naïve North American adult plasma samples. Seropositivity was established when the median florescence intensities were divided by mean +3SD of the naive North American MFI values to get the arbitrary units; individuals with arbitrary units above one (AU>1) were considered as sero-positive or responders while those with arbitrary unit less than one (AU<1) were considered as sero-negative or non responders.

3.6.4 PCR and HbSS typing

3.6.4.1 Genomic DNA extraction

The blood samples collected in EDTA tubes were centrifuged at 2500X g for 10 minutes to get the buffy coat collected in micro-centrifuge tube. Genomic DNA was obtained using the QIAamp 96 DNA blood kit (Qiagen, Valencia, CA). DNA was extracted by pipetting 20 µL QIAGEN protease (proteinase K) into the bottom of a 1.5 ml micro-centrifuge tube and 200 µL buffy coat added. This was followed by addition of buffer AL, mixed by pulse vortexing and incubated at 56 ^{o}C for 10 minutes. Absolute ethanol (96-100%) was then added and pulse vortexed for 15 seconds. The mixture was applied into the QIAamp mini spin column and centrifuged at 6000X g for 10 minutes after which the filtrate was discarded. DNA adhered to the spin column was washed with 500 µL of buffer (wash buffer 1 and 2), centrifuged at 6000X g for one minute, and 20000X g for 3 minutes respectively. Finally, 200 µL of the elution buffer was then added and incubated at room temperature for 1 minute before centrifuging at 6000X g for 1 minute. The

extracted DNA was quantified using the nanodrop 2000 spectrophotometer (Inquaba biotec) and stored at -20º C.

3.6.4.2 Amplification and digestion of genomic DNA

The extracted genomic DNA fragment (457 base pairs) 10-100 ng from each sample were amplified using PCR. The forward primers 5'GATATATCTTAGAGGGAGGGCTGAG-3' and reverse primers AGACCAATAGGCAGAGAGAGTCAG-3' 1 µL each premixed with dNTPs, with 12.5 µL Gotaq DNA polymerase (Promega, USA) and 2 µL template DNA formed a final volume of 25 µL PCR reaction mixture. The PCR amplification reactions were performed in a 96- well plate thermocycler (My Cycler ^{TM} Bio rad, USA) with the following settings: Initial denaturation at 94 ºC for 2 minutes, denaturation 94 ºC for 30 seconds, annealing at 62 ºC for 30 seconds, extension at 72 ºC for 30 seconds and final extension at 72 ºC for 5 minutes and the PCR amplicons were then purified and used for the digestion, 10 µL of the digestion master mix containing 1 µL of Neb3 buffer, DdeI enzyme (NEB, UK Ltd) and PCR water was aliquoted in PCR tubes after which the 10 µL of purified DNA amplicons were added and centrifuged at 1200 rpm for 2 minutes and then incubated in a thermocycler with the following reaction conditions: incubation at 37 ºC for 8 hours, inactivation for 65 ºC for 20 minutes and final hold at 4 ºC. The digested products were loaded on a 2% agarose gel for visualization with a UV transilluminator.

3.7 Statistical analysis

Statistical analyses were performed using Graph Pad Prism 6 (Graph Pad Software, La Jolla, CA). The prevalence and levels of fetal hemoglobin were reported in terms of mean, median and range. Unpaired t tests was used to compare the mean fetal hemoglobin levels in females and males and in the different age groups; it was also used to compare the mean IgG responses to both the pre-erythrocytic and blood stage *P. falciparum* antigens in the SCD patients and the non-SCD individuals when categorized into age groups of 5-10 and 11-30 years and also into males and females. Chi-square test was used to compare the proportions of responders and non-responders both in SCD patients and non-SCD individuals. Spearman's rank correlation and linear regression was used to establish the relationship between the levels of fetal hemoglobin and the IgG responses to the multiple *P. falciparum* antigens. The multivariate regression analysis was used for testing the model of association between age, HbF and the IgG responses to *P. falciparum* antigens. In all cases the P value <0.05 was considered significant.

3.8 Ethical considerations

All procedures in this study were carried out in accordance with international guidelines for the protection of human subjects. Ethical approval was obtained from Maseno University Ethical Review Committee and research permit by NACOSTI (Appendix V and VII). Informed assent and consent forms for individuals aged above 13-17 years and those below and above 18 years

respectively were obtained and signed (Appendix II and III). The study participants were free to withdraw consent at anytime.

CHAPTER 4: RESULTS

4.1 Demographic characteristics

The study population comprised of 100 SCD patients and 70 non-SCD individuals aged 5-30 years. Study individuals were categorized into two age groups namely 5-10 years (61%) and 11-30 years (39%). The overall mean age was 10.75 years. The females were 53% with a mean age of 10.28 years while the males were 47% with a mean age of 11.28 years (5-30). There was no significant difference in the mean age between males and females ($P > 0.05$). The population was made up of small-scale farmers who mostly depend on fishing as their main source of livelihood. The housing types included both the semi-permanent and temporary structures consisting mainly of grass thatched and mud walled structures.

4.2 Prevalence and levels of fetal hemoglobin in SCD patients

For the SCD patients, the fetal hemoglobin levels were determined and quantified using Betke method. A high HbF level was defined as having a value higher than 10% while those with less than 10% were categorized to have low level of HbF values. The SCD patients with high level of fetal hemoglobin (HbF > 10%) were 77 % (n=77) while 23% (n=23) had low level of fetal hemoglobin (Hb F<10%; Table 4.1). The males with high HbF were 74.46% (n=35) with a mean fetal hemoglobin of 25.17% while the females with high HbF were 79.25% (n=42) with a mean of 21.49%, however, there was no significant difference between the HbF levels in males and females ($P > 0.05$; Table 4.1). When HbF values were compared in relation to the gender, males with low HbF were 25.53% (n=12) with

a mean fetal hemoglobin of 5.54% while the females with low HbF were 20.75% (n=11) with a mean fetal hemoglobin of 5.25%. There was no significant difference between the HbF levels in males and females (P > 0.05; Table 4.1).

Table 4.1: Prevalence of low and high fetal hemoglobin (HbF) in Sickle cell disease patients by gender

	Gender					
	Males		**Females**		**N**	
Levels	**n**	**Mean HbF %**	**n**	**Mean HbF %**		**P value**
<10%	12	5.54	11	5.25	23	0.73[a]
>10%	35	25.17	42	21.49	77	0.06[b]
Total N (%)	47		53		100	

N= number of individuals,
[a] = Males vs females with low HbF
[b] = Males vs females with high HbF
Total N (%) - number of both males and females
HbF - Fetal hemoglobin
P< 0.05 significant, Unpaired T- test

4.2.1 Levels of Fetal hemoglobin in sickle cell disease patients

The Mean ± SEM HbF levels for the study population was 19.08% ± 1.066 with a median of 18.33 and range of 1.44-56.25% respectively with the 25^{th} and 75^{th} percentile of 11.78% and 26.29% respectively (Table 4.2). Females (n=53) demonstrated a high level of fetal hemoglobin with a Mean ± SEM of 19.19 % ±1.456 with a range of 2.15-45.88%. The males (n=47) had a Mean ±SEM of 18.95% ±1.582 with a range of 1.44-56.25%. However, the difference was not significant ($P > 0.05$; Table 4.2). The males aged 5-10 years had mean HbF level of 16.5% but as age increased the mean HbF level increased to 22.05%. This however, was not significant ($P > 0.05$). In contrast the females demonstrated a decrease in fetal hemoglobin levels where at age 5-10 years the mean level of fetal hemoglobin was 19.42% whereas at age 11-30 years the mean was 18.68 %. However, the difference was not significant ($P > 0.05$; Table 4.2).

Table 4.2: Fetal hemoglobin levels in sickle cell disease patients

Gender	Age (Years)	N (%)	Mean HbF	P value
Males	5-10	26 (55.3%)	16.5	0.07[a]
	11-30	21 (44.7%)	22.05	
Females	5-10	37 (69.8%)	19.42	
	11-30	16 (30.1%)	18.68	0.82[b]
Males vs Females	5-30	47(47%)	18.95	
		53(53%)	19.19	0.91[c]

P value < 0.05 significant, Unpaired T test
[a]= Males age 5-10 vs 11-30 years
[b]= Female age 5-10 vs 11-30years
[c] =All females vs all males
HbF - Fetal hemoglobin

4.3 IgG levels and seropositivity in sickle cell disease patients and non-sickle cell disease individuals.

4.3.1 Prevalence of IgG responses to Pre-erythrocytic *Plasmodium falciparum* antigens

The IgG responses to the eleven *P. falciparum* antigens were varied among the SCD patients and non-SCD individuals (Figure 4.1). Pre-erythrocytic antigens demonstrated more than 80% of the seropositive individuals, where AMA-1-3D7 had the highest seropositive SCD patients 95% (n=100) in SCD patients and 100% (n=70) in non-SCD. In contrast, CSP had a highest number of seronegative individuals both in SCD patients at 60% (n=100) and in non-SCD individuals at 50% (n=70; Figure 4.1). Seropositive SCD patients were more than the non-SCD

individuals only in LSA-NRC with 92%. Other antigens like AMA-1-FVO, AMA-1-3D7 and CSP had high IgG responses in non-SCD than in the SCD patients (Figure 4.1).

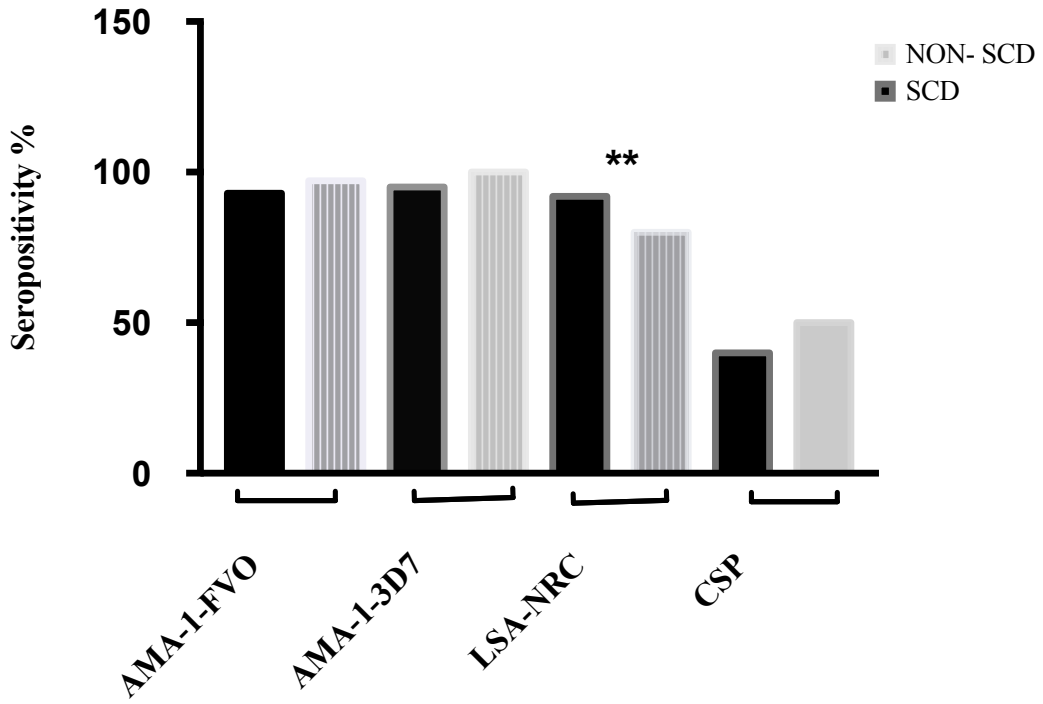

Figure 4.1: IgG responses to pre-erythrocytic *P. falciparum* antigens in SCD patients and non-SCD individuals with % seropositivity.

**Significant

4.3.2 Prevalence of IgG responses to blood stage *Plasmodium falciparum* antigens

The blood stage antigens also demonstrated high number of responders with more than 75% per antigen both in SCD patients and non-SCD individuals. There was a higher number of seropositive individuals to *P. falciparum* antigens GLURP-R2,

MSP-3-FVO and EBP-2 in SCD patients than in non-SCD individuals, while MSP-1-42-FVO, MSP-1-42-3D7, EBA-175 and GLURP-R0 had higher levels of seropositive non-SCD individuals than the SCD patients (Figure 4.2). GLURP-R2 had the highest number of seropositive SCD patients 94% (n=100), while GLURP-RO had the lowest seropositive SCD patients 64% (Figure 4.2).

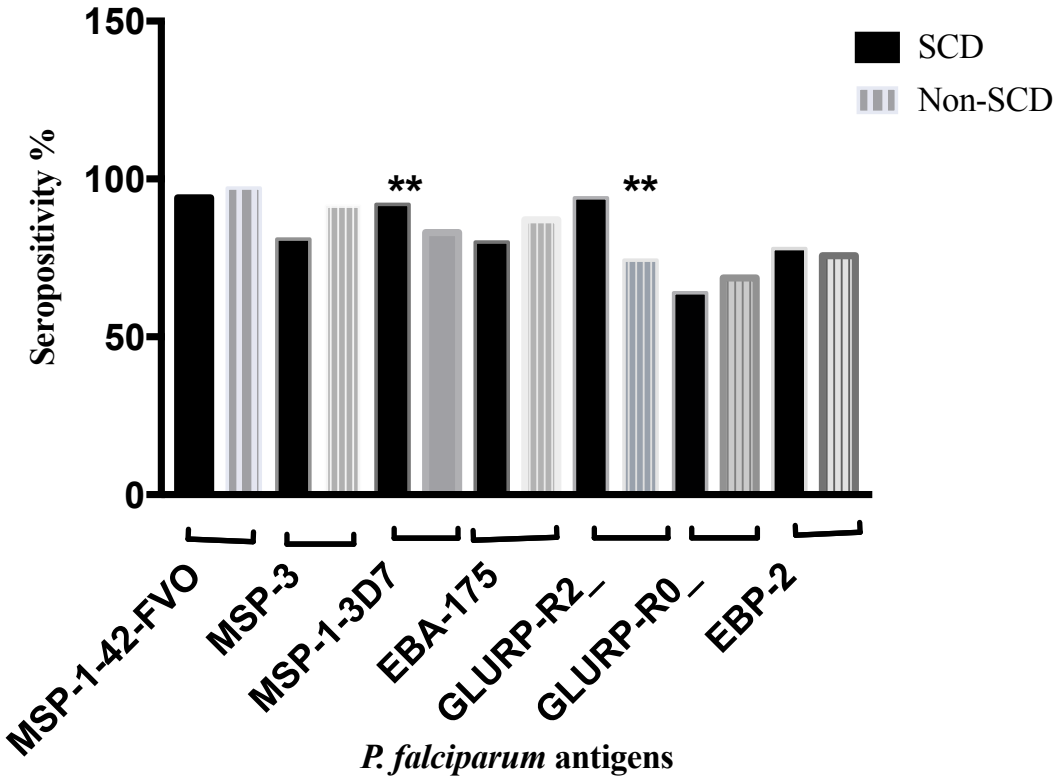

Figure 4.2: The IgG responses to blood stage *P. falciparum* antigens in SCD patients and non-SCD individuals with % seropositivity.

**Significant

4.3.3 IgG responses in seropositive sickle cell disease patients and non-sickle cell disease individuals

When the proportion of seropositive individuals in SCD and non-SCD were compared using chi square the difference in the numbers was expressed differently in each antigen where MSP-3, AMA-1-3D7, GLURP-R2 and LSA-NRC showed a significant difference between the sickle cell disease patients and non sickle cell disease individuals ($P< 0.05$; Table 4.3) respectively. Other antigens like AMA-1-FVO, MSP-1-3D7, MSP-1-FVO, EBA-175, GLURP-R0 and EBP-2 were not statistically different between the SCD and non-SCD individuals ($P> 0.05$; Table 4.3).

Table 4.3: Proportion of responders in both sickle cell disease patients and non sickle cell disease individuals in a population from MEA

P. falciparum antigens	SCD Responders N=100	Non-SCD Responders N=70	χ^2	P value
AMA-1-FVO	93 (93)	68 (97.1)	1.41	0.23
MSP-1-42-FVO	94 (94)	68 (97.1)	0.91	0.34
MSP-3-FVO*	81 (81)	64 (91.4)	3.57	0.05
MSP-1-42-3D7	92 (92)	58 (82.9)	3.32	0.06
AMA-1-3D7	95 (95)	70 (100)	3.61	0.05
EBA-175	80 (80)	61 (87.1)	1.49	0.22
GLURP-R2*	94 (94)	52 (74.3)	13.2	0.03
GLURP-R0	64 (64)	48 (68.6)	0.38	0.53
EBP-2	78 (78)	53 (75.7)	0.12	0.72
LSA-NRC-3D7	92 (92)	56 (80)	5.26	0.02
CSP	40 (40)	35 (50)	1.67	0.19

*Significant, Chi square test, N- number of individuals
SCD-Sickle cell disease, AMA-Apical membrane antigen, MSP-Merozoite surface protein, EBA-Erythrocyte binding antigen, GLURP-Glutamate rich protein, LSA-Liver stage antigen, EBP-2 erythrocyte binding protein, CSP-Circumsporozoite protein

4.3.3.1 IgG responses to Pre-erythrocytic *P. falciparum* antigens in seropositive sickle cell disease patients and non-sickle cell disease individuals

The mean IgG levels in seropositive SCD patients and non-SCD individuals were varied. There was a statistical difference in the overall mean IgG levels to the pre-

erythrocytic *P. falciparum* antigens between the SCD patients and non-SCD individuals with the non-SCD mean IgG levels being higher than in the SCD patients (P< 0.05) with LSA-NRC demonstrated high IgG levels in the SCD than the non-SCD individuals (P< 0.05; Table 4.4). The antigen LSA-NRC had the highest mean IgG level and range in SCD patients (14.35, 1.17-40.34; Table 4.4), while AMA-1-3D7 had the highest mean IgG level and range in non-SCD individuals (44.47, 1.76-88.71; Table 4.4). All the pre-erythrocytic antigens demonstrated a statistical difference between the seropositive SCD patients and non-SCD individuals (P< 0.05), however, CSP was not statistically different between SCD patients and non-SCD individuals (P> 0.05; Table 4.4).

Table 4.4: IgG levels in seropositive SCD patients and non-SCD individuals

Pre-erythrocytic *P.falciparum* antigens	**SCD Mean IgG levels AU>1 (Range) N=100**	**Non-SCD Mean IgG levels AU>1 (Range) N=70**	**P value**
AMA-1-FVO	10.10 (1.00-32.15)	27.63(1.01-61.73)	<0.0001
LSA-NRC-3D7*	14.35 (1.17-40.34)	6.28 (1.12-21.39)	<0.0001
CSP	2.70 (1.07-10.42)	2.86 (1.02-12.70)	0.7
AMA-1-3D7**	10.48 (1.01-30.91)	44.47 (1.76-88.71)	<0.0001
Mean IgG levels	**10.51**	**23.77**	**<0.0001**

AU- Arbitrary Units
* Highest IgG level in SCD
** Highest IgG level in non-SCD
AMA-Apical membrane antigen, LSA-Liver stage antigen, CSP-Circumsporozoite protein
N- Number of individuals

4.3.3.2 IgG responses to blood stage *P. falciparum* antigens in seropositive sickle cell disease patients and non-sickle cell disease individuals

The mean IgG levels in seropositive SCD patients and non-SCD individuals were varied. There was no statistical difference in the overall mean IgG levels to the blood stage *P. falciparum* antigens between the SCD patients and non-SCD individuals (P> 0.05; Table 4.5). The antigen MSP-1-42-FVO had the highest mean IgG level and range both in SCD patients and non-SCD individuals (33.37, 2.98-55.50 and 28.78, 1.19-102.9; Table 4.5) respectively. All the blood stage antigens demonstrated no statistical difference between the seropositive SCD patients and non-SCD individuals (P> 0.05), however, MSP-3-FVO was statistically different between SCD patients and non-SCD individuals (P< 0.05; Table 4.5).

Table 4.5: IgG levels in seropositive SCD patients and non-SCD individuals

Blood stage *P. falciparum* antigens	SCD Mean IgG levels AU>1 (Range) N=100	Non-SCD Mean IgG levels AU>1 (Range) N=70	P value
MSP-1-42-FVO**	33.37 (2.98-55.50)	28.78 (1.19-102.9)	0.13
MSP-3-FVO*	6.53 (1.07-40.01)	8.96 (1.14-39.33)	0.05
MSP-1-3D7	14.41 (1.18-24.27)	13.24 (1.47-29.77)	0.36
EBA-175	7.57 (1.06-22.27)	9.07 (1.09-27.80)	0.22
GLURP-R2	17.03 (1.13-36.77)	14.78 (1.11-45.74)	0.28
GLURP-R0	8.43 (1.04-25.98)	7.95 (1.11-55.17)	0.77
EBP-2	3.62 (1.03-10.03)	4.06 (1.07-12.30)	0.31
Mean IgG levels	**13.73**	**12.91**	**0.36**

P< 0.05 significant, Unpaired T test
AU Arbitrary Units
SCD –Sickle cell disease
* Statistically different in SCD and non-SCD
** Highest IgG level in SCD and non-SCD
MSP- Merozoite surface protein, EBA-Erythrocyte binding antigen, GLURP-Glutamate rich protein, EBP-Erythrocyte binding protein
N- Number of individuals

4.3.4 IgG levels in sickle cell disease patients and non sickle cell disease individuals

The MFI values from the SCD and non-SCD groups were log transformed and the means compared using unpaired t-test. The SCD patients generally exhibited higher levels of IgG responses to each of the eleven *P. falciparum* antigens as compared to the non-SCD group (Table 4.6 and Table 4.7). However, when

grouped to the respective parasite asexual stages such as pre-erythrocytic and blood stage antigens, the mean IgG responses for the pre-erythrocytic antigens for both the SCD patients and the non-SCD individuals were not statistically different ($P > 0.05$; Table 4.6). The SCD patients had a higher mean IgG response of 3.59 while the non sickle cell disease had a mean IgG response of 3.54. Separate pre-erythrocytic antigens like LSA-NRC, CSP and AMA-1-3D7, demonstrated significant difference between the SCD patients and the non-SCD individuals ($P < 0.05$), with AMA-1-FVO and AMA-1-3D7 demonstrating high IgG levels in the non-SCD individuals. However, AMA-1-FVO was not statistically different ($P > 0.05$; Table 4.6).

Table 4.6: The IgG levels (Log MFI) to pre-erythrocytic antigens in sickle cell disease patients and non-sickle cell disease individuals

	GROUPS		
	SCD N=100	NON SCD N=70	
Pre-erythrocytic antigens	**Mean ±SEM**	**Mean ±SEM**	**P value**
LSA-NRC-3D7	3.69±0.06	3.33±0.06	<0.0001
CSP	3.17±0.05	2.93±0.06	0.01
AMA-1-3D7	3.79±0.05	4.00±0.05	0.01
AMA-1-FVO	3.75±0.05	3.88±0.06	0.09
Mean IgG response	**3.59±0.02**	**3.54±0.04**	**0.19**

P< 0.05 significant; Unpaired T test

MFI- median fluorescence intensity

SEM Standard error of the mean, AMA-Apical membrane antigen, LSA-Liver stage antigen, CSP- Circumsporozoite protein

N- Number of individuals

The mean IgG response to blood stage antigens was significantly different in the SCD patients when compared with the non-SCD individuals (P< 0.05; Table 4.7). The SCD patients exhibited higher mean IgG levels 3.63±0.03 (n=100) than the non- SCD individuals with a mean IgG level of 3.30±0.03 (n=70). Antigens like MSP-1-42-3D7, GLURP-R2, GLURP-R0 and EBP-2 were the blood stage antigens that demonstrated significant difference in the SCD patients when compared with the non-SCD individuals (P< 0.05), with SCD demonstrating higher responses in all these antigens than the non-SCD individuals. In contrast

MSP-3 and EBA were not significantly different in the SCD when compared with the non-SCD (P> 0.05; Table 4.7).

Table 4.7: IgG levels (Log MFI) in *P. falciparum* blood stage antigens in sickle cell disease patients and non sickle cell disease individuals

	GROUPS		
	SCD N=100	NON-SCD N=70	
Blood stage antigens	**Mean ±SEM**	**Mean ±SEM**	**P value**
MSP-3-FVO	3.09±0.05	3.08±0.07	0.8
MSP-1-42-3D7	4.01±0.05	3.60±0.07	<0.0001
EBA-175	3.55±0.06	3.44±0.07	0.21
GLURP-R2	3.89±0.06	3.25±0.09	<0.0001
GLURP-R0	3.39±0.07	2.86±0.07	<0.0001
EBP-2	3.83±0.05	3.57±0.06	0.006
Mean IgG response	**3.63±0.03**	**3.30±0.03**	**<0.0001**

P< 0.05 Significant, Unpaired T test
MFI- Median fluorescent intensity
SEM- Standard error of mean
MSP- Merozoite surface protein, EBA-Erytrocyte binding antigen, GLURP-Glutamate rich protein, EBP-Erythrocyte binding protein
N- Number of individuals

4.3.5 IgG responses in males and females in sickle cell disease patients and non- sickle cell disease individuals

4.3.5.1 IgG responses to pre-erythrocytic *P. falciparum* antigens in males and females with sickle cell disease

Generally the males exhibited a higher IgG response than the females to each of the pre-erythrocytic antigens with a Mean IgG level of 3.65 and 3.55 respectively (Table 4.8). However, these IgG responses to the *P. falciparum* pre-erythrocytic antigens showed no statistical difference (P> 0.05; Table 4.8).

Table 4.8: IgG responses to *P. falciparum* pre-erythrocytic antigens in males and females in SCD patients

	GENDER		
Pre-erythrocytic antigens	**Mean IgG (Log MFI) Females (N=53)**	**Mean IgG (Log MFI) Males (N=47)**	**P value**
LSA-NRC	3.65 ±0.06	3.73±0.06	0.45
CSP	3.16±0.06	3.18±0.06	0.84
AMA-1-FVO	3.67±0.07	3.85±0.06	0.07
AMA-1-3D7	3.7±0.07	3.85±0.06	0.13
Mean IgG responses	**3.55±0.06**	**3.51±0.06**	**0.55**

P <0.05 significant; Unpaired T test
AMA-Apical membrane antigen, LSA-Liver stage antigen, CSP-Circumsporozoite protein
N- Number of individuals

4.3.5.2 IgG responses to blood stage *P. falciparum* antigens in males and females with sickle cell disease

The IgG response to the blood stage antigens was expressed differently in both males and females with SCD (Table 4.9). There was no significant difference between males and females in all the antigens. However, the mean IgG response between the males and females was significantly different with the males having higher mean IgG levels than the females (P< 0.05; Table 4.9).

Table 4.9: IgG responses to *P. falciparum* blood stage antigens in males and females in SCD patients

	GENDER		
Blood stage antigens	**Mean IgG (Log MFI) Female (N=53)**	**Mean IgG (Log MFI) Males (N=47)**	**P value**
MSP-3	3.18±0.07	3.02±0.07	0.12
MSP-1-3D7	4.07±0.07	3.96±0.07	0.26
EBA-175	3.53±0.07	3.58±0.08	0.65
GLURP-R2	3.82±0.08	3.97±0.07	0.19
GLURP-R0	3.49±0.10	3.29±0.09	0.16
EBP-2	3.82±0.06	3.84±0.07	0.78
Mean IgG responses	**3.57±0.03**	**3.69±0.03**	**0.02**

P< 0.05 significant; Unpaired T test
MFI- Median fluorescent intensity
MSP- Merozoite surface protein, EBA-Erytrocyte binding antigen, GLURP-Glutamate rich protein, EBP-Erythrocyte binding protein
N- Number of individuals

4.3.5.3 IgG responses to pre-erythrocytic *P. falciparum* antigens in males and females in sickle cell disease and non-sickle cell disease individuals

The IgG response to pre-erythrocytic antigens in females of both the SCD and non -SCD was compared for each antigen, there was a significant difference in all the three antigens AMA-1- 3D7, LSA-NRC and CSP ($P< 0.05$). However, AMA-1-FVO was not statistically different between the SCD and non-SCD individuals, the mean IgG responses for all the pre-erythrocytic antigens in SCD females were not significantly different from the non-SCD ($P> 0.05$; Table 4.11). In the males CSP and LSA-NRC showed a significant difference ($P< 0.05$), while AMA-1-FVO and AMA-1-3D7 showed no statistical difference when the SCD patients were compared with the non-SCD individuals, the mean IgG responses for all the pre-erythrocytic antigens in males was not statistically different between the SCD and non-SCD individuals ($P> 0.05$; Table 4.10).

Table 4.10: The IgG responses to *P. falciparum* pre-erythrocytic antigens in males and females in SCD patients and non-SCD individuals

		GROUPS		
		SCD	NON-SCD	
Gender	**Pre-erythrocytic antigens**	**Mean IgG (Log MFI)**	**Mean IgG (Log MFI)**	**P value**
Males	LSA-NRC	3.73±0.08	3.34±0.06	0.005
	CSP	3.18±0.06	2.97±0.07	0.03
	AMA-1-FVO	3.85±0.09	3.89±0.07	0.62
	AMA-13D7	3.85±0.06	3.99±0.06	0.12
Mean IgG response		**3.65±0.04**	**3.59±0.05**	**0.1**
Females	LSA-NRC	3.65±0.07	3.31±0.11	0.01
	CSP	3.16±0.06	2.89±0.08	0.01
	AMA-1-FVO	3.67±0.07	3.87±0.09	0.09
	AMA-13D7	3.7±0.07	4.01±0.08	0.005
Mean IgG response		**3.51±0.06**	**3.55±0.04**	**0.69**

P <0.05 significant, Unpaired T test
AMA-Apical membrane antigen, LSA-Liver stage antigen, CSP-Circumsporozoite protein
MFI- Median fluorescent intensity, SCD- Sickle cell disease

4.3.5.4 IgG responses to blood stage *P. falciparum* antigens in males and females in sickle cell disease and non sickle cell disease individuals

For the blood stage antigens there was a significant difference between females SCD patients and females non-SCD individuals in MSP-1, GLURP-R2, GLURP-R0 and EBP-2 ($P < 0.05$; Table 4.11). However, there was no significant difference in MSP-3 and EBA-175 ($P > 0.05$), this was similar in the males where there was a statistical difference between the males SCD patients and the males non SCD individuals in MSP-1, GLURP-R2, GLURP-R0 and EBP-2 ($P > 0.05$; Table 4.11), however there was no difference in MSP-3 and EBA-175 ($P > 0.05$; Table 4.11). The mean IgG response for blood stage antigens in males and females both in SCD patients and non-SCD individuals was significant ($P > 0.05$; Table 4.12).

Table 4.11: IgG responses to *P. falciparum* blood stage antigens in males and females in SCD patients and non-SCD individuals

		GROUPS		
		SCD	Non-SCD	
Gender	**Blood stage antigens**	**Mean IgG (Log MFI)**	**Mean IgG (Log MFI)**	**P value**
Males	MSP-3	3.18±0.06	3.15±0.07	0.74
	MSP-1-3D7	4.07±0.06	3.71±0.07	0.007
	EBA-175	3.58±0.08	3.47±0.09	0.35
	GLURP-R2	3.97±0.08	3.19±0.14	<0.0001
	GLURP-R0	3.49±0.10	2.92±0.10	0.02
	EBP-2	3.84±0.07	3.62±0.07	0.03
Mean IgG responses		**3.63±0.04**	**3.37±0.03**	**0.01**
Females	MSP-3	3.02±0.07	2.99±0.10	0.82
	MSP-1-3D7	3.96±0.07	3.49±0.13	0.005
	EBA-175	3.53±0.88	3.42±0.11	0.37
	GLURP-R2	3.82±0.09	3.33±0.13	0.001
	GLURP-R0	3.29±0.09	2.79±0.11	0.009
	EBP-2	3.82±0.06	3.51±0.09	0.005
Mean IgG responses		**3.57±0.04**	**3.25±0.05**	**<0.0001**

P< 0.05 Significant, Unpaired T test, MFI- Median fluorescent intensity

4.3.6 Relationship between age and the IgG responses in SCD patients and non-SCD individuals

Using the unpaired t-test, the mean IgG response for each of antigen was compared with the respective age groups; 5-10 and 11-30 years age groups in the SCD patients and non-SCD individuals (Table 4.12). The IgG responses to pre-erythrocytic antigens showed no significant difference with age in the SCD patients group ($P > 0.05$; Table 4.12). However, in the non-SCD group, there was a significant difference on IgG responses to CSP and AMA-1-FVO antigens ($P< 0.05$). There was no significant difference for LSA-NRC and AMA-1-3D7 ($P> 0.05$; Table 4.12). The mean IgG response comparison between the age groups in the SCD patients and non-SCD patients was only significant in age group 5-10 ($P< 0.05$). However, in age group 11-30 there was no statistical difference ($P> 0.05$).

Table 4.12: Relationship between age and the IgG responses to *P. falciparum* pre-erythrocytic antigens in sickle cell disease patients and non sickle cell disease individuals

Age (Years)		**5-10**	**11-30**	
Group	**Antigens**	**Mean IgG (Log MFI)**	**Mean IgG (Log MFI)**	**P value**
SCD	LSA-NRC	3.62±0.07	3.81±0.08	0.08
	CSP	3.14±0.06	3.29±0.07	0.41
	AMA-1-FVO	3.73±0.07	3.85±0.07	0.24
	AMA-1-3D7	3.69±0.07	3.85±0.07	0.14
	Mean IgG	**3.55±0.06[a]**	**3.68±0.06[b]**	**0.01,0.03[a]**
NON SCD	LSA-NRC	3.22±0.07	3.14±0.08	0.12
	CSP	2.77±0.09	3.07±0.07	0.006
	AMA-1-FVO	3.86±0.11	4.12±0.06	0.007
	AMA-1-3D7	3.77±0.09	3.97±0.06	0.09
	Mean IgG	**3.41±0.04[a]**	**3.65±0.03[b]**	**0.02,0.59[b]**

P < 0.05 significant; Unpaired T test

[a] =Age comparison SCD vs non- SCD (5-10 vs 5-10Years) Pre-erythrocytic antigens

[b]=Age comparison SCD vs non-SCD (11-30 vs 11-30 Years) Pre-erythrocytic antigens

N= SCD 5-10 years (63), 11-30 years (37), MFI- Median fluorescent intensity

Blood stage antigens in SCD patients showed a varied significance difference where EBA-175, GLURP-R2 and EBP-2 showed age-dependent IgG responses to malaria ($P< 0.05$; Table 4.13). In contrast, the IgG responses to MSP-1-42 (3D7 and FVO), MSP-3 and GLURP-R0 were not significant ($P> 0.05$; Table 4.13). In the non-SCD individuals EBA-175, GLURP-R2 and GLURP-R0 demonstrated age-dependent IgG responses ($P< 0.05$; Table 4.13). However, the IgG responses to MSP-3, MSP-1-3D7 and EBP-2 were not significant ($P> 0.05$). The mean IgG response comparison per each age group in both SCD and non-SCD (5-10 vs 5-10 and 11-30 vs 11-30) was significant respectively ($P< 0.05$; Table 4.13).

Table 4.13: The relationship between age and the IgG responses to *P. falciparum* blood stage antigens in sickle cell disease patients and non sickle cell disease individuals

Age (Years)		**5-10**	**11-30**	
Group	**Antigens**	**Mean IgG (Log MFI)**	**Mean IgG (Log MFI)**	**P value**
SCD	MSP-1-42-FVO	4.05±0.07	4.15±0.06	0.39
	MSP-3-FVO	3.04±0.06	3.19±0.07	0.14
	MSP-1-42-3D7	3.98±0.07	4.07±0.05	0.43
	EBA-175	3.44±0.07	3.76±0.06	0.005
	GLURP-R0	3.84±0.08	3.97±0.08	0.29
	GLURP-R2	3.28±0.09	3.56±0.10	0.04
	EBP-2	3.73±0.06	3.99±0.06	0.005
	Mean IgG responses	**3.72±0.05[a]**	**3.82±0.04[b]**	**<0.0001, 0.01[a]**
NON SCD	MSP-3-FVO	3.01±0.11	3.13±0.08	0.38
	MSP-1-42-3D7	3.47±0.12	3.72±0.09	0.09
	EBA-175	3.18±0.11	3.66±0.10	0.03
	GLURP-R0	2.83±0.12	3.61±0.09	<0.0001
	GLURP-R2	2.66±0.09	3.04±0.07	0.009
	EBP-2	3.48±0.09	3.64±0.10	0.17
	Mean IgG responses	**3.12±0.10[a]**	**3.47±0.11[b]**	**<0.0001, 0.01[b]**

4.4 Relationship between levels of fetal hemoglobin, IgG responses and age

4.4.1 Levels of Fetal hemoglobin and IgG responses

Positive and significant correlation between the levels of fetal hemoglobin and the IgG responses to the pre-erythrocytic antigens was demonstrated by only LSA-NRC ($r = 0.26$, $P< 0.05$; Figure 4.3a; B). Other antigens for example CSP demonstrated positive correlations between fetal hemoglobin and IgG responses but were not significant. However, AMA-1-FVO, CSP and AMA-1-3D7 demonstrated negative correlations between fetal hemoglobin levels and IgG levels which were not significant ($P> 0.05$; Figure 4.3 a; A, C and D). The blood stage antigens, which include; EBA-175, GLURP-R2 and EBP-2 demonstrated positive correlations between the levels of fetal hemoglobin and the IgG responses, however, MSP-1-42-FVO demonstrated a negative correlation between fetal hemoglobin levels and IgG levels but all this were not significant ($P> 0.05$; Figure 4.3 b; F, G, H and E).

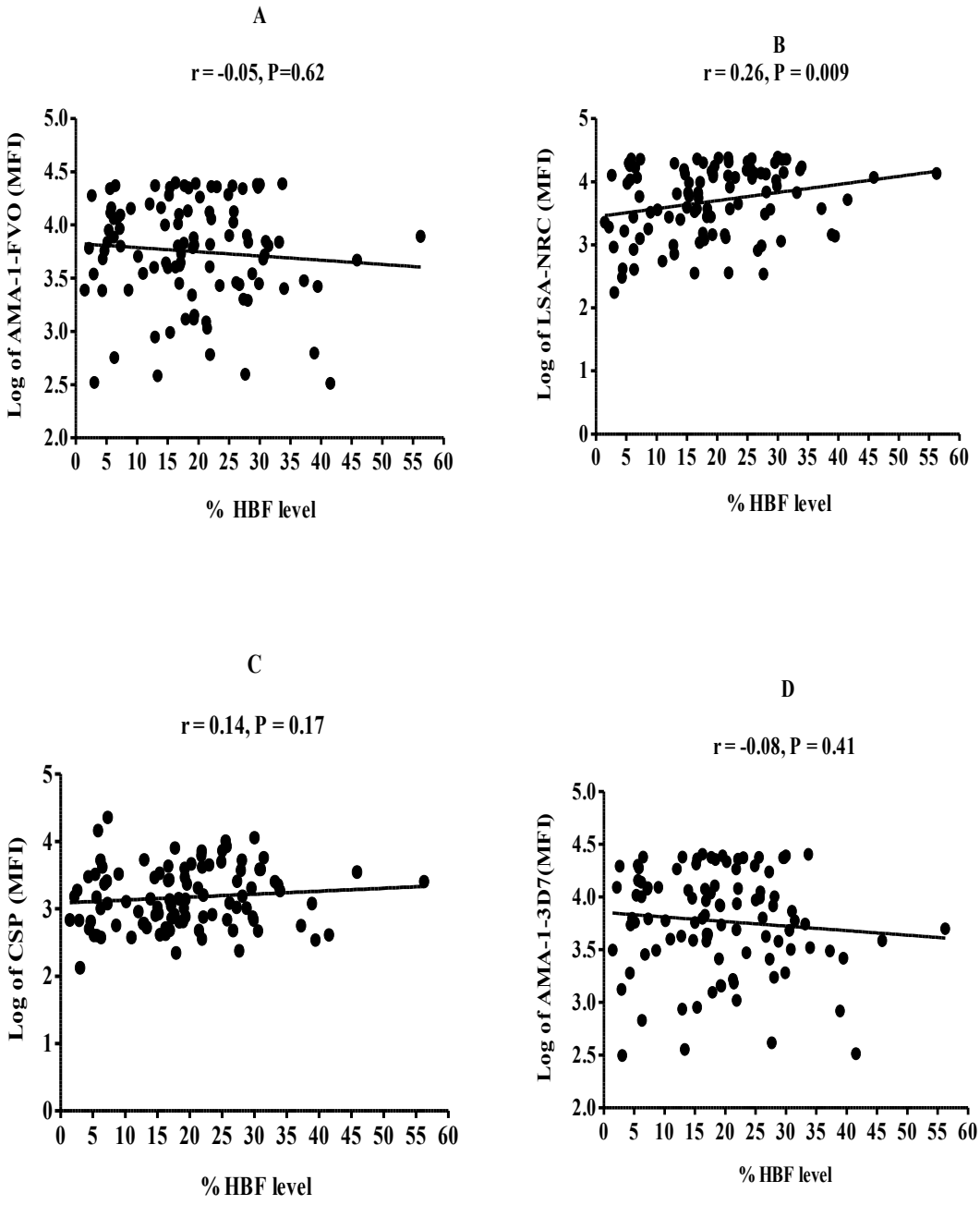

Figure 4.3a: The relationship between the levels of fetal hemoglobin (range 1.44-56.25) and IgG responses to pre-erythrocytic *Plasmodium falciparum* antigens. (A=AMA-1-FVO-1, B=LSA-NRC, C=CSP and D=AMA-1-3D7).

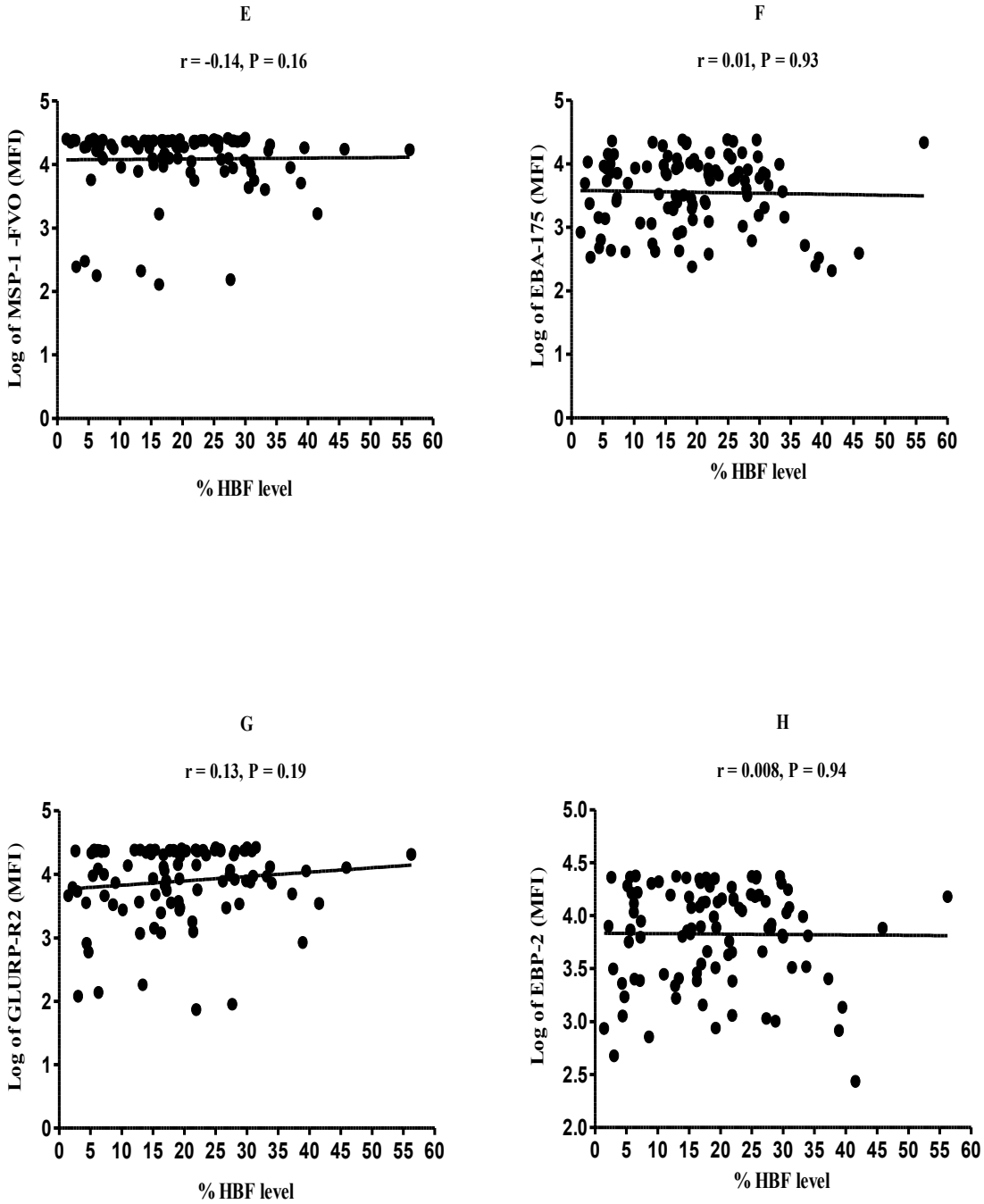

Figure 4.3b: The relationship between the levels of fetal hemoglobin (range 1.44-56.25) and IgG responses to blood stage *P. falciparum* antigens (E=MSP-1-42-FVO, F= EBA-175, G= GLURP-R2, H= EBP-2)

When categorized into males and females the coefficient of correlation was very low, however, in males GLURP-R2 and LSA-NRC demonstrated positive correlations with the levels of fetal hemoglobin that were significant (P< 0.05; Figure 4.4 A and B) respectively. The females on the other hand showed very weak correlations between levels of HbF and the IgG responses (Data not shown).

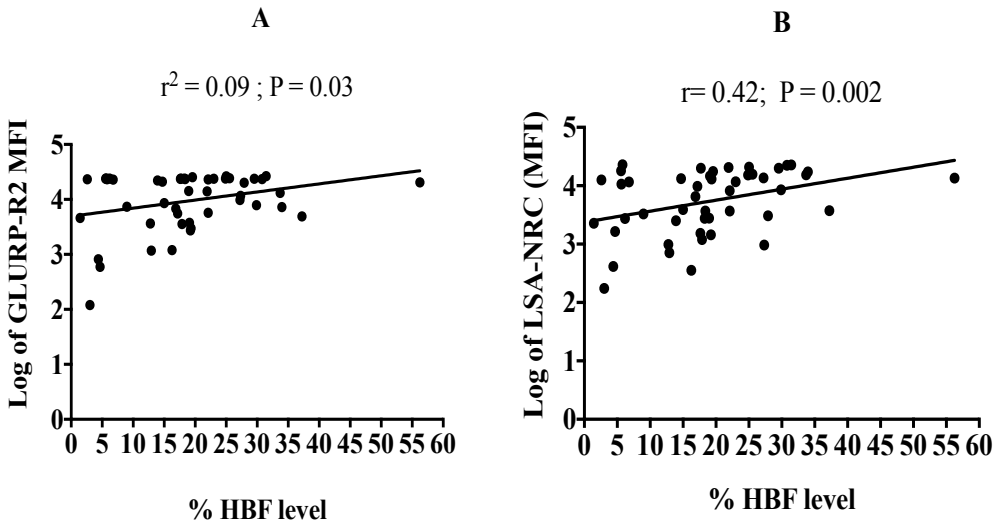

Figure 4.4: The relationship between the levels of fetal hemoglobin and the IgG responses to *Plasmodium falciparum* antigens in males, A=GLURP-R2 and B=LSA-NRC.

4.4.2 Relationship between Fetal hemoglobin and age in SCD patients

The levels of fetal hemoglobin increased as age increased (r =0.17, P< 0.05; Figure 4.5) in SCD patients. The results of linear regression between age and levels of fetal hemoglobin further indicate a strong correlation (r^2 =0.06, P < 0.05; Figure 4.5).

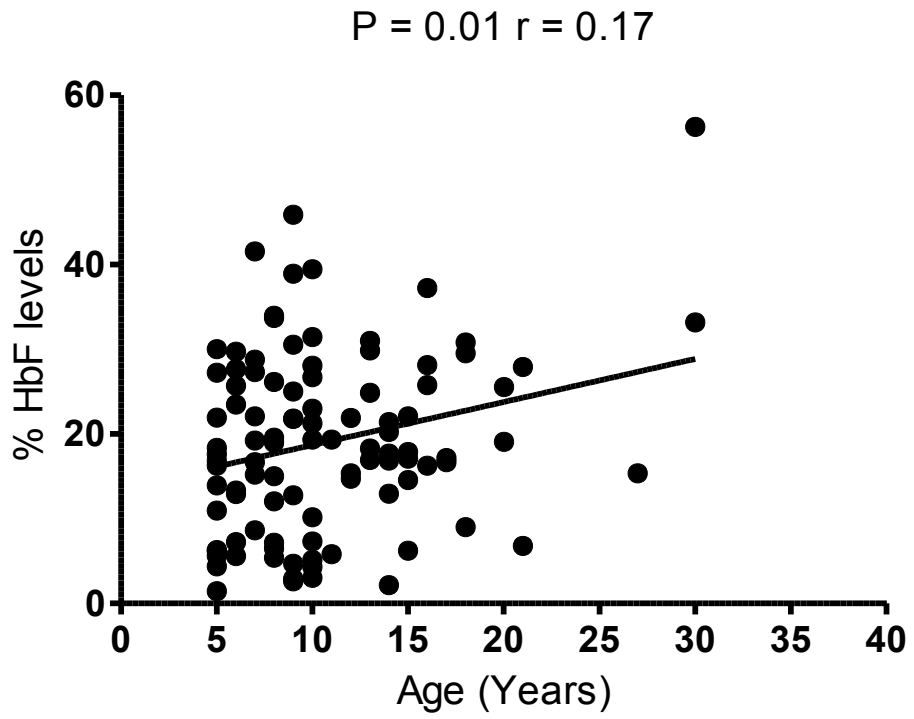

Figure 4.5: Relationship between age and the level of fetal hemoglobin (%) in sickle cell disease patients aged 5-30 years old living in a malaria endemic area, **n= 100.**

4.4.2.1 Relationship of levels of fetal hemoglobin and age in by gender.

The relationship of age and the levels of fetal hemoglobin were expressed differently in the separate sexes; male and female SCD patients, the males fetal hemoglobin levels increased as age increased, this was significant (P< 0.05; Figure 4.4 B), after linear regression the age is shown to predict up to 19% of the levels of fetal hemoglobin (P<

0.05; Figure 4.4A), however in the females there was a weak significant correlation between age and the levels of fetal hemoglobin (P> 0.05; Figure 4.4 A).

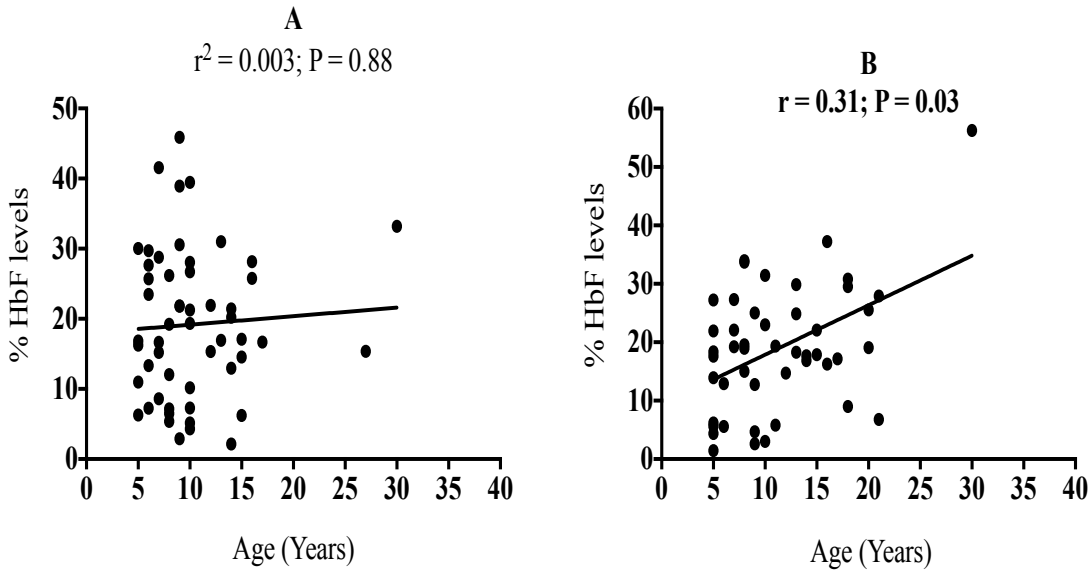

Figure 4.6: Relationship of levels of fetal hemoglobin (%) and age in both female (A) and male (B) sickle cell disease patients aged 5-30 years old living in a malaria endemic area, n= 100.

4.4.3 Testing for a model for Age, HbF and IgG responses in SCD patients

The multivariate model for age and the antibody responses to EBA-175 indicated that the levels of fetal hemoglobin as a negative effect on the age-dependent antibody responses (r =-0.01, P< 0.05; Table 4.14). For EBP-2 the age-dependent antibody is not affected by the levels of fetal hemoglobin (r= 0.00, P< 0.05; Table 4.14), the age-dependent antibody responses to GLURP-RO was affected negatively, however, the effect was not significant (r= -0.01, P >0.05; Table 4.14).

The relationship of antibody responses to LSA-NRC with the levels of fetal hemoglobin was positively affected by age ($r = 0.01$, $P < 0.05$; Table 4.14).

Table 4.14: Multivariate model of age, levels of fetal hemoglobin and the IgG responses in SCD patients

Antigen	Mean MFI	Age	Mean HbF	Coefficient of determination (r)	P value
EBA-175	3.55	5-30	19.08	-0.01	0.02
GLURP-RO	3.39	5-30	19.08	-0.01	0.08
EBP-2	3.83	5-30	19.08	0	0.03
LSA-NRC	3.69	5-30	19.08	0.01	0.02

P <0.05 significant; multivariate regression analysis
EBA- Erythrocyte binding antigen, GLURP-Glutamate rich protein, EBP-Erythrocyte binding protein, LSA-Liver stage antigen, MFI- Median fluorescent intensity, HbF-Fetal hemoglobin

CHAPTER 5: DISCUSSION

5.1 Prevalence and levels of fetal hemoglobin

The study reports high prevalence of fetal hemoglobin in SCD patients from Western Kenya. The observed prevalence is based on previously used definition of HbF levels, where a value greater than 10% and below 10% is considered as high and low prevalence of fetal hemoglobin respectively (Mpalampa *et al.,*2012). Approximately 77% of the SCD patients aged 5- 30 years (n= 100) had high levels of HbF and only 23% had low levels of fetal hemoglobin. Compared to other studies, the prevalence of fetal hemoglobin in this population appears higher than other populations. For instance a study in Uganda, reported that 37% of SCD patients had a high level of HbF (Mpalampa *et al.,* 2012). The Senegal SCD patients demonstrated 25% with high HbF levels (Diop *et al.*, 1999). The high levels of HbF in Dakar/ Senegal group, however has been associated with the Senegal haplotype, which is thought to be responsible for high levels of fetal hemoglobin (Akinsheye *et al.,* 2012).

The findings in this study thus suggest that there must be a haplotype that is responsible for the high levels of which needs to be described. A study from Nigerian SCD patients, reported that the proportion with high levels of HbF was 17% (Fatunde and Scott-Emuakpor, 1992). In this study the proportion with a low prevalence of fetal hemoglobin were 23% of which females were n=11 with a mean prevalence of fetal hemoglobin of 5.25%, while the males were n=12 with a mean fetal hemoglobin of 5.54%. Those individuals categorized within the high

prevalence the females were more than the males in number n=42 with a mean fetal hemoglobin of 21.49%, however, the males were n=35 but with a higher mean prevalence of fetal hemoglobin of 25.17%.

The mean± SEM of fetal hemoglobin in this study population was 19.08% ± 1.06 with a range of 1.44-56.25%. Other studies done in Saudi Arabia (El-Hazmi, 1992), Nigeria (Fatunde and Scott-Emuakpor, 1993) and Congo (Mouélé *et al.,* 1999) reported a mean fetal hemoglobin level of 9.1%, 9.5 and 8.8% respectively making the current study to have the highest mean HbF. This might be due to the fact that these individuals in Western have a haplotype that is responsible for the high fetal hemoglobin as reported in the Senegal group (Diop *et al.,* 1999).

Although the prevalence reported in the study is not consistent with the findings of other studies, the participants in this study had a mean age of 10.75 and a age range of 5 to 30 range years. Other studies, in particular from Saudi Arabia and Uganda had age ranging from 9.8 and 9.3 years respectively (Pembrey *et al.,* 1978; Mpalampa *et al.*, 2012). With a more older population in this study that have survived beyond 5 years, high prevalence was expected because HbF provides some survival advantage to SCD patients (Pembrey *et al.,* 1978). Other studies which have reported higher levels of fetal hemoglobin include studies with the Arab- Indian (AI) haplotype with a mean of 19.2 ±7.006 and a range of 3.6-39.6% and has been reported to be 3-4 folds higher than those of African origin (Ngo *et al*., 2013). Saudi Arabia (Pembrey *et al.,* 1978) also reported a mean HbF of 25.56%. Betke method as used in most of the studies mentioned above and as

defined by the ICSH has been shown to be highly variable in the different laboratory set-ups. Betke technique is also reported to be inaccurate at high HbF levels, where it gives falsely low values of HbF when it rises above 50% (Ducrocq *et al.,* 1998). This may also be a factor as to why this study reports high levels of HbF.

When the females and males were compared, the females demonstrated a higher level of fetal hemoglobin mean ± SD (19.09±10.60) levels than the males mean ± SD (18.95±10.85). This was also seen in previous studies in Nigeria (Olufemi *et al.,* 2011) where the males had a mean ± SD of 4.71 ± 3.49 and the females had a mean of 4.99. However, the difference was not significant. Another study in Nigeria (Olaniyi and odetunde, 2010) showed no statistical difference in the levels of fetal hemoglobin between males and females. In contrast, a study in Nigeria (Kotila *et al*., 2000) demonstrated that the males had slightly higher levels of fetal hemoglobin than the female counterparts with the mean± SD HbF for the males 7.6±3.9 while the female had a mean± SD of 6.7± 3.6, however, the difference was not significant.

The study reports an increased mean level of fetal hemoglobin as age increased in males 5-10 years (16.5%) to 11-30 years (22.05%). However, this was not significant and was in contrast with the study in Nigeria where the levels of fetal hemoglobin in males decreased as age advanced (Olufemi *et al.,* 2011). The females had their mean level of fetal hemoglobin decreasing as age increased i.e. age group 5-10 years (19.42%) decreased to (18.68%) in age group11-30 years,

however, the difference was not significant. Though not significant but may also be linked to the physiological changes the females go through as they approach puberty. This was in contrast with a study in Nigeria (Olufemi *et al.,* 2011) where as age advanced the levels of fetal hemoglobin also increased in the females. The finding in this study therefore agrees with other studies that gender does not influence the levels of HbF and it is not a potential confounder that can be controlled. The finding further suggests that further search for genes associated with the prevalence or levels of HbF should not be a concern with the imbalance of X chromosomes in the study population.

5.2 IgG levels and seropositivity

The SCD patients and non SCD group demonstrated different IgG responses and levels to the eleven *P. falciparum* antigens. Given that this was a study done in a malaria endemic area both the SCD patients n=100 and non-SCD individuals n=70 showed high seropositivity to *P. falciparum* antigens. When IgG responses were categorized in to pre-erythrocytic and blood stage infections, the SCD patients demonstrated high numbers of seropositive individuals, especially LSA-NRC demonstrated high numbers of seropositive individuals than in the non-SCD individuals. On the other hand AMA-1-3D7 demonstrated only seropositive non-SCD individuals. Antibody response to LSA-NRC (1.00-40.34), though low was statistically different between the SCD and non-SCD with the SCD responding both in high numbers and levels of IgG. This suggests that high IgG response to LSA-NRC a vaccine candidate in SCD patients is protective, supporting other

studies that indicate that antibody responses to LSA-1 provides protection against malaria (John *et al.,* 2005). However, the non-SCD individuals demonstrated both high numbers of seropositive individuals with high levels of antibody responses to the pre-erythrocytic antigens. The highest IgG response was demonstrated in AMA-1-3D7 (1.01-88.71), which has also been suggested to be protective against clinical malaria (John et *al.,* 2005).

Antibody responses to almost all blood stage antigens showed that there was no statistical difference in the IgG levels between the seropositive SCD patients and the non-SCD individuals. This gives a clear picture that the IgG responses to blood stage *P. falciparum* antigens in SCD patients is not in any way special and that no specific vaccine to prevent the blood stage antigen should be administered to the SCD patients. In contrast there was a statistical difference between seropositive SCD patients and non-SCD individuals in MSP-3-FVO with the non-SCD patients having a higher IgG level. The IgG response to MSP-1-42-FVO was the highest both in SCD and non-SCD individuals. EBP-2 was similarly demonstrated in both groups where the seropositive individuals in the SCD and non SCD was 78 (n=100) and 53 (n=70) respectively and also both had a lower antibody level ranging from 1.03-10.03 and 2.97-12.30 respectively. When the proportion of seropositive individuals both in SCD and non-SCD were compared, the pre-erythrocytic antigens; LSA-NRC and AMA-1-3D7 showed no significant difference. This indicates that the level of protection to clinical malaria is the same in these individuals. The blood stage antigens i.e. MSP-3 and GLURP-R2

also indicated no statistical difference in the number of seropositive individuals. GLURP-R2 had the highest SCD seropositive patients, suggesting that more studies on LSA-NRC, MSP-3 and GLURP-R2 should be done to explain this difference in IgG responses.

The mean IgG responses to pre-erythrocytic and blood stage *P. falciparum* antigens was compared using unpaired t-test, this showed varied differences in the SCD and non-SCD individuals. The SCD individuals demonstrated higher mean IgG responses to both the pre-erythrocytic and blood stage antigens. This supports other studies which indicate that high antibody responses to pre-erythrocytic antigens is an indicator of protection against clinical malaria in malaria endemic areas (John *et al.*, 2003). The blood stage mean IgG responses were statistically different in the two groups with the SCD having high mean IgG response compared to the non-SCD individuals. Thus the study reports that SCD individuals are more protected against clinical malaria than the non-SCD. The IgG responses to the blood stage antigens i.e. MSP-1-42, GLURP-R2 and GLURP-R0 indicated a clear difference of responses between the SCD and non SCD group with the SCD having high levels of the above antigens. High antibody levels against blood stage antigens; which have been shown to be the major cause of the pathogenesis of malaria infection, have been described to be protective against clinical malaria (Meraldi *et al.,* 2004).

This study was also able to establish gender difference in antibody responses to *P. falciparum* antigens in the SCD and non-SCD individuals. The IgG response to

both pre-erythrocytic and blood stage *P. falciparum* antigens was variable in males and females with SCD and the non SCD individuals, but in all cases males had higher levels of IgG responses than females. Both the pre-erythrocytic and blood stage antigens that were significantly different between the males with SCD patients and non-SCD were also significantly different in females with SCD and non-SCD individuals. The males generally exhibited higher antibody levels than the females in both the SCD and non-SCD individuals. This may be attributed to the fact that the males and females have a different physiological make up and therefore the difference in antibody response to these antigens but no study has clarified this finding.

The study also determined whether there was a relationship between age and IgG responses to *P. falciparum* antigens in the SCD patients just as in the normal population where *P. falciparum* antigens have demonstrated age-related antibody response (Dobaño *et al.,* 2012). The antigens were grouped into pre-erythrocytic and blood stage antigens and their relationship to age determined first with the SCD patients and then with the non-SCD individuals. The study findings indicate that there was an age related antibody responses to *P. falciparum* antigens where as one advances in age so do the antibody levels in their body system.

This study therefore supports other studies done in malaria endemic areas where children are more prone to mortality and morbidity due to malarial infection as compared to the adult groups with immunity to malaria developing by the age of 3-5 years usually with reduced episodes of severe malaria (Newton *et al.,* 1997).

These findings further support the fact that individuals living in malaria endemic areas are protected against malaria due to repeated exposures and the presence of non-sterile clinical immunity. Sickle cell disease patients aged 5-10 years are more protected against the pre-erythrocytic antigens than the non-SCD individuals of the same age group. This might further explain the fact that SCD patients are susceptible to malaria and do not survive past the age of five years in malaria endemic areas, however, this group has high antibody levels and thus survival for the age 5 to 30 years (Platt, 1994). When age group 5-10 was compared to the age group 11-30 years with SCD, the age group 5-10 years had less antibody levels. Age groups 11-30 showed no difference in antibody levels between the SCD and non-SCD.

The blood stage antigens expressed a similar trend where the antibody levels increased with age in the SCD patients and non-SCD individuals. The SCD individuals demonstrated high mean antibody response than the non-SCD individuals. This therefore explains the survival advantage of the susceptible HbSS individuals in this malaria endemic zone where by in addition to the chemoprophylaxis given to them the SCD individuals are more protected than the non SCD individuals supporting previous studies on the resistance of malaria by the HbSS individuals as compared to the HbAA individuals (Aluoch, 1997; Eridani, 2013).

Levels of exposure to malaria infection has been linked to the levels and prevalence of antibody responses (Kilombero Malaria Project, 1992; Orlandi-

Pradines *et al.,* 2006; John *et al.,* 2008). The findings in this study population suggest that levels and prevalence of antibody responses in the sickle cell disease patients and non-SCD individuals is different, therefore, the reason for this difference needs to be established.

High levels of IgG to pre-erythrocytic antigens like CSP, LSA-1, AMA-1 have been shown to correlate with protection from *P. falciparum* malaria infection in adults (John *et al*., 2005) and protection from clinical malaria in children (Keh *et al.,* 2012). Antibody responses to AMA-1 has been linked to protection against clinical malaria (Osier *et al.,* 2008; Fowkes *et al.,* 2010). The blood stage antigens also demonstrated the same trend of seropositivity and antibody levels. There was a high number of seropositive individuals both in the SCD patients and the non-SCD individuals. The antibody levels in the SCD group were lower ranging from 1.03-55.50 as compared to the non SCD group with a range of 1.07-102.9.

The blood stage development of the malaria parasite in the human has always been shown to be the major cause of morbidity and mortality with the expression of the malaria symptoms. High IgG responses to these blood stage *P. falciparum* antigens have been associated with protection against clinical malaria (Stanisic *et al.,* 2009). Other studies support the fact that high antibody responses to antigens like EBA-175 (Dziegiel *et al.*, 1993; John *et al.,* 2005; Osier *et al.*, 2008; Crompton *et al.,* 2010; McCarra *et al.,* 2011; Kangoye *et al.,* 2014), GLURP, MSP-1, MSP-3; Medeiros *et al.*, 2013) are linked to protection against clinical malaria.

Differences in the levels of IgG responses to *P. falciparum* antigens in SCD and non SCD groups is of major research interests. Recently a study reported that, specific IgG responses to *Pf*EMP-1 and HbF found in individuals with genetic disorder of the beta-globin chain of hemoglobin like SCD and β-thalassemia, co-operate to prevent the cytoadherence of the infected red blood cells to the microvasculature by the formation of abnormal knobs. This facilitates the splenic clearance of infected red blood cell, reducing the parasitemia and clinical severity of malaria (Fairhurst *et al.,* 2012).

5.3 The levels of fetal hemoglobin and IgG responses

This study reports an association of fetal hemoglobin and IgG responses to LSA-NRC, a pre-erythrocytic *P. falciparum* antigen. High antibody levels to LSA-NRC (1.01-40.34) have been shown to be associated with protection against clinical malaria (Noland *et al.,* 2008). When gender was considered, the males demonstrated that, levels of fetal hemoglobin associated with both LSA-NRC and GLURP-R2 to provide protection against clinical malaria in the sickle cell disease patients.

Maternal IgG responses specific to *Pf*EMP-1 antigen co-operates with fetal hemoglobin to enhance malaria resistance in infants (Amaratunga *et al.,* 2011: Kangoye *et al.,* 2014). The *P. falciparum* erythrocyte membrane protein -1 (*Pf*EMP-1) has been shown to be the most virulent factor of *P. falciparum* which enhances the cytoadherence of the *P. falciparum* infected red blood cells to the

microvascular endothelial cells (MVECs) thereby causing the pathogenesis of malaria (Amaratunga *et al.,* 2011). Hemoglobin variants for example HbSS, HbC and HbAS have been shown to display an abnormal *Pf*EMP-1 knob thus preventing the cytoadherence of infected *P. falciparum* red blood cells from adhering to the MVECs (Fairhurst *et al.,* 2012). The IgG response to *P. falciparum* antigens in co-operation with fetal hemoglobin usually expressed in SCD individuals is able to provide protection against complicated and severe malaria. This study therefore confirms the findings of previous studies on malaria protection in individuals with the hemoglobin variants (Amaratunga *et al.,* 2011; Fairhurst *et al.,* 2012; Krause *et al.*, 2012). Mmbando *et al.* (2015) further explains that individuals with the HbS gene and withhigh levels of HbF are protected against malaria.

The mean fetal hemoglobin levels increased as age increased in this population where the spearman's rho(r) was 0.17 and was statistically significant; this was in contrast with the Nigerian study where generally the mean HbF levels declined with age (Olaniyi and odetunde, 2010). The increase in mean HbF levels was predicted by age up to 6% where the coefficient of correlation (r^2) was 0.06 and was significant. This explains the survival advantage expressed in those individuals expressing high levels of fetal hemoglobin. They have been shown to have an increased life expectancy than those with a low fetal hemoglobin level (Platt *et al.,* 1994) who die early in age (Mpalampa *et al.,* 2012).

When categorized into the females and males, the males r=0.31 demonstrated up to 19% (r^2 = 0.199) of their level of fetal hemoglobin being predicted by age, indicating that males with SCD can survive longer as compared to the females, however, this might not be the case because survival of the SCD patients is dependent majorly on early diagnosis and care given to them (Modell, 2008). These findings also report more survival advantage in males living in this area than the females, their fetal hemoglobin correlates with the IgG responses to both LSA-NRC and GLURP-R2 that have been shown to protect against clinical malaria.

A model of age, levels of fetal hemoglobin and the IgG responses, the study found the effect of the levels of fetal hemoglobin on the relationship between age and IgG responses was negative for EBP-2 and GLURP-R2 however, it had a positive effect on LSA-NRC which has demonstrated a co-operation with levels of fetal hemoglobin in providing protection against malaria. The study provides a platform to investigate how IgG response to LSA, vaccine candidate can interact with HbF in providing protection against *P. falciparum* malaria, it would be interesting to further assess how the levels HbF play a role in protection especially in follow up and cohort studies.

5.4 Hypothesis Testing

This study reports that there was a significant difference in the IgG responses to multiple *P. falciparum* vaccine candidate antigens between the SCD and non-SCD

individuals in a malaria endemic area. The finding of this study therefore rejects the null hypothesis.

The finding of this study also report that there was no relationship of fetal hemoglobin with the IgG responses to multiple pre-erythrocytic and blood stage *P. falciparum* vaccine candidate antigens except for LSA-NRC. This finding therefore accepts the second null hypothesis.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i. The SCD patients in this malaria endemic area had high prevalence of 77% and high levels of fetal hemoglobin ranging from 1.44-56.25%. Fetal hemoglobin levels determine the life expectancy of the SCD patients.

ii. The non-sickle cell disease generally had high antibody levels to *P. falciparum* antigens as compared to the SCD individuals. However, when grouped into the pre-erythrocytic and blood stage antigens, the SCD and non-SCD individuals showed varied IgG levels to each of the pre-erythrocytic antigens with the IgG responses to pre-erythrocytic antigens being statistically different while the IgG responses to blood stage antigens exhibited no difference. For instance non-SCD patients had high IgG responses to pre-erthrocyic antigens than the SCD individuals except for LSA-NRC.

iii. Fetal hemoglobin levels positively correlated with the IgG responses to LSA-NRC in the SCD patients' population thus co-operating in providing protection against malaria. The IgG responses to some antigens were negatively correlated with the levels of fetal hemoglobin.

6.2 Recommendations

6.2.1 Recommendation for Practice and Policy

i. The antigens exhibiting high IgG responses like LSA-NRC should be investigated further for their efficacy in vaccine development.
ii. Fetal hemoglobin should be considered a special entity in malaria protection in SCD patients.
iii. The management of SCD patients should ensure the levels of fetal hemoglobin are maintained or even raised in these patients.

6.2.2 Recommendations for future research

i. The high levels of HbF in SCD patients needs to be investigated further so as to be linked genetically to a haplotype which is responsible for the high HbF in Western Kenya.
ii. Further rigorous study design such a longitudinal design on the same population can provide a robust association between fetal hemoglobin and antibodies to P. *falciparum* antigens and other immunological aspects like cytokines among others.

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APPENDICES

APPENDIX I: Map of Study area

Map adapted from wwwnc.cdc.gov

APPENDIX II: Assent Form

English Assent for Human Investigational Studies

Study title: **PREVALENCE OF FETAL HEMOGLOBIN AND ANTIBODY RESPONSES TO *Plasmodium falciparum* ANTIGENS IN SICKLE CELL DISEASE PATIENTS IN WESTERN KENYA**

Principal Investigator: George Ayodo

Research Staff: Medical staff at the hospital; including a clinical officer and lab technologist, pharmacist and research assistants

Why are we doing this study? We are doing a research study to understand causes of disease of the blood. To do understand this, we are requesting you to participate in this study by giving us your blood sample of about 10-20 drops. With this, we will test for those things in the blood that cause the disease of the blood. If we understand how this blood disease is caused, we will able to know how to cure it.

Why am I being asked to be in the study? We are inviting you to be in the study because you have symptoms that could mean you have disease of the blood.

What if I have questions? You can ask questions if you do not understand any part of the study. If you have questions later that you don't think of now, you can talkto me again or ask your nurse to call Dr. George Ayodo at 0737 773 914.

If I am in the study what will happen to me? If you decide that you want to be part of this study, we will get a small blood sample from you. We are asking to get about 10-20 drops of your blood from your finger with a small lancet (finger prick). The needle prick will hurt for just a short time. The small amount of blood that we will take (10-20 drops of blood) will not harm you. We will test your blood for the presence of disease of blood, if confirmed then you will get treatment at the hospital.

Will I be hurt if I am in the study? The risks of this study are: pain, a little bleeding or blood clotting at the area where blood will be drawn from, There is also a very small risk of infection, but we will take great care to make sure that the area is very clean, and we have not had a problem with infection in previous studies.

Will the study help me? The study won't directly help you. The study may help us understand disease of blood better, and eventually come up with better ways of preventing it. This won't help you, but may help your family members and others in the future.

Do I have to be in this study? You do not have to be in this study, if you do not want to be. If you do not want to be in this study, you will still get regular treatment at the Ministry of Health clinic as always. It is also OK if you change your mind after you have been enrolled for this study and nobody will be angry or upset. We are discussing the study with your parents and you should talk to them about it too.

What happens after this study? When we have finished this study we will write a report of our findings. This report will not include your name or that you are in the study.

Assent: If you decide you want to be in this study, please print/write your name. If you decide that you don't want to be in the study, even if you have been enrolled for the study, then all you have to do is tell us, and we will not include you in the study.

Signature or Fingerprint* of child	Date of assent	Printed name of participant

*If the participant, parent or guardian is unable to read and/or write, an unrelated witness should be present during the informed consent discussion. After the written informed consent form is read and explained to the participant, parent or guardian, and after they have orally consented to their or their child's participation in the study, and have either signed the consent form or provided their fingerprint, the witness should sign and date the consent form. By signing the consent form, the witness attests that the information in the consent form and any other written information were explained to and understood by the participant, parent or guardian and that informed consent was freely given by the participant, parent or guardian.

Signature of person witnessing consent	Date	Printed name of person witnessing consent

Signature of person obtaining consent	Date	Printed name of person obtaining consent

(Must be study investigator or individual designated in the Checklist to obtain consent.)

_____ Date_____

Signature of Principal Investigator or Designated Study Official

(Affirming subject eligibility for the study and that informed consent has been obtained.)

APPENDIX III: Consent Form

English Consent for Human Investigational Studies

Study title: **PREVALENCE OF FETAL HEMOGLOBIN AND ANTIBODY RESPONSES TO *Plasmodium falciparum* ANTIGENS IN SICKLE CELL DISEASE PATIENTS IN WESTERN KENYA**

Investigator: Dr. George Ayodo

Location of the study: Bondo and Rarienda Constituency, Siaya County.

Purpose:

Sickle Cell Disease is a blood genetic disorder that causes pain, acute chest syndrome and early death. The risk of this disease is variable from one patient to another. One of the factors that influence its risk is the Fetal hemoglobin (HbF). This is part of the blood that goes down as one grows old and studies have confirmed that those who suffer from sickle cell disease but have high level of it do to suffer from severe form the disease. How this factor comes to be high or low in some individuals is not known. We therefore want to disentangle this, by establishing levels of HbF in people suffering from sickle cell disease. We are searching for genes that make these levels be different from individuals. We are also establishing the role of HbF in malaria resistance and also finding out if there is any other product blood that can be used to diagnose Sickle Cell Disease

Procedure:

We would kindly ask you or child to support us in our work. We will ask you or child questions on your medical history such as do you get symptoms like acute chest syndrome and pain crises or do these symptoms affects your family members. Following the interview, if we consider you for further investigations, we will request ¼ teaspoonful of your or your child's blood at the time of your clinic visit to ascertain if you have the disease or not. If you have the disease then we will set a day so that you donate 3-4 tablespoonfuls of blood (10-12mls). With these blood samples, we will do the test for HbF, genes and metabolites. Of course, these tests will be free of costs.

Risks and Benefits

There are minimal risks to having a few drops of blood drawn from you in this study. The risk includes bleeding, slight pain, bruising and possible infection. All

these are uncommon events that have occurred in very few adults or children previously studied by the research group. If you are pregnant, we will not include you in the study. The direct benefit for you and your child in this study is confirming the SCD that will help your doctor manage your sickness as provided by the ministry of health. The result will also benefit the community in the future as may help them to understand SCD more.

Confidentiality

We will maintain the privacy and confidentiality of medical records. All information you give us will be treated as confidential and in compliance with legal data protection requirements. You are free to withdraw from the study at any time. The result of the study using your blood will be assigned a study number to preserve your identity. The principal will keep a database linking you or your child's personal identifiers to the study number investigator and relevant key personnel. Only key study personnel and institutional review boards will be allowed access to the medical information collected in this study.

Summary of your rights as a participant in a research work

Your / your child's participation in this research is voluntary. Refusing to participate will not involve any penalty. If you decide to enroll in this study, you can withdraw yourself or your child anytime you want. If information generated in the study is published or presented your child's identity will not be revealed.

Reimbursement

The study participants or guardian or parent will be reimbursed Ksh 200 for any visit to health facility that relate to this study.

Questions

Under the circumstance, the sponsor of the study will pay for the injuries resulting directly from being in the study. If you or your child experiences physical injury or illness as a result of participating in this research study, you should contact the Investigator, Dr. George Ayodo at Box 1578 at 0737 773 914.

Contact information: ----------hasdescribed to you what is going to be done, the risks, hazards, and benefits involved, and can be contacted at ----------.

If you have any questions then you can call Dr. George Ayodo at Centre for Global Health and Child Development (Milimani Estate, Off Tom Mboya Street or Center of Global Health Research (KEMRI-Kisian) P.O Box 130 in Kisumu at 0737 773 914.

If you consent, then Please specify in what way your sample may be used. After your physician/counselor has explained the following questions to you, please answer them by ticking **Yes** or **No**.

I consent that my sample will be used for the investigation of SCD.	**O Yes**	**O No**
I wish that my sample be destroyed upon completion of the study.	**O Yes**	**O No**
I consent that my sample will be stored to be used for future research projects on SCD.	**O Yes**	**O No**
I consent that my sample may be shipped to other researchers for investigations relating to SCD, provided the sample does not reveal my identity.	**O Yes**	**O No**
I consent that a member of the research team (or any designated person) will contact me.	**O Yes**	**O No**

Signature

Signing below indicates that you have been informed about the research in which you voluntarily agree to participate with your child; that you asked any question about the study; and that the information given to you has permitted you to make a fully informed and free decision about yourself /your child's participation in the study. By signing this consent form, you do not waive any legal rights, and the investigators are not relieved of any liability they may have. You can withdraw yourself / your child from this study any time. You will be offered a copy of this consent form and it will be provided to you if you would like to have one.

Printed name of the participant

Signature or fingerprint of Participant or child signature: if this form is used to obtain assent if the participant is a minor or legally incompetent**.**

----------Date----------

Parents or Legal Guardian signature

Relationship to participant ----------

If the participant, parent or guardian is unable to read and or write, a related witness should be present during the informed consent discussion. After the written informed form is read and explained to the participant, parent or guardian, and after they have orally consented to their or their child's participation in the study, and have either signed the consent form, the witness attests that the information in the consent form and other written information were explained to and understood by the participant, parent or guardian and that informed consent was freely given by the participant, parent or guardian.

---------Name of the Person Witnessing Consent (Printed)

---------Signature of the Person Witnessing Date /Time

----------Date---------- ----------

--------Signature of the Clinician obtaining consent Printed Name

---------- Date ----------

Signature of the Principal Investigator (Affirming subject eligibility for the study and informed consent has been obtained.

APPENDIX IV: Data collection form

Fetal hemoglobin study

*Serial number of the data collection form*_____

*Serial number of the consent form*_____

*Study ID of the family*_____

_____

*Study ID of the patient*_____

_____

1. Details of the patient:

*a) Name of the patient:*_____

_____

*b) Age*_____ *Gender*_____

*c) Location*_____*Sulocation*_____*Village*___

_____

*d) Telephone contact*_____ *Guardian's telephone contact*_____

2. Patient's information:

a) *Is the patient in severe anemic condition?*

Yes or No

b) Has the patient got blood transfusion in the last 4 months?

Yes or No

c) Is the patient on treatment with Hydroxyurea?

Yes or No

d) Does the patient have any chronic illness?

Yes or No

e) Has the patient been tested for sickle cell elsewhere?

Yes or No

g) Was the result positive?

Yes or No

3. Sample collection at the field:

a) Is blood sample collected from the patient?

Yes or No

b) Estimate of the amount of blood _____ml

c) Is the tube labeled with patient ID and date?

Yes or No

4. **Sample processing at the Lab**

a) *Ref sample OD_____Test sample OD_____ HbF level_____%*

b) *Ref sample OD----------Test sample OD----------HbF level_____%*

c) *DNA concentration _____ng/ul*

d) *Is HbS typing positive?*

Yesor No

e) *Has DNA been stored at -20^{0}C*

Yes or No

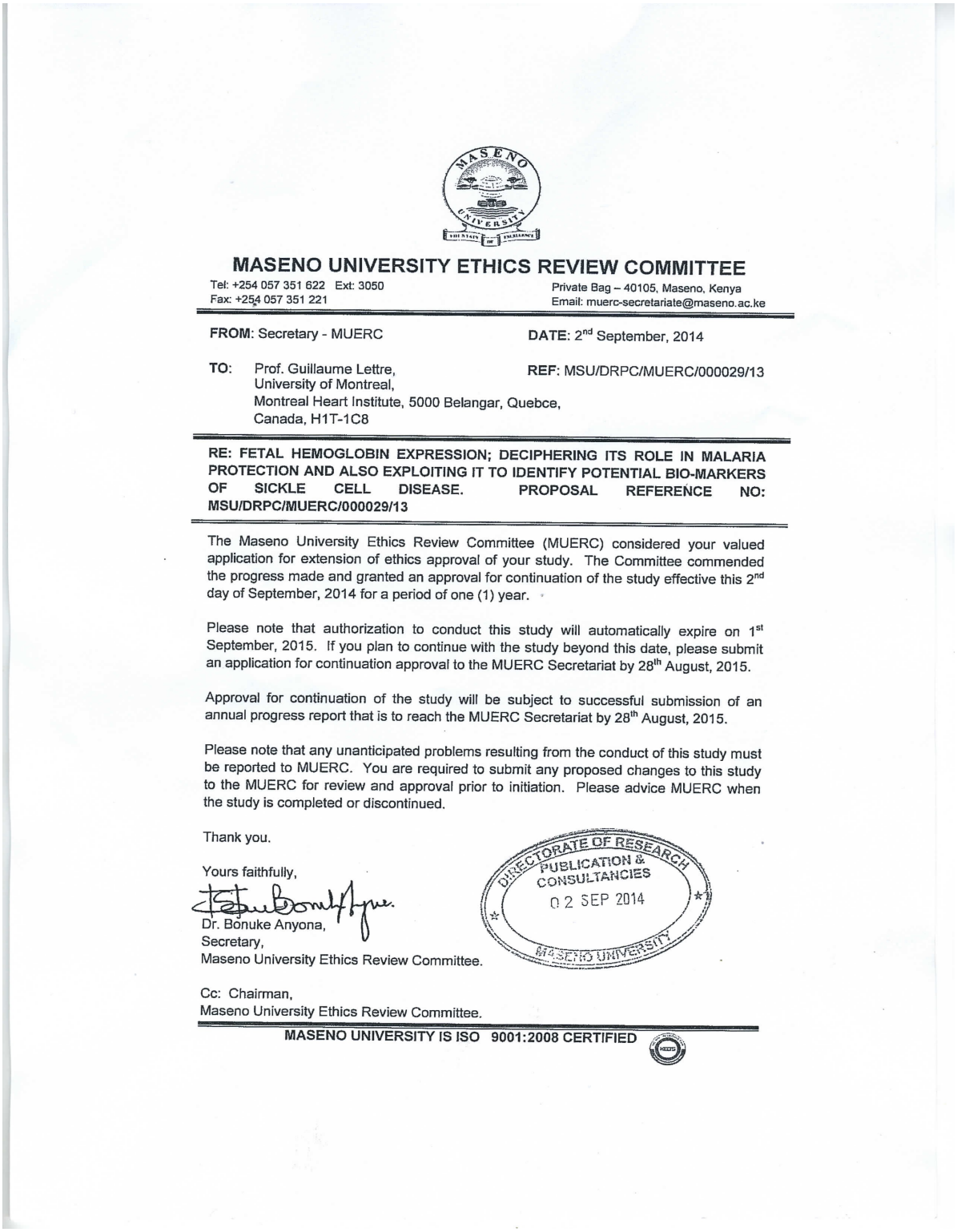
f) *Has plasma been stored at -20^{0}C*

Yes or No

g) *Are the antibodies present*

Yes or No

APPENDIX V: Maseno University Ethical approval



MASENO UNIVERSITY ETHICS REVIEW COMMITTEE

Tel: +254 057 351 622 Ext: 3050
Fax: +254 057 351 221

Private Bag – 40105, Maseno, Kenya
Email: muerc-secretariate@maseno.ac.ke

FROM: Secretary - MUERC

DATE: 2nd September, 2014

TO: Prof. Guillaume Lettre,
University of Montreal,
Montreal Heart Institute, 5000 Belangar, Quebce,
Canada, H1T-1C8

REF: MSU/DRPC/MUERC/000029/13

RE: FETAL HEMOGLOBIN EXPRESSION; DECIPHERING ITS ROLE IN MALARIA PROTECTION AND ALSO EXPLOITING IT TO IDENTIFY POTENTIAL BIO-MARKERS OF SICKLE CELL DISEASE. PROPOSAL REFERENCE NO: MSU/DRPC/MUERC/000029/13

The Maseno University Ethics Review Committee (MUERC) considered your valued application for extension of ethics approval of your study. The Committee commended the progress made and granted an approval for continuation of the study effective this 2nd day of September, 2014 for a period of one (1) year.

Please note that authorization to conduct this study will automatically expire on 1st September, 2015. If you plan to continue with the study beyond this date, please submit an application for continuation approval to the MUERC Secretariat by 28th August, 2015.

Approval for continuation of the study will be subject to successful submission of an annual progress report that is to reach the MUERC Secretariat by 28th August, 2015.

Please note that any unanticipated problems resulting from the conduct of this study must be reported to MUERC. You are required to submit any proposed changes to this study to the MUERC for review and approval prior to initiation. Please advice MUERC when the study is completed or discontinued.

Thank you.

Yours faithfully,

Dr. Bonuke Anyona,
Secretary,
Maseno University Ethics Review Committee.

DIRECTORATE OF RESEARCH PUBLICATION & CONSULTANCIES
02 SEP 2014
MASENO UNIVERSITY

Cc: Chairman,
Maseno University Ethics Review Committee.

MASENO UNIVERSITY IS ISO 9001:2008 CERTIFIED

APPENDIX VI: Approval of Research Proposal

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: kubps@yahoo.com
dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 810901 Ext. 57530

Internal Memo

FROM: Dean, Graduate School

DATE: 29th June, 2015

REF: 156/24062/13

TO: Ms. Webala Akinyi Brenda
C/o Zoological Sciences Dept.
KENYATTA UNIVERSITY

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that the Graduate School Board at its meeting of 17th June, 2015 approved your M.Sc. Research Proposal Entitled "Prevalence of Fatal Hemoglobin and Antibody Responses to *PLasmodium falciparum* Antigens in Sickle Cell Disease Patients in Western Kenya".

You may now proceed with your Data collection, subject to clearance with the Principal Secretary, Higher Education, Science and Technology.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed supervision Tracking Forms per semester. The form has been developed to replace the progress Report Forms. The Supervision Tracking Forms are available at the University's Website under Graduate School webpage downloads.

Thank you.

REUBEN MURIUKI
FOR: DEAN, GRADUATE SCHOOL

c.c. Chairman, Zoological Sciences Dept.

Supervisors:

1. Dr. Michael Gicheru
   C/o Zoological Sciences Dept.
   KENYATTA UNIVERSITY

2. Dr. George Ayodo
   Kenya Medical Research Institute (KEMRI)
   University of Minnesota Research Projects
   Kisumu
   C/o Zoological Dept.
   KENYATTA UNIVERSITY

RM/cao

APPENDIX VII: Research Permit (NACOSTI)

CONDITIONS

1. You must report to the County Commissioner and the County Education Officer of the area before embarking on your research. Failure to do that may lead to the cancellation of your permit
2. Government Officers will not be interviewed without prior appointment.
3. No questionnaire will be used unless it has been approved.
4. Excavation, filming and collection of biological specimens are subject to further permission from the relevant Government Ministries.
5. You are required to submit at least two(2) hard copies and one(1) soft copy of your final report.
6. The Government of Kenya reserves the right to modify the conditions of this permit including its cancellation without notice.

REPUBLIC OF KENYA

National Commission for Science, Technology and Innovation

RESEARCH CLEARANCE PERMIT

Serial No. A 6664

CONDITIONS: see back page

THIS IS TO CERTIFY THAT:
MISS. WEBALA BRENDA AKINYI
of KENYATTA UNIVERSITY, 0-50205
WEBUYE,has been permitted to conduct research in *Kisumu County*

on the topic: *PREVALENCE OF FETAL HEMOGLOBIN AND ANTIBODY RESPONSES TO PLASMODIUM FALCIPARUM ANTIGENS IN SICKLE CELL DISEASE PATIENTS IN WESTERN KENYA*

for the period ending:
18th September,2016

Permit No : NACOSTI/P/15/3369/7393
Date Of Issue : 22nd September,2015
Fee Recieved :Ksh 1,000

.....
Applicant's Signature

.....
Director General
National Commission for Science, Technology & Innovation