COMPARATIVE MEASUREMENT OF FETAL HAEMOGLOBIN LEVELS AND THEIR ASSOCIATION WITH ANTIBODY RESPONSES TO *Plasmodium falciparum* ANTIGENS AMONG SICKLE CELL PATIENTS IN BONDO SUB-COUNTY, WESTERN KENYA

BY

MBOYA IMELDA AWUOR

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DECLARATION

I declare that this thesis is my own original work and has not been presented to any institution of higher learning for the award of a degree certificate.

IMELDA AWUOR MBOYA

MSC/PH/00077/2014

Signature..... Date.....

This thesis has been submitted for examination with our approval as supervisors:

Bernard Guyah, PhD

Department of Biomedical Science and Technology

Maseno University

Signature……. Date…….

George Ayodo, PhD

Centre for Global Health Research

Kenya Medical Research Institute

Signature…….. Date……..

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DEDICATION

To all my family members

ABSTRACT

Plasmodium falciparum malaria infection remains one of the greatest global public health problems. However, studies have shown that fetal haemoglobin (HbF) protects against malaria in sickle cell disease (SCD) and HbF cooperates with antibodies to provide protection. Associations between HbF and antibodies have been done only on infants and therefore protection could be due to maternal immunity. Children at 5 years old have developed their own immunity and also HbF levels do not significantly change at this age. Bondo in western Kenya is one of the regions that have been affected by SCD recording a prevalence of 1.6% against a prevalence of 1.8 in western Kenya. Association of HbF levels and antibody responses to pre-erythrocytic and erythrocytic stage antigens of *P. falciparum* malaria has not been studied. Levels of HbF have been determined using Betke method but it produces falsely elevated values. High Performance Liquid Chromatography (HPLC) is more sensitive and precise but, its results have not been compared with that of Betke to determine the association of HbF levels with antibodies to malaria. Therefore this study sought to compare tests for HbF levels and relate them with the antibody responses to pre-erythrocytic and erythrocytic stage antigens of *P. falciparum* malaria infection among sickle cell patients above 5 years old in Bondo sub-county, Western Kenya. The specific objectives were; to correlate HbF levels using HPLC and Betke; to determine the association between HbF and antibodies to pre-erythrocytic stage antigens of *P. falciparum* malaria and to determine the association between HbF and antibodies to blood stage antigens of *P. falciparum* malaria. A cross sectional study design and purposive sampling was used to select 100 SCD patients. Using a sample size of 100, detectable effect sizes were obtained at 80% power and α of 0.05. HbF values were determined using HPLC and Betke methods and the quantification of IgG antibodies against *P. falciparum* recombinant antigens was done by the Multiplex Suspension Array Technology. Using pearson's correlation test to compare HbF levels obtained by HPLC and Betke, the study reports that there was no statistically significant correlation between the two methods ($r = -0.016$) and therefore either method can be used to measure HbF levels. Linear regression showed that there was an association between HbF and antibodies to pre-erythrocytic antigens CSP ($p = 0.040$) and LSA ($p = 0.002$) when Betke method was used. However no associations were found when HPLC method was used. Fetal haemoglobin also showed associations with antibodies to erythrocytic antigens, GLURP-R2 when both Betke ($p = 0.010$) and HPLC ($p = 0.049$) methods were used and associations with erythrocytic antigens AMA-1-FVO ($P = 0.001$), AMA-1-3D7 ($P = 0.002$), MSP-3-FVO ($p = 0.03$), and EBP-2 ($p = 0.044$) when HPLC method was used. The study found other associations that could not be established based on Betke. The findings of this study have shown that HbF associates with antibodies to pre-erythocytic and erythrocytic antigens of *P. falciparum* malaria and therefore could influence protection against malaria infection. Association between HbF and antibody responses provides useful information that informs development of vaccine for malaria on individuals with SCD.

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ABBREVIATIONS AND ACRONYMS

AMA	:	Apical membrane antigen
CSP	:	Circumsporozoite protein
DNA	:	Deoxyribose nucleic acid
EBA	:	Erythrocyte binding antigen
GLURP	:	Glutamate rich protein
HbA	:	Adult haemoglobin
HbAS	:	Sickle cell trait haemoglobin
HbF	:	Fetal haemoglobin
HbS	:	Sickle cell haemoglobin
hHPFH	:	heterocellular hereditary persistence of fetal haemoglobin
HPLC	:	High performance liquid chromatography
LSA	:	Liver stage antigen
MSP	:	Merozoite surface protein
PBNT	:	1x Phosphate buffered solution (PBS), 0.1% Bovine serum albumin (BSA), 0.05% Tween 20, 0.05% Sodium (Na) azide.
P. falciparum	:	*Plasmodium falciparum*
***Pf*EMP-1**	:	*Plasmodium falciparum* erythrocyte membrane protein 1
PCR	:	Polymerase chain reaction
SCA	:	Sickle cell anaemia
SCD	:	Sickle cell disease
TRAP	:	Thrombospondin related anonymous protein
VSA	:	Variant surface antigens
WHO	:	World health organization

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CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria infection especially *Plasmodium falciparum* remains the single most important threat to public health at a global scale, accounting for more than 90% of the world's malaria mortality (Snow, 2015). The majority of the at-risk population in Kenya (17 million people) lives in areas of epidemic and seasonal malaria transmission. Bondo in western Kenya is one of the regions where malaria transmission is intense throughout the year with *P. falciparum* prevalence at 20% to 40%. Children under five are particularly susceptible to malaria illness, infection and death as they have not developed any immunity to disease (WHO, 2016). The factors determining which children die and which survive are complex, but they are likely related to both the host and the parasite. Of the host-specific factors, the sickle cell trait (HbAS) remains the best described, having been shown to confer strong protection against *P. falciparum* malaria (Williams *et al.,* 2005); nevertheless, the protective mechanisms at work remain incompletely understood. Approximately 300,000 children are born every year with sickle cell disease (SCD) in the world, with over 75% of this population in sub-Saharan Africa (Makani *et al.*, 2013). The prevalence of SCD in western Kenya is estimated to be 1.8% (Suchdev *et al.*, 2011). Bondo in western Kenya is one of the regions that has been affected by SCA recording a prevalence of 1.6% (Suchdev *et al.*, 2014). Bondo also has an established clinic (Lwak Health Center) for SCD patients with specific clinic dates where the patients from all over the sub-county seek for management.

Fetal hemoglobin (HbF) is the main hemoglobin component in the foetus produced from the sixth week of gestation and during the rest of fetal life (Edoh *et al.*, 2006; Olaniyi *et al.*, 2010). It is present at levels of 65 to 90% at birth and usually drops to less than 1% by 6 to

12 months of age. However, HbF levels remain elevated in SCA (Andrea *et al.*, 2009), due to genetic selection and is the most powerful modulator of the clinical features of SCA (Akinsheye *et al.*, 2011). The levels do not significantly change after the age of 5 years (Bruno *et al.*, 2015). Measurement of HbF is clinically useful in the study and diagnosis of some globin gene disorders where its levels may vary considerably and in a number of acquired conditions associated with mild increase in HbF. Moreover, HbF is known to inhibit the polymerization of HbS and different agents able to increase HbF production have been introduced for therapeutic aim. A reliable monitoring of HbF concentration during the treatment and follow up of patients with SCD is therefore required (Andrea *et al.*, 2009).

Several methods have been developed for determination of HbF levels (Rosnah *et al.*, 2007), but they always give different results. The classical method for the determination of HbF is based on alkali denaturation by Betke (Andrea *et al.*, 2009). Betke is reliable, easy to perform and has an acceptable accuracy and reproducibility in the clinically relevant range of 1 to12% of HbF. Currently, the most common approach to the quantification of HbF is based on the separation of haemoglobin from other haemoglobin fractions by cation exchange high performance liquid chromatography (HPLC) (Andrea *et al.*, 2009). High performance liquid chromatography is a more sensitive and precise method for detecting abnormal haemoglobins. These methods often provide different results depending on the level of HbF. However, most hospitals in Kenya are not able to determine HbF levels using HPLC because it is quite expensive. Moreover, few studies have been done to compare HbF levels of HPLC and Betke method (Andrea *et al.*, 2009; Mallick *et al.*, 2015; Tan *et al.*, 1993). However, these studies have been done on patients with malignancies and thalassemias and no studies done in as far as HbF in adult sickle cell anaemia is concerned.

The mechanism by which sickle cell trait imparts resistance to malaria is unknown although a number of factors are involved. Red cells of patients with SCA when infected with the *P. falciparum* parasite deform most probably because the parasite reduces the oxygen tension within the erythrocytes to very low levels as it carries out its metabolism (Luzzatto, 2012). Deformed HbAS red cells are removed by phagocytes when passing through splenic sinusoids (Luzzatto, 2012). Infant resistance to malaria has previously been attributed to poor parasite growth in HbF-containing RBCs (Amaratunga *et al.*, 2011). Fetal haemoglobin and maternal *Pf*EMP-1-specific IgG work cooperatively to impair the cytoadherence of parasitized RBCs in the first few months of life, thus limiting the ability of *P. falciparum* to multiply and cause inflammation (Amaratunga *et al.*, 2011). Infants under six months of age therefore, have a low rate of infection and incidence of severe disease compared to older children, despite the relative immaturity of their immune systems (Billig *et al.*, 2012). Persons living in malaria holoendemic areas are thought to have experienced repeated natural infections since childhood and developed antibody responses to multiple pre-erythrocytic antigens.

Antibodies to blood stage antigens acting primarily on the parasites undergoing growth in the red blood cells are associated with protection against high density parasitemia and morbidity. Disease-controlling immunity to malaria is associated with the development of *Pf*EMP-1-specific antibodies (Amaratunga *et al.*, 2011). Most studies on HbF protection from malaria have been done on infants but the mechanism of protection is poorly understood. Although antibodies to blood stage antigens have been shown to have protection against malaria, no studies have been able to associate HbF values with their antibody responses to *P. falciparum* infection in older sickle cell patients whose maternal immunity has been depleted. This study therefore aimed to study the association of HbF

with the antibody responses to infection with *P. falciparum* malaria among sickle cell patients above 5 years.

1.2 Statement of the Problem

Sickle cell disease patients are protected from malaria infection. This could be due to the presence of elevated levels of HbF. However measurement of HbF levels is a challenge because they have been determined using several methods and these methods often provide different results. Of these methods, HPLC is more accurate than Betke method yet Betke method is more commonly used due to its cost effectiveness. Studies have not been done in western Kenya to compare HbF levels among sickle cell patients above 5 years old. The relationship between HbF and immune response is also poorly understood. Fetal hemoglobin cooperates with antibodies to provide protection against malaria; however, understanding this protection provided by HbF alone has been complicated because of the effect of maternal antibodies since most studies have been done on infants. Also immunity against malaria is acquired generally by the age of 5 years. Investigations have not been undertaken on SCD patients without maternal antibodies and their association with HbF on protection against malaria among the SCD patients.

1.3 Objectives

1.3.1 General Objective

To compare tests for HbF levels by HPLC and Betke and relate them with the antibody responses to *P. falciparum* malaria antigens among sickle cell patients above 5 years old in Bondo sub-County, western Kenya.

1.3.2 Specific Objectives

a) To correlate HbF levels of sickle cell disease patients above 5 years using HPLC and Kleihauer Betke methods.

b) To determine the association between HbF measured by HPLC and antibodies to selected pre-erythrocytic stage antigens of *P. falciparum* malaria among sickle cell patients (above 5 years in Bondo sub-county, western Kenya).

c) To determine the association between HbF measured by HPLC and antibodies to selected blood stage antigens of *P. falciparum* malaria among sickle cell patients (above 5 years in Bondo sub-county, western Kenya).

1.3.3 Hypotheses

a) There is no correlation between HbF levels by HPLC and Betke methods.

b) There is no association between HbF levels and antibodies to pre-erythrocytic stage antigens of *P. falciparum* malaria among sickle cell patients.

c) There is no association between HbF levels and antibodies to blood stage antigens of *P. falciparum* malaria among sickle cell patients.

1.4 Significance of the Study

The study provides important findings that informs, when a specific method can be used to test HbF levels. This provides useful information towards the best approach to determine HbF levels when values are high in areas with high prevalence of HbF. Past report has shown that HbF cooperates with antibodies at a younger age. The findings of this study therefore reconcile the past report given that this study focuses on individuals above 5 years old. Using HPLC method, the study found other associations that could not be established based on Betke method for testing HbF. Association between HbF and antibody response provides useful information that can help towards development of vaccines for malaria targeting individuals with SCD.

CHAPTER TWO

LITERATURE REVIEW

2.1 General Introduction

Malaria is a major public health problem, and continues to be an important vector-borne parasitic disease worldwide (Omondi *et al.*, 2017). Globally, 3.2 billion people are at risk of malaria (WHO, 2015) and 90% of all malaria deaths in the world today occur in Africa south of the Sahara. This is because the majority of infections in Africa are caused by *P. falciparum*, the most dangerous of the four human malaria parasites ("The burden of malaria in Africa," 2003). Malaria remains a major cause of morbidity and mortality in Kenya with more than 70% of the population at risk of the disease ("Kenya Malaria Operational Plan ", 2014). Majority of the population at risk (17 million people) lives in areas of epidemic and seasonal malaria transmission. However, an estimated 12 million people live in endemic areas, one-third of whom (~4 million people) live in areas where parasite prevalence is estimated to be equal to or greater than 40%. Endemic regions like western part of the country and the coastal region experiencing continuous transmission throughout the year and malaria is the leading cause of death in children under 5 years of age (Omondi *et al.*, 2017). Immunity against severe malaria is acquired gradually; generally by the age of 5 years (Griffin *et al.*, 2014). Bondo in western Kenya is one of the regions that has been affected by malaria recording a prevalence of 58% among children below 10 years (Jane *et al.*, 2010).

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, which results in an abnormal haemoglobin, HbS molecule (Aloni *et al.*, 2013). About 300,000 infants are born every year with major Hb disorders including more than 200,000 cases of sickle cell anaemia (SCA) in Africa (WHO, 2006). The

prevalence of SCD in western Kenya is estimated to be 1.8% (Suchdev *et al.*, 2011). Bondo in western Kenya is one of the regions that has been affected by SCA recording a prevalence of 1.6% (Suchdev *et al.*, 2014). Bondo also has an established clinic (Lwak Health Center) for SCD patients with specific clinic dates where the patients from all over the sub-county seek for management.

On deoxygenation, sickle haemoglobin undergoes a conformational change that promotes intracellular polymerisation, which leads to distortion of the normal biconcave erythrocyte disc into the distinctive and pathological crescent shape. The resulting haemolytic anaemia manifests as recurrent vaso-occlusion and organ damage that together cause substantial morbidity and early mortality (Ndeezi *et al.*, 2016). The recurrent pain and complications caused by the disease can interfere with many aspects of the patient's life, including education, employment and psychosocial development (Chakravorty *et al.*, 2014). The greatest burden is seen in sub-Saharan Africa, where more than 75% of all sickle cell disease occurs, with this proportion projected to increase by 2050 (Ndeezi *et al.*, 2016). No global data regarding the precise numbers of children born with SCD exist because, in contrast to Europe and North America, newborn screening for SCD is not available in most resource-poor countries with the highest predicted burdens. Despite the numbers born with SCD in resource-poor countries, remarkably, few detailed studies have described the clinical course and complications of the disease (Chakravorty *et al.*, 2014). Current, up-to-date information on the burden of mortality from SCD in Africa among populations with access to currently available treatments and preventive interventions is lacking (Scott *et al.*, 2011).

2.2 Sickle Cell Disease and Malaria

The relationship between SCD and malaria is complex, and remains a subject of controversy (Makani *et al.,* 2010). Sickle cell haemoglobin carriers are protected from malaria infection. Despite this advantage, individuals with SCD exhibit significant morbidity and mortality due to malaria (Kotila *et al.,* 2007), although the evidence for this is limited (Sadarangani *et al.,* 2009). Malaria is a precipitating factor for the frequent vasso-occlusive crises experienced by SCD patients and is responsible for hospital admissions (Kotila *et al.,* 2007). Prevalence of parasitaemia is lower in persons with SCA than in persons without SCA (Makani *et al.,* 2010). The sickle cell trait provides carriers with a high degree of protection against severe *P. falciparum* malaria during early life, which explains the relatively high penetrance of this mutation - in some areas reaching 30% - in sub-Saharan African communities exposed to high rates of infection with *P. falciparum* (Gerardo *et al.,* 2005).

In Africa, SCA is estimated to contribute to an equivalent of 5% of under-five deaths, with only half of the affected children living beyond their fifth birthday (Mpalampa *et al.,* 2012). The prevalence of sickle cell trait in western Kenya is estimated to be approximately 17.4%. This high level of sickle cell carrier state in this region is thought to be maintained by the high malaria prevalence. Prevalence of *P. falciparum* infection among children below 10 years has been recorded high in Bondo sub-county with a prevalence of 58% (Jane *et al.,* 2010). Descriptions of the natural history and clinical spectrum of SCD in Africa are surprisingly scarce and, as a result, most treatment recommendations for the management are based on studies conducted in resource-rich countries (Sadarangani *et al.,* 2009).

The HbS allele apparently does not prevent infection but results in impaired entry and growth of the parasites during the erythrocytic stage of development, enhanced removal of the parasitized variant RBCs and reduced rosette formation (Walter *et al.*, 2013). When heterozygote's are infected, the merozoites, which have a high metabolic rate, consume a lot of oxygen and this leads to sickling under low oxygen tension. The spleen removes these sickled cells before the micro-organisms have a chance to produce a large infectious population in the body. It is this selective heterozygote advantage that maintains HbS gene at a higher level in malarial than in non-malarial environments (Walter *et al.*, 2013). Although the principle of SCT protection against severe malaria as the factor accounting for the high levels of SCT in some parts of the world is now generally accepted, the details of this interaction are much less known (Elguero *et al.*, 2015).

2.3 Fetal Haemoglobin Levels and Sickle Cell Disease

Fetal haemoglobin consists of two α and two γ chains and is the major type of Hb in the fetus and the newborn (Adekile *et al.*, 1993; Andrea *et al.*, 2009). It is produced during the sixth week of gestation and during the rest of fetal life (Andrea *et al.*, 2009). The level of HbF in a newborn baby varies from 65 to 90% at birth but drops to less than 1% by 6 to 12 months of age (Andrea *et al.*, 2009). In normal adults, synthesis of HbF is restricted to a small population of erythrocytes termed F cells. Babies with SCA are born with a high level of HbF (85-95%) and its decrease after birth is considerably slower than in normal infants. The levels of HbF in children born with SCA vary considerably but appear rather constant in each patient after the age of 5-7 years (Adekile *et al.*, 1993). Fetal hemoglobin influences the clinical course and disease severity of malaria in SCA and populations with high HbF levels have less severe disease with fewer complications and better survival (Mpalampa *et al.*, 2012; Makani *et al.*, 2011).

Increased production of HbF can ameliorate the severity of SCD (Persson *et al.*, 2013). Fetal haemoglobin levels can be evaluated by counting the number of F cells, that is, adult erythrocytes that contain measurable amounts of this haemoglobin. The HbF concentration and its distribution in red blood cells are major genetic modulators of SCD and high levels of this haemoglobin dilute the amount of HbS thereby inhibiting or delaying the polymerization process, the result of which is fewer harmful events (Carrocini *et al.*, 2011). The strong relationship existing between percentage HbF level and disease severity in SCA suggests that baseline measurement of percentage HbF is paramount in predicting important aspects of clinical course (Olaniyi *et al.*, 2010). The determination of HbF is therefore an important step in haemoglobin studies as detection and quantification of increased levels are important for genetic counselling and prognosis in β-thalassaemia and SCA.

An increased level of HbF may be associated with a milder phenotype. Moreover HbF is known to inhibit the polymerization of HbS (Andrea *et al.*, 2009). Since the range of HbF may vary from 1% to 95%, no single method is accurate for its quantitation over the whole range (Old *et al.*, 2012). Several laboratory techniques have been developed for both qualitative and quantitative hemoglobin analysis but they are laborious, have long analysis time and are operator-dependent (Rosnah *et al.*, 2007). The methods available for HbF determination include alkali denaturation, immunological techniques (by immunodifusion, ELISA and immunoradiometric), Immunoelectrophoresis, or column chromatography (Prehu *et al.*, 1998; Old *et al.*, 2012). The classical method for the determination of HbF is that based on the alkali denaturation (Andrea *et al.*, 2009). This method is reliable, easy to perform and has an acceptable accuracy and reproducibility in the clinically relevant range (1-12%) of HbF (Prehu *et al.*, 1998). The method relies on the resistance to denaturation by alkali of HbF compared to HbA, the denaturation being activated by the ionisation of buried, weakly acidic side chains present in HbA and not in HbF. Alkali denaturation is a

kinetic test to be performed only under well standardized conditions in order to obtain reproducible results (Andrea *et al.*, 2009). This method is also difficult to use for large series of samples (Prehu *et al.*, 1998). Further, the staining intensity of HbF-containing cells can also be very variable and is not uniform in adults and neonates, as well as in conditions like *β*-thalassaemia and SCD (Italia *et al.*, 2007).

Flow cytometry is more objective, quantitative, reproducible, sensitive, and specific than the Betke method. High performance liquid chromatography is the most common approach to the quantification of HbF which is based on separation of Hb from other Hb fractions. It gives the best estimation of HbF over the whole range (Old *et al.*, 2012). While by Betke, resistance of all HbF is measured, in HPLC the acetylated fraction is eluted before the main fraction of HbF, thus leading to a value significantly lower (Andrea *et al.*, 2009). High performance liquid chromatography has been shown to have a high degree of precision and reproducibility (Eastman *et al.*, 1996; Tan *et al.*, 1993), and has made haemoglobin abnormality detection much more accurate, faster and automated. In the past, many of the ion-exchange column methods and electrophoresis methods could not separate HbF from other Hb variants. Most of the current ion-exchange HPLC methods separate normal levels of HbF into separate peaks. Some of these methods can also separate HbF when HbF levels are elevated (Randie *et al.*, 2009).

Although HPLC is more sensitive than Betke method, no studies have been done to compare HbF levels in adults with sickle cell anaemia.

2.4 Fetal Haemoglobin and Antibodies to Malaria Antigens

The level at which HbF persists varies widely between patients and between geographic areas but patients maintaining higher levels tend to have less hemolysis and mild clinical courses of malaria (Serjeant, 2013). Incidence of *p. falciparum* parasitemia in children

younger than 5 years is lower than in children older than 5 years. This could be due to protection from passive maternal immunity (Makani *et al.,* 2010). The presence of HbF may provide a physiologic mechanism for protection against clinical malaria. Some studies suggest that parasite growth is restricted in erythrocytes containing higher levels of HbF (Dobbs *et al.*, 2016). The HbF retards the expansion of a *Plasmodium* population because *Plasmodium* parasites do not survive as well in HbF-containing RBCs as in those containing HbA (Billig *et al.,* 2012). Fetal hemoglobin and *Pf*EMP-1-specific IgG substantially attenuate parasite virulence by weakening cytoadherence *in vivo*, which prevents the sequestration of most parasitized RBCs at the late ring stage and thus enables their removal from the bloodstream by the spleen (Amaratunga *et al.*, 2011). HbF and maternal immune IgG could produce the dramatic reductions in parasite densities and malaria episodes observed in young infants – consistent with the proposed cooperative effects of HbS and acquired immunity. Protective effects of maternal immune IgG and HbF might be reconstituted by the contemporaneous development of humoral immunity and expression of HbS in some African infants, which would cooperate to extend malaria resistance into their early childhood (Amaratunga *et al.*, 2011).

2.5 Antibodies to Pre-erythrocytic Stage Antigens of *Plasmodium falciparum*

The pre-erythrocytic stage *Plasmodium* parasite is a metabolically highly active but symptomatically silent preparatory phase of the life cycle (Duffy, 2012). Pre-erythrocytic stage vaccine targets sporozoites and liver stage parasites (Nega, 2016). Intervening to kill the parasite at this stage would prevent the symptomatic blood stage of infection, and is an attractive vaccine target for several reasons: the number of infected hepatocytes is extremely low, in the range of dozens to hundreds; human parasites like *P. falciparum* take nearly a week to complete development in hepatocytes, providing sufficient time for

elimination; unlike Plasmodium-infected red blood cells, the infected hepatocyte is capable of presenting parasite antigens to immune effector cells (Duffy, 2012).

Circumsporozoite protein (CSP) is the most abundant protein which is currently known as leading antigen for development of an anti-sporozoite vaccine. It entirely blocks infection establishment in the liver and was found to be capable of preventing clinical malaria as well as transmission. Liver stage antigens (LSAs) are candidate vaccines targeting infected hepatocytes or antigens in the liver. LSAs induced cytotoxic and cytokine –secreting CD4+ and CD8+ T cells in-vitro that killed parasites growing in the liver. These effector cells can be stimulated by dendritic cells in the lymph nodes draining the mosquito's sporozoite injection sites and then travelling to the liver where they recognize infected cells while presenting sporozoite antigens on the cell surface (Nega, 2016).

In areas where levels of transmission of *P. falciparum* are high and stable, the age-related acquisition of high-level immunoglobulin G (IgG) antibodies to pre-erythrocytic circumsporozoite protein (CSP) and liver-stage antigen 1 (LSA-1) has been associated with protection from clinical malaria. Antibody responses to pre-erythrocytic antigens contribute to or correlate with protection against *P. falciparum* infection (John *et al.*, 2005). Kenyan children with high levels of IgG antibodies to the pre-erythrocytic antigens CSP, LSA-1, and TRAP have a lower risk of developing clinical malaria than children with lower levels of these antibodies (John *et al.*, 2005).

2.6 Antibodies to Erythrocytic Stage Antigens of *Plasmodium falciparum*

Malaria disease develops during the blood-stage of infection, when the merozoite form of the parasite invades erythrocytes and replicates inside them. Intervention at this stage of the parasite's development through vaccination is likely to reduce malaria-related clinical symptoms (Mazumdar *et al.*, 2010). After repeated exposure to *P. falciparum* infection,

natural immunity is acquired that appears to prevent clinical symptoms by controlling blood-stage parasite replication. This provides a strong rationale that the development of an effective malaria vaccine is achievable. Antibodies are an important component of acquired human immunity against malaria, and key targets of these antibodies include antigens expressed by merozoites. Antibodies that target merozoite antigens are believed to be important in mediating acquired immunity. Immunity generated by candidate blood-stage vaccines function, in part, by directly inhibiting invasion of erythrocytes (Duraisingh *et al.*, 2003). As a major interface between host and pathogen, the merozoite surface is a major target for the development of a malaria vaccine. A number of potential vaccine candidate antigens identified so far are located on or associated with the surface of the merozoite or in apical organelles (Joos *et al.*, 2015).

Plasmodium falciparum invades erythrocytes by using multiple receptor–ligand interactions defined as invasion pathways. The first *P. falciparum* ligand identified that binds to erythrocytes with high affinity was EBA-175 (Duraisingh *et al.*, 2003). The EBAs are sequestered in the micronemes and include EBA175, EBA140, EBA181, and EBL1. Antibodies to EBA175, EBA140, and EBA181 are strongly associated with protective immunity in children (Persson *et al.*, 2013). Erythrocyte binding antigen 175 is a *P. falciparum* protein that binds the major glycoprotein found on human erythrocytes, glycophorin A, during invasion (Tolia *et al.*, 2005; Duraisingh *et al.*, 2003). Antibodies to the F_2 domain of EBA-175 can partially inhibit invasion of *P. falciparum* merozoites into human erythrocytes (Duraisingh *et al.*, 2003). Antibodies to the EBAs may exert a protective effect by inhibiting erythrocyte invasion, thereby facilitating control of parasitemia and decreasing associated morbidity (Richards *et al.*, 2010).

Merozoite surface proteins (MSPs) are attractive candidate antigens for vaccine development and several current vaccine candidates are recombinant MSP analogues. Merozoite surface proteins are expressed by mature intrahepatic forms and as such, are possible targets of cellular effectors (Joos *et al.*, 2015). Merozoite surface proteins displayed onto the surface of invasive merozoites are directly accessible to host immune effectors in the blood, such as antibodies, complement, neutrophils, or monocytes (Joos *et al.*, 2015). Antibodies against various recombinant MSPs have been associated with protection against clinical episodes of *P. falciparum* malaria in endemic settings. The exact function of such antibodies is still poorly understood (Joos *et al.*, 2015). The presence of high titers of cytophilic antibodies, IgG3, against the conserved region of MSP-3 has been correlated with protection against the parasite (Mazumdar *et al.*, 2010).

The Glutamate Rich Protein is a protein expressed in the hepatic, asexual and sexual stages of the parasite life cycle. GLURP of *P. falciparum* is the target of cytophilic antibodies which are significantly associated with protection against clinical malaria (Hermsen, 2007). Several immuno-epidemiological studies using sera and clinical data from various sites have consistently identified high anti-R0-GLURP immunoglobulin G (IgG) levels as significant predictors of protection against high levels of parasitaemia, and febrile malaria episodes (Lusingu *et al.*, 2005).

Apical membrane antigen is an integral membrane protein that can be divided into three domains on the basis of intra domain disulfide bonds. It is expressed in the late schizont stage of malaria parasite and is required for merozoite invasion of erythrocytes and sporozoite invasion of hepatocytes. Antibodies against AMA1 have been shown to block parasite invasion of human erythrocytes. Natural immune responses (both humoral and

cellular) against the AMA1 antigen have been observed in populations exposed to *P. falciparum* malaria (Ebrahimzadeh *et al.*, 2014).

CHAPTER THREE

METHODOLOGY

3.1 Study Area

The study was conducted at Lwak health center in Bondo sub-county, western Kenya where the prevalence of SCD is 1.6% (Suchdev *et al.*, 2014), against that of western Kenya which is 1.8% (Suchdev *et al.*, 2011). Bondo is located in Siaya County and has a population of 309,190 (Ochomo *et al.*, 2014). It borders Siaya to the North, Kisumu to the East and Lake Victoria to the South. It lies between 0° 26° to 0° 90° and at longitude 33° 58° E and 34° 35° W and covers a total of 1,972km2 out of which 972km2 is land mass while the rest 1,000km2 is water surface ("Center for media & democracy source watch ", 2012). The altitude of the district rises from 1,140 m in the eastern parts to 1, 400 m above sea level in the west (Ochomo *et al.*, 2014). The majority of the ethnic group living there are the luo. The area is holoendemic for malaria and HbF levels among these populations are unknown. Bondo has an established clinic (Lwak Health Center) for SCD patients with specific clinic dates where the patients from all over the sub-county seek for management. The study recruited 100 SCD patients. All the patients visited Lwak Health Center for their clinics where 8ml of blood sample was withdrawn from each of them in order to carry out the study.

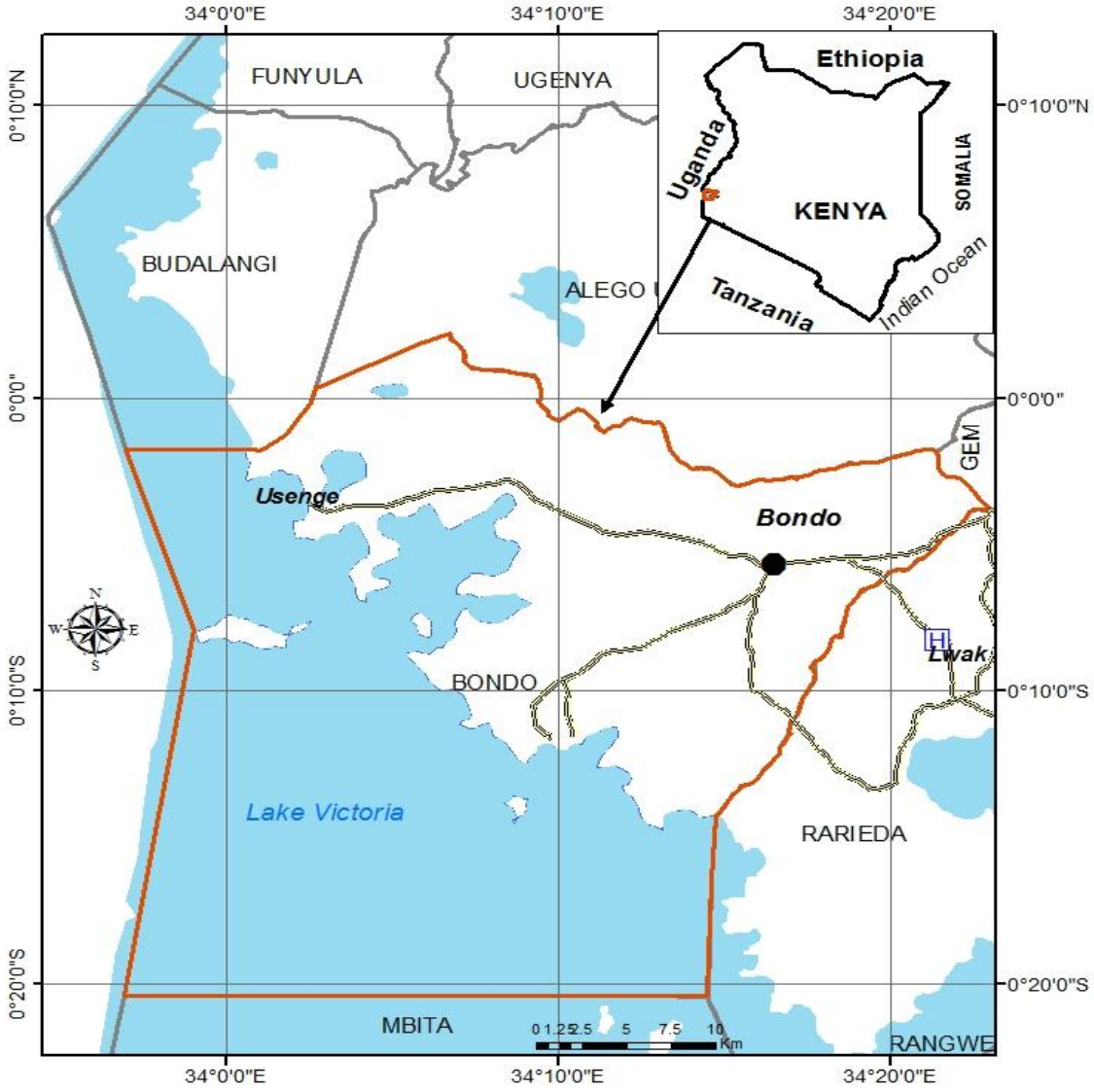

Figure3.1: Map of Study Area

3.2 Study Design

The study design adopted was cross sectional.

3.3 Study Population

The target population included confirmed SCD patients aged above 5 years attending the clinic during the study period. Polymerase chain reaction testing for HbSS was used to confirm patients who had been registered to the health facility. Patients who had been

referred from other peripheral health facilities were also included in the study once they had been tested and confirmed to have SCD.

3.3.1 Inclusion Criteria

a) SCD patients aged above 5 years old.

b) Patients from a common genetic pool (from one ethnic group).

c) Patients who had consented to the study.

3.3.2 Exclusion Criteria

a) SCD patients on treatment with hydroxyurea since hydroxurea increases levels of HbF (Kirkham, 2015).

b) Patients who had been transfused within the last 4 months.

c) Patients who had chronic infections and were severely anaemic.

3.4 Sampling Method

Purposive sampling was used to select a sample size of 100 SCD patients due to genetic selection making the size of the target population small. 200 µl of blood was used for the PCR confirmatory test after which 6ml of blood was withdrawn from each participant. The 500 µl of the blood from the SCD patients was used for determining HbF levels and 1 ml of plasma was used for antibody testing and quantification.

3.5 Sample Size

The study recruited 100 SCD patients. With the amount of fixed sample size, the detectable effect sizes of 0.28 (a two-tailed test) was expected with the 80% power and α (false-positive rate) of 0.05. The detectable effect antibody response was assumed on prevalence of antibody titres.

3.6 Laboratory Procedures

3.6.1 Genomic DNA Extraction of HbSS

Blood samples collected in EDTA tubes were centrifuged at 2500 xg 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 of QIAGEN protease K into the bottom of a 1.5 ml micro-centrifuge tube. 200 µl of the buffy coat was added followed by addition of buffer AL which was mixed by pulse vortexing and incubated at 56 ^{o} C for 10 minutes. Absolute ethanol (96%) was added and pulse vortexed for 15 seconds. The mixture was applied into the QIAamp mini spin column and centrifuged at 6000xg for 10 minutes after which the filtrate was discarded. DNA which adhered to the spin column was washed with 500 µl of buffer (wash buffer 1 and 2), centrifuged at 6000xg for 1 minute, and 20000xg for 3 minutes respectively. Finally, 200ul of the elution buffer was added, incubated at room temperature for 1 minute and centrifuged at 6000xg for 1 minute. The quantity and purity of the extracted DNA were done using the nanodrop 2000 spectrophotometer (Inquaba biotec) and stored at -20 ^{o} until use.

3.6.2 Amplification and Digestion of Genomic DNA

The extracted genomic DNA fragment from each sample was amplified using PCR amplification of a 457bp fragment. The wild type allele produced two fragments of 268 and 189bp and the mutant allele was not digested (only one fragment of 457bp). The 1 µl of each forward (5'- GATATATCTTAGAGGGAGGGCTGAG-3') and reverse (5'-AGACCAATAGGCAGAGAGAGTCAG-3') primers was premixed with dNTPs. 12.5 µl of Gotaq DNA polymerase (Promega, USA) was added to 2 µl of the DNA template which formed a final volume of 25 µl PCR reaction mixture. The PCR amplification reactions was performed in a 96- well plate thermocycler (My Cycler ^{TM} Biorad, 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. PCR amplicons was purified and used for digestion. 10 µl of the digestion master mix containing 1 µl of Neb3 buffer, D*deI* enzyme (NEB, UK Ltd) and PCR water was aliquoted in PCR tubes after which 10 µl of purified DNA amplicons was added and centrifuged at 1200 rpm for 2 minutes and then incubated in a thermocycler with the following 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 pre-stained 2% agarose gel for visualization with a UV transilluminator.

3.6.3 Determination of Fetal Hemoglobin Levels by Betke

Two independent tests were carried out to determine the levels of HbF in the patients and a third test was carried out when the difference between test 1 and test 2 was greater than five. The test relies on the resistance to denaturation of the HbF by alkali being activated by the buried weakly acidic side chains of the haemoglobin molecule (Betke *et al*., 1959). Blood samples were centrifuged at 2500xg for 10 minutes to obtain the hemolysate. The hemoblobin obtained in the hemolysate was first transformed into a more stable form of cyanmethemoglobin by adding 1.3ml of drabkin's solution to the hemolysate which was left to stand for 5 minutes. 50µl of 1.2 N NaOH was added to 2.8 ml of cyanmethemoglobin. The reaction was stopped immediately after 2 minutes by the addition of 0.5 ml of saturated ammonium sulphate solution and then filtered. The optical density of the filtrate and control solution which was isotonic sodium chloride solution were measured at 540 nm on a spectrophotometer ELISA reader (Molecular devices Sunnyvale, CA). The percentage of fetal haemoglobin were calculated by the following formula,

$$\%\text{HbF level} = \frac{\text{OD 540 nm of the test solution X 100}}{\text{OD 540 nm of control solution (NaCl) X 20.}}$$

High HbF was defined as fetal hemoglobin ≥ 10% as used in earlier studies (El-Hazmi, 1992).

3.6.4 Determination of Fetal Hemoglobin Levels by HPLC

Levels of fetal haemoglobin were determined using the Biorad VARIANT β-Thalassemia short program that utilizes the principle of HPLC. 5µl of blood sample was pipetted into separate 1.5ml sample vials. 1.0ml of haemolysis reagent was added to each sample vial and the contents mixed. The sample vials were then loaded into wells and were sequentially injected into the analysis stream at 6.5 minute intervals. Two dual piston pumps and a pre-programmed gradient controlled the elution buffer mixture passing through the analytical catridge. HbF was separated based on their ionic interaction with the cartridge material. A dual wavelength filter (415 and 690 nm) monitored the haemoglobin elution from the catridge. The separated HbF passed through the flow cell of the filter photometer where the changes in absorbance at 415 nm were measured. The additional filter at 690 nm was used to correct the background absorbance. Any changes in absorbance were monitored and displayed as a chromatogram of absorbance versus time. Analysis data from the detector was processed by the built-in-integrator.

3.6.5 Antibody Testing

Antibody testing and quantification was carried out in the University of Minnesota/KEMRI Laboratories, Kisumu. Quantification of IgG antibodies against *P. falciparum* pre-erythrocytic antigens (LSA-1 and CSP) and *P. falciparum* erythrocytic antigens (AMA-1-FVO, AMA-1-3D7, MSP-42-FVO, MSP-3-FVO, MSP-42-3D7, EBA-175, GLURP-R2, GLURP-RO and EBP-2) was done by the Multiplex Suspension Array Technology (SAT) referred by the commercial name Bio-plex or Luminex based upon the traditional flow cytometry technology. The volume of working solution (50µl / well) was

determined together with the number of beads that would result in 1000 beads/region/well or 5000 beads/region/well. The bead stocks were then combined in a 15 ml amber tube and diluted with a phosphate buffer, bovine serum albumin, sodium azide tween 20 solution (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 (obtained from centrifuged anti-coagulated blood) was added to each 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 microtiter shaker (IKA® MTS, Walmington, 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 100µl/well of PBNT before the beads were resuspended in 50µl 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 microtiter 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 100µl/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.

3.7 Data Management and Analysis

Data collected was entered and stored into an excel sheet for processing. Statistical analysis was done using Graph pad Prism 6 and SPSS. Fetal haemoglobin levels were reported in median and range. Mann whitney test was used to compare the median HbF levels obtained in SCD patients while unpaired t tests were used to compare mean IgG responses to pre-erythrocytic and erythrocytic antigens in the SCD patients. Pearson's correlation test was used to correlate the HbF levels obtained by HPLC and Kleihauer Betke methods. Linear regression analysis was used to determine the association between HbF and antibodies to pre-erythrocytic stage antigens and erythrocytic stage antigens of *P. falciparum* malaria. In all cases, p values <0.05 was considered statistically significant.

3.8 Ethical Considerations

Ethical approval was obtained from Maseno University Ethical Review Committee (Appendix VI) and authorization to collect data from the School of graduate studies (Appendix VII). All procedures were carried out in accordance with the international guidelines for the protection of human subjects. A basic background about the study was given. Informed consent (Appendix IV) and informed assent (Appendix III) was obtained before the study from all the participants. The study participants were also free to withdraw consent at any given time they wished to do so. The study participants were assigned a study number so that the specimen could not be linked to an individual (Appendix V) and the information and laboratory results were kept confidential. Data was stored under password protection and only key study personnel had access to the information.

CHAPTER FOUR

RESULTS

4.1 Demographic and Clinical Characteristics of Sickle Cell Disease Patients

The number of females enrolled in the study was 53 while that of males was 47. The age range of the patients was 5-30 years and with a mean of 10.75 (Table 4.1). The mean age of males was slightly higher than that of the females (11.28 vs. 10.28). The population consisted of 10 patients who were above 18 years old and 90 patients below 18 years old.

Table 4.1: Demographic information of SCD patients

	Males	**Females**	**Total**
Mean Age (range)	11.28 (5 – 30)	10.28 (5 – 30)	10.75 (5 – 30)
Gender (%)	47 (%)	53(%)	100 (%)

Table 4.1: The SCD patients were between 5-30 years of age with a mean age of 10.75. The number of males was 47 with a mean age of 11.28, while the number of females was 53 with a mean age of at 10.28.

4.2 Fetal Hemoglobin Levels

4.2.1 Detection of Fetal Hemoglobin Levels by Betke Method

Fetal hemoglobin levels were determined by Betke. The minimum level of HbF for the study population was 1.44% with the females having a minimum level of 2.15% while the males having a minimum level of 1.44%. The maximum level of HbF for the study population was 56.25% with the females having a maximum level of 45.88% while the males having a maximum level was 56.25%. The median HbF for the study population was 17.67 with the females having a median of 16.68 while the males having a median of 18.29 (Table 4.2).

Table 4.2: Fetal hemoglobin levels determined by Betke Method

Gender	HbF Range	Median
Males	1.44 – 56.25	18.29
Females	2.15 – 45.88	16.68
Total	1.44 – 56.25	17.67

Table 4.2: Levels of HbF for the males were between 1.44 and 56.25 with a median of 18.29. Levels of HbF for the females were between 2.15 and 45.88 with a median of 16.68. Levels of HbF for both males and females were between 1.44 and 56.25 with a median of 17.67

High HbF was defined as HbF above 10%. High HbF levels were reported in 77% of SCD patients while low levels were reported in 23% of the SCD patients. Out of the 53 female patients, 77% (n=41) had high levels of HbF and out of the total 47 male patients, 77% (n=36) also had high levels of HbF. This means that the percentage levels of HbF were the same for both the male and the female patients. The number of males with low HbF levels was 11 while the number of females was 12. Percentage levels for low HbF levels were also the same for the male and female patients at 23% (Table 4.3). The median HbF level for the study population for HbF greater than 10% was 19.34 while that of HbF less than 10% was 5.63.The median for high HbF levels of male patients was 22.01% while that of the female patients was 18.93%. The median for low HbF levels of the male patients was 5.57% while that of the female patients was 6.12%. (Table 4.3)

Table 4.3: High and Low Fetal hemoglobin levels determined by Betke Method

	Males			Females			Total		P values
	N	%	Median	N	%	Median	N	Median	(Median)
HbF > 10%	36	77	22.01	41	77	18.93	77	19.34	0.140
HbF < 10%	11	23	5.57	12	23	6.12	23	5.63	0.430
Total N	**47**	**100**			**53**	**100**		**100**	

Table 4.3: Mann whitney test; no significant difference in median HbF values > 10% in males versus female (p = 0.140) and in HbF values < 10% (p = 0.430). High HbF levels (HbF > 10%) were reported in 77% of SCD patients with a median of 19.34; the number of males being 36 with a median of 22.01, while the number of females being 41 with a median of 18.98. Low HbF levels (HbF < 10%) were reported in 23% of the SCD patients with a median of 5.63; the number of males being 11 with a median of 5.57, while the number of females being 12 with a median of 6.12.

4.2.2 Detection of Fetal Hemoglobin Levels by HPLC Method

Levels of HbF were also determined using HPLC. The minimum level of HbF was 0.7% while the maximum level was 21.4%. The minimum level of HbF for the females was 0.7% while the maximum was 21.4%. The minimum level of HbF for the males was 0.9% while the maximum was 19%. The median HbF of the study population was 5.65. The median HbF for the females was 5.7 while that of the males was 5.6. (Table 4.4)

Table 4.4: Fetal hemoglobin levels determined by HPLC method

Gender	HbF range	Median
Male	0.9 – 19.0	5.6
Female	0.7 – 21.4	5.7
Total	0.7 – 21.4	5.65

Table 4.4: Levels of HbF for the males were between 0.9 and 19.0 with a median of 5.6. Levels of HbF for the females were between 0.7 and 21.4 with a median of 5.7. Levels of HbF for both males and females were between 0.7 and 21.4 with a median of 5.65

High levels of HbF were reported in 18% of SCD patients while low levels were reported in 82% of the SCD patients. Out of the 53 female patients, 18.87% (n=10) had high levels HbF while 17.02% (n=8) of the total 47 male patients had high levels of HbF. On the other hand, low levels of HbF were observed in 81.13% (n=43) of the female patients and 82.98% (n=39) of the male patients. The median HbF level for the study population for HbF levels greater than 10% was 12.5 while the median HbF levels for values less than 10% was 4.95 (Table 4.5). The median for high HbF levels of male patients was 12% while that of the female patients was 12.9%. The median for low HbF levels of the male patients was 5% while that of the female patients was 4.9% (Table 4.5).

Table 4.5: High and Low Fetal hemoglobin levels determined by HPLC method

	Males			**Females**			**Total**		**P-value**
	N	**%**	**Median**	**N**	**%**	**Median**	**N**	**Median**	**(Median)**
HbF > 10%	8	17	12.0	10	19	12.9	18	12.5	0.593
HbF < 10%	39	83	5.0	43	81	4.9	82	4.95	0.474
Total N	**47**	**100**		**53**	**100**			**100**	

Table 4.5: Mann whitney test; no significant difference in median HbF values > 10% in males versus female (p = 0.593) and in HbF values <10% (p = 0.474). High HbF levels (HbF > 10%) were reported in 18% of SCD patients with a median of 12.5; the number of males being 8 with a median of 12.0, while the number of females being 10 with a median of 12.9. Low HbF levels (HbF < 10%) were reported in 82% of the SCD patients with a median of 4.95; the number of males being 39 with a median of 5.0, while the number of females being 43 with a median of 4.9.

4.2.3: Correlation of Fetal Hemoglobin Levels by HPLC and Betke Methods

The relationship between Betke and HPLC was determined using Pearsons r correlation. Graph pad prism 5 was used to analyze this correlation. There was no correlation between HPLC and Betke and no statistical significance was found (r = -0.016, p = 0.87; Figure 4.1).

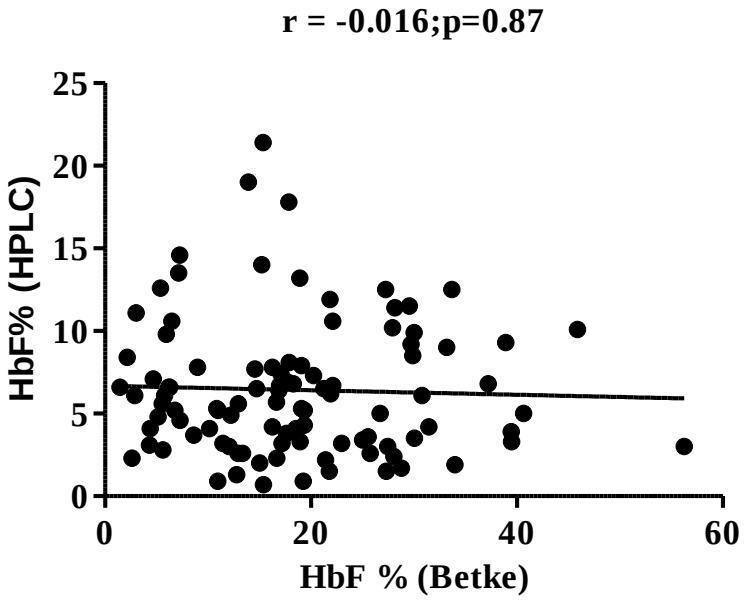

Figure 4.1: Pearsons r correlation of HbF concentrations measured by HPLC method against HbF concentrations measured by Betke's method. There was no correlation and no statistical finding between HPLC and Betke. Correlation coefficient r = -0.016; $p = 0.87$.

4.3. Pre-erythrocytic IgG Levels in SCD patients

Median flourescence intensity (MFI) values from SCD patients were log transformed using $\log_{10}$. The mean ± SEM for LSA-NRC was 3.689 ± 0.056 while that of CSP was 3.170 ± 0.045 (Table 4.6). The mean MFI values for each of the pre-erythrocytic IgG levels in males were higher than that of females (Table 4.7).

Table 4.6: Mean MFI values for pre-erythrocytic IgG levels in SCD patients

Pre-erythrocytic antigens	Mean ± SEM
LSA-NRC	3.689 ± 0.056
CSP	3.170 ± 0.045

Table 4.6: The mean MFI values for LSA-NRC was 3.689 ± 0.056 while that of CSP was 3.170 ± 0.045

Key: SEM - Standard error of mean, LSA- NRC- Liver stage antigen with C- and N-terminal flanking domains and two of the 17 amino acid repeats from the central repeat region, CSP - Circumsporozoite protein

Table 4.7: Mean MFI values for pre-erythrocytic IgG levels in male and Female SCD patients

Pre-erythrocytic antigens	Males	Females	P value
LSA-NRC	3.73 ± 0.06	3.65 ± 0.06	0.45
CSP	3.18 ± 0.06	3.16 ± 0.06	0.84

Table 4.7: The mean MFI values for males were higher than that of females, but not statistically significant, unpaired T test.

4.4 Mean MFI Values for Erythrocytic IgG Levels in SCD Patients

The highest mean ± SEM was expressed by MSP-42-FVO (4.087 ± 0.052) while the lowest mean was expressed by MSP-3-FVO (3.098 ± 0.050) (Table 4.8). There was no statistical significance in mean MFI values for erythrocytic IgG levels for males and females (Table 4.9).

Table 4.8: Mean MFI values for erythrocytic IgG levels in SCD patients

Erythrocytic antigen	Mean ± SEM
AMA-1- FVO	3.753 ± 0.049
AMA-1-3D7	3.771 ± 0.048
MSP-42-FVO	4.087 ± 0.052
MSP-3-FVO	3.098 ± 0.050
MSP-42-3D7	4.009 ± 0.048
EBA-175	3.554 ± 0.057
GLURP-R2	3.890 ± 0.059
GLURP-RO	3.387 ± 0.069
EBP-2	3.828 ± 0.046

Table 4.8: The highest mean MFI value for erythrocytic IgG level was expressed by MSP-42-FVO while the lowest mean was expressed by MSP-3-FVO.

Key: SEM – Standard error of mean, AMA - Apical membrane antigen, MSP- Merozoite surface protein, EBA – Erythrocyte binding antigen, GLURP – Glutamate rich protein, EBP – Erythrocyte binding protein

Table 4.9: Mean MFI values for erythrocytic IgG levels in male and female SCD patients

Erythrocytic antigen	Males	Females	P value
AMA-1- FVO	3.85 ± 0.06	3.67 ± 0.07	0.07
AMA-1-3D7	3.85 ± 0.06	3.70 ± 0.07	0.13
MSP-42-FVO	3.92 ± 0.07	3.86 ± 0.07	0.52
MSP-3-FVO	3.02 ± 0.07	3.18 ± 0.07	0.12
MSP-42-3D7	3.96 ± 0.07	4.07 ± 0.07	0.26
EBA-175	3.58± 0.08	3.53 ± 0.07	0.65
GLURP-R2	3.97 ± 0.07	3.82 ± 0.08	0.19
GLURP-RO	3.29 ± 0.09	3.49 ± 0.10	0.16
EBP-2	3.84 ± 0.07	3.82 ± 0.06	0.78

Table 4.9: *P*- < 0.05 significant, unpaired T test, there was no statistical finding in mean MFI values for males and females.

4.5 Association between HbF Levels and IgG Antibodies to Pre-erythrocytic Stage Antigens of *P. falciparum* Malaria

Linear regression analysis was used to determine associations between HbF and IgG antibodies to pre-erythrocytic antigens of *P. falciparum* malaria among SCD patients. Statistical significance were demonstrated by CSP ($p = 0.040$) and LSA ($p = 0.001$) when Betke method was used to measure levels of HbF while there was no statistical significance demonstrated by CSP ($p = 0.175$) and LSA ($p = 0.118$) when HPLC method was used to measure levels of HbF (Table 4.10).

Table 4.10: Association between HbF values and IgG antibodies to pre-erythrocytic antigens of *P. falciparum* malaria among the sickle cell population.

Pre-erythrocytic antigens	Betke p-value	HPLC p-value
CSP	**0.040**	0.175
LSA-NRC	**0.001**	0.118

Table 4.10: Linear regression analysis of HbF values and IgG antibodies to pre-erythrocytic antigens of *P. falciparum* malaria. Statistical significance was demonstrated by CSP (p = 0.040) and LSA-NRC (p = 0.001) with the Betke method while with HPLC there were no statistical findings.

Key: CSP = Circumsporozoite protein, LSA= Liver stage antigen

4.6 Association between HbF Values and IgG Antibodies to Erythrocytic Stage Antigens of *P. falciparum* Malaria

Statistical significance was only demonstrated by GLURP-R2 (p = 0.010) when HbF values were measured by Betke method while 5 antibodies to erythrocytic antigens were statistically significant when HPLC method was used which included AMA-1-FVO (p = 0.001), AMA-1-3D7 (p = 0.002), MSP-3-FVO (p = 0.03), GLURP-R2 (p = 0.049) and EBP-2 and (p = 0.044) respectively (Table 4.11).

Table 4.11: Association between HbF levels and antibodies to erythrocytic antigens of *P. falciparum* malaria

Erythrocytic antigens	Betke p-value	HPLC p-value
AMA-1-FVO	0.270	**0.001**
AMA-1-3D7	0.272	**0.002**
MSP-42-FVO	0.129	0.474
MSP-3-FVO	0.126	**0.030**
MSP-42-3D7	0.323	0.366
EBA-175	0.188	0.089
GLURP-R2	**0.010**	**0.049**
GLURP-RO	0.146	0.068
EBP-2	0.100	**0.044**

Table 4.11: Linear regression analysis of HbF values and IgG antibodies to erythrocytic antigens of *P. falciparum* malaria. Statistical significance was demonstrated by GLURP-R2 (p = 0.010) with the Betke method while with HPLC statistical significance was demonstrated by AMA-1-FVO (p = 0.001), AMA-1-3D7 (p = 0.002), MSP-3-FVO (p = 0.03), GLURP-R2 (p = 0.049) and EBP-2 (p = 0.044).

CHAPTER FIVE

DISCUSSION

5.1 Correlation of Fetal Hemoglobin Levels by HPLC and Betke

This study sought to compare levels of HbF by HPLC and Betke among SCD patients above 5 years old. Findings of this study have shown that there is no significant difference in HbF measurement obtained by HPLC and those obtained by Betke for this study population.

From the results, there is no correlation between HbF levels obtained by HPLC and Betke. This is in disagreement with a previous study carried out in Carlifornia, USA, on children and adult patients with haemoglobinopathies and thalassaemic disorders in which linear regression analysis of HbF measured by HPLC showed excellent correlation with values obtained by Betke (Khera *et al.*, 2015). Fetal haemoglobin levels measured by HPLC also showed good correlation with HbF levels measured by Betke's method in a similar study conducted in Australia on patients with thalassaemias and haemoglobin H disease (Tan *et al.*, 1993). Similarly, a study conducted on diabetic patients in Brazil showed that HbF strongly correlated between the results obtained by HPLC and those obtained by Betke (Pardini *et al.*, 1999).

Different findings from the previous findings might have been attributed to various reasons. One is that the previous studies included all age groups while in the current study, the age group had been specified and two is that the previous studies looked at patients who had various forms of genetic diseases and HbF levels would vary among patients with haemoglobinopathies, thalassaemic disorders and haemoglobin H disease. The patients in the current study were all SCD patients. The findings of this study also differed from a study carried out on paediatric patients whereby, good correlation of HbF levels measured

by HPLC and Betke were observed when the level of HbF was greater than 2.0% with a statistical difference (Tatu *et al.*, 1997). Different findings from this study might have been attributed to two reasons; one is that the current study did not include paediactric patients but rather older patients and two is that it did not look at only levels above 2.0% but all the HbF values obtained from the patients.

The study therefore demonstrated that Betke method can still be used to determine HbF levels in remote setups. However, HPLC method would be a better option to use when HbF values obtained by Betke are above 12%, since it gives more accurate values than Betke as shown in previous studies.

5.2 Association between HbF and Antibodies to Pre-erythrocytic Antigens of *P. falciparum* Malaria

Findings of this study have shown that there is an association between HbF and antibodies to pre-erythrocytic antigens of *P. falciparum* malaria. This is in agreement with a study done in Mali which found that HbF and maternal immune IgG work cooperatively in infants to impair cytoadherence of parasitized RBCs in vivo, thereby suggesting protection from malaria (Amaratunga *et al.*, 2011). However the current study differs because associations were found with antibodies to pre-erythrocytic antigens while in the previous study done in Mali associations were found with maternal antibodies. Associations between HbF and antibodies to pre-erythrocytic antigens of *P. falciparum* malaria were only found when Betke method was used and not when HPLC method was used. This finding is different from the previous study which was done on cord blood erythrocytes that contained 79-100% of HbF in which asoociations were shown when HPLC method was used to measure HbF (Amaratunga *et al.*, 2011). The different findings in the techniques might be

attributed to the difference in levels of HbF in cord blood as compared to the levels of HbF in SCA patients.

Previous findings have demonstrated that high levels of IgG antibodies to the pre-erythrocytic antigens CSP and LSA-1 have been demonstrated to be strongly associated with a reduction in the risk of multiple related but distinct outcomes of *P. falciparum* infection in Kenyan children and adults (John *et al.*, 2008). High level of IgG antibody to LSA-1 was correlated with protection from clinical malaria (John *et al.,* 2008). However, a similar study demonstrated that antibody to pre-erythrocytic antigen CSP had not been proven to be correlated with protection against infection or clinical malaria among individuals in malaria-endemic areas in earlier studies (Zhou *et al.*, 2002). The findings of these studies however did not compare antibody levels with HbF levels. Moreover, the studies were done on all age groups unlike the current study which did not look at infants and children below 5 years. Also, the study did not consider the aspect of SCD which was done by the current study. The findings of the current study found associations with CSP and LSA suggesting that HbF could cooperate with erythrocytic antigens in protecting SCA patients from *P. falciparum* malaria.

5.3 Association between HbF and Antibodies to Erythrocytic Antigens of *P. falciparum* Malaria

The findings of this study showed that there was an association between HbF and IgG antibodies to erythrocytic stage antigen GLURP-R2 when HbF levels were measured by both Betke and HPLC method. Other antibodies to erythrocytic antigens AMA-1 FVO, AMA-1 3D7, MSP-3 FVO and EBP-2 only demonstrated associations with HbF when HPLC method was used. These findings are in disagreement with a similar study done in Ghana which showed that there is normal invasion and development of *P. falciparum* in

HbF rich RBCs and that HbF offers no protective factor against malaria during the first months of life. Though erythrocytes rich in HbF seem to be permissive to infection with *P. falciparum,* reduced cytoadherence may particularly in the presence of IgG lead to lower parasite densities due to increased clearance of infected cells in the spleen (Sauerzopf *et al.*, 2014). Different findings may be contributed by the protective role of maternal antibodies IgG antibodies as the study was done on a group of infants. Also the previous study did not look at the cooperative effect of HbF and antibodies but rather on the invasion of HbF in RBCs.

In other studies including infants and young children, serologically measured antibodies against targets such as AMA1, have been associated with protection from clinical malaria (Dobbs *et al.*, 2016). Apical membrane antigen-1-3D7 has been found to be associated with a reduced risk of clinical malaria indicating its potential as a candidate antigen for vaccine development (Dodoo *et al.*, 2011). Stronger responses to AMA-1 have been associated with protection against *P. falciparum* infection (Greenhouse *et al.*, 2011). The findings of the previous studies on AMA-1 in protection against malaria are different from the findings of the current study in that the current study did not look at their protective role but rather the associations between HbF and the antibodies that would influence protection against malaria infection. The current study however did not look at the parasitaemia levels in SCD patients.

Antibodies against various recombinant MSPs have been associated with protection against clinical episodes of *P. falciparum* malaria in endemic settings (Joos *et al.*, 2015). Immunoglobulin G3 antibodies to merozoite surface protein 2 (MSP2) have been linked to resistance to high parasitaemia and clinical disease (Riley *et al.*, 2001). Antibodies against GLURP may contribute to the control of parasite multiplication and reduction in febrile

malaria incidence in children living in an area of intense malaria transmission (Lusingu *et al.*, 2005). Antibodies to EBA's are strongly associated with protection from symptomatic malaria and high-density parasitemia (Richards *et al.*, 2010). Increased levels of antibodies towards, EBA175 have been found in HbAS children living in areas of low malarial transmission in Burkina Faso but not in children in an area of higher transmission (Verra *et al.*, 2007). The previous studies however did not determine HbF levels to establish its relationship to these antibodies. The previous study was also done in an area of low malaria transmission. The current study was done in an area where malaria is endemic and the population targeting even older SCD patients made it unique in its new findings where the levels of antibodies to erythrocytic antigens and HbF were associated. Previous studies have suggested a contribution of acquired immunity to protection in HbAS children, evidenced by the observation that the level of protection against malaria increases with age to a greater extent in HbAS than in HbAA children.

A recent study did not find any association between HbF and antibodies to erythrocytic antigens of *P. falciparum* malaria (Webala *et al.*, personal communication). Different findings here might have been attributed to the use of a different technique which is Betke only unlike the current study which compared results with both HPLC and Betke. The findings of the current study therefore suggest that HbF could cooperate with pre-erythrocytic antigens in protecting SCA patients from *P. falciparum* malaria.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of Findings

The study showed that there is no significant difference in estimation of HbF levels measured by HPLC and HbF levels measured by Betke methods among SCD patients. However, close cluster levels was observed at lower levels of HbF (<10%) than higher levels of HbF (>10%). The study has also shown that there is an association between HbF and antibodies to pre-erythrocytic antigens of *P. falciparum* malaria when Betke method is used to measure levels of HbF while there is no association when HPLC method is used. There was also an association with antibody to an erythrocytic antigen GLURP-R2 when both Betke and HPLC methods were used to measure HbF levels. The study also demonstrated that there are associations between HbF and antibodies to erythrocytic antigens AMA-1-FVO, AMA-1-3D7, MSP-3-FVO and EBP-2 of *P. falciparum*

6.2 Conclusions

a) Lack of association in HbF levels between HPLC and Betke methods suggest that either method can be used to determine HbF levels and it is important to consider that levels may have effect on the measurements.

b) Association between HbF and antibody responses to pre-erythrocytic antigens LSA and CSP highlights the role of HbF in cooperating with IgG antibodies to erythrocytic antigens in influencing protection against *P. falciparum* malaria.

c) Association between HbF and antibody responses to erythrocytic antigens, AMA-1-FVO, AMA-1-3D7, MSP-3-FVO, GLURP-R2 and EBP-2 highlights the role of HbF in cooperating with IgG antibodies to erythrocytic antigens in influencing protection against *P. falciparum* malaria.

6.3 Recommendations from this Study

a) The study recommends the use of Betke method for measurement of HbF levels in remote areas since it is cost effective as compared to HPLC.

b) HbF and antibodies to pre-erythrocytic antigens should be considered as an important entity in vaccine development in SCD patients.

c) HbF and antibodies to erythrocytic antigens should be considered an important entity in vaccine development in SCD patients

6.4 Recommendations for Further Research

a) Antibodies to pre-erythrocytic antigens CSP and LSA need to be investigated with HbF levels among SCD patients and age matched normal children to determine their protective role in prevention against *P. falciparum.*

b) Antibodies to erythrocytic antigens of *P. falciparum* malaria AMA-1-FVO, AMA-1-3D7, MSP-3-FVO, GLURP-R2 and EBP-2 need to be investigated with HbF levels among SCD patients and age matched normal children to determine their protective role in prevention against *P. falciparum.*

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APPENDICES

APPENDIX I: Genomic DNA Extraction of HbSS

PATIENT ID	DNA (ng/ul)	Volume of DNA (ul)
001	98.4	150
002	59.6	150
003	48.8	150
004	46.2	150
005	116.4	150
006	39	150
007	117.2	150
009	39.4	150
010	31.7	150
011	55.1	150
013	102.9	150
015	40.5	150
016	41.3	150
017	32.6	150
018	120.8	150
019	74.4	150
020	38.2	150
023	74.3	150
024	45.5	150
025	27.5	150
026	39.3	150
027	28.7	150
028	168.8	150
029	74.4	150
030	151.1	150
031	39	150
033	96.4	150
034	134.1	150
036	57.4	150
037	51.7	150
038	75.5	150
040	61.9	150
041	71.2	150
042	32.1	150
043	31.3	150
045	63.5	150
046	32.8	150

048	36.1	150
049	47.9	150
051	49.3	150
052	62.4	150
053	132.7	150
055	36.4	150
057	56.2	150
061	73.3	150
062	47.9	150
067	39.4	150
068	36.3	150
071	50.6	150
074	68.3	150
075	88.7	150
076	36.4	150
077	38.9	150
078	126.2	150
079	57.8	150
081	88.8	150
082	247.4	150
084	127.9	150
085	146.1	150
087	75	150
091	45.9	150
093	31.2	150
094	155.1	150
095	80	150
096	83.5	150
097	48.6	150
098	149.2	150
099	30.5	150
100	40.5	150
104	68.3	150
106	62.4	150
107	70	150
108	53.6	150
112	70.5	150
113	60.7	150
115	95.8	150
116	36.8	150
117	30.2	150
118	33	150

119	50.8	150
123	97.5	150
125	41.9	150
127	220.2	150
128	157.9	150
129	109.6	150
130	35.8	150
131	36.3	150
132	48.2	150
133	36	150
134	36.5	150
135	30.1	150
136	45.4	150
137	41.2	150
138	28.9	150
139	27.5	150
140	39.2	150
141	49	150
142	35.4	150
143	57.5	150
144	31.2	150

APPENDIX II: Amplification and Digestion of Genomic DNA

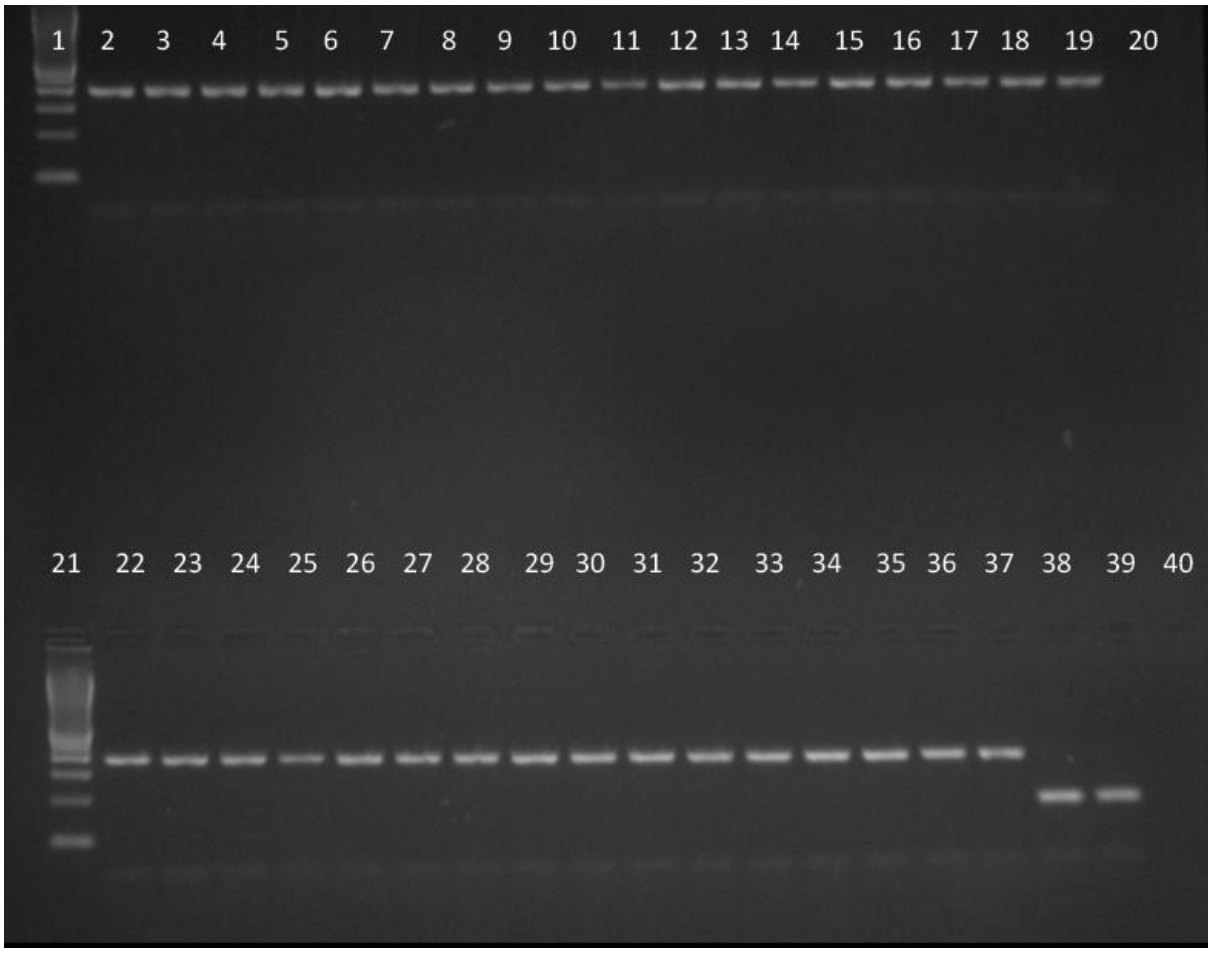


Figure of DNA products visualized under UVtransilluminator: 1 and 21 = low molecular weight DNA ladder; 2-20 = tested samples; 22-37 = tested samples; 38 and 39 = CEU gDNA control; 40 = negative control. All the test samples were positive except number 20 which was repeated.

APPENDIX III: Assent Form

English Assent for Human Investigational Studies

Study title: Comparative measurement of Fetal haemoglobin levels and their association with antibody responses to *Plasmodium falciparum* antigens among sickle cell patients in Bondo sub-County, western Kenya.

Principal Investigator: Imelda Awuor Mboya

Research Staff: Medical staff at the hospital; including a clinical officer and a laboratory technologist.

Why are we doing this study? We are doing a research study to understand causes of blood disease. To 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.

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.

Will the study help me? The study may help us understand disease of blood better, and eventually come up with better ways of preventing it. This 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.

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 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*	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 IV: Consent Form

English Consent for Human Investigational Studies

Study title: Comparative measurement of Fetal haemoglobin levels and their association with antibody responses to *Plasmodium falciparum* antigens among sickle cell patients in Bondo sub-County, western Kenya.

Principal Investigator: Imelda Awuor Mboya

Location of the study: Bondo Sub County, Western Kenya.

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 not suffer from severe form of 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 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 you 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. 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 it 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.

You / 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.

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, Imelda Mboya at Box at 072515905.

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

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 in, 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 V: Data Collection Form

1. 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*_____

*a) Name of the patient:*_____

*b) Age*_____ *Gender*_____

c)

*Location*_____*Sublocation*_____*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*

f) 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_____Hb F level_____%*

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

c) *DNA concentration _____ng/ul*

d) *Is HbS typing positive?* *Yes* *or*

No

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

No

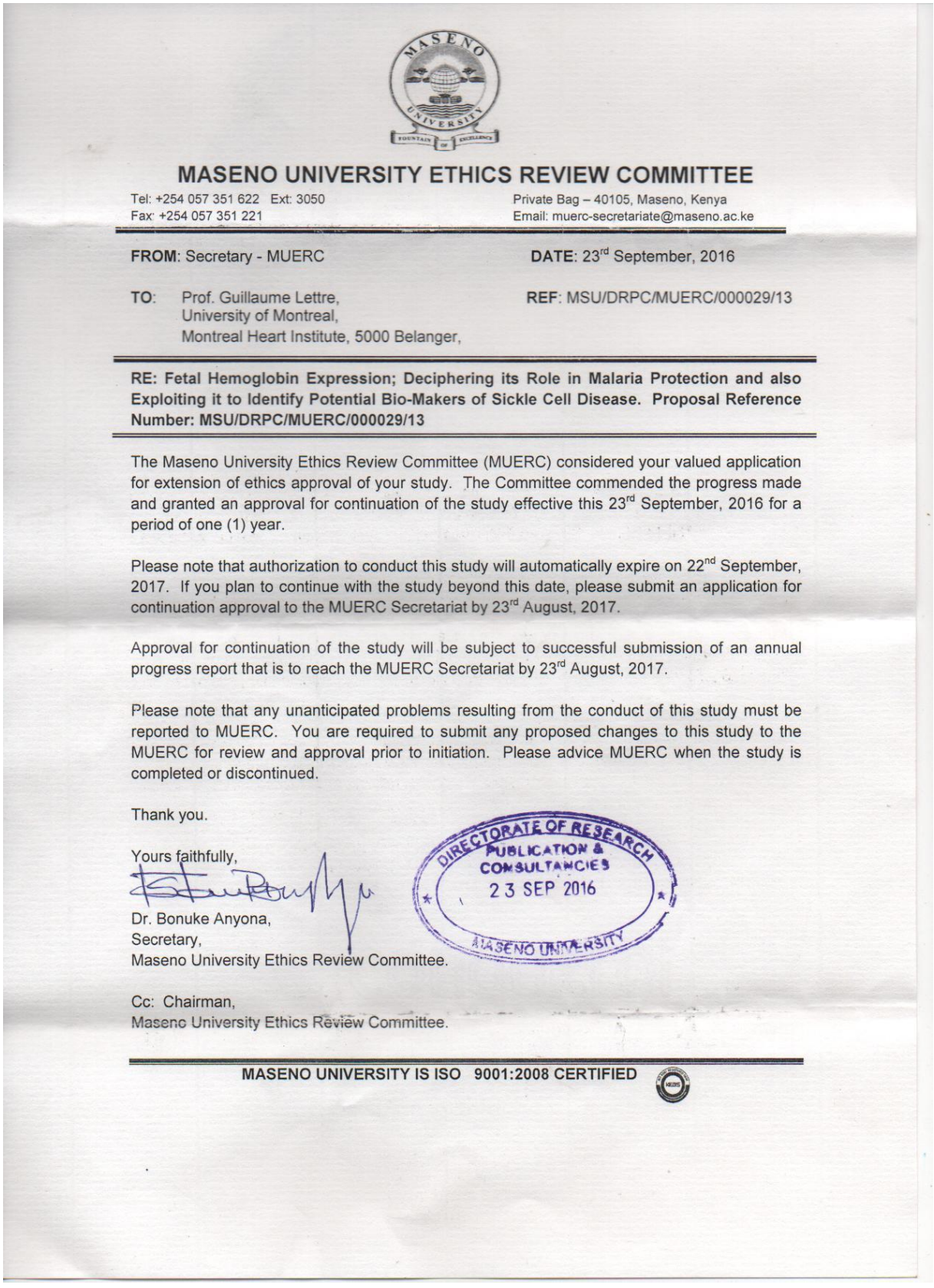
f) *Has plasma been stored at -20^{0}C* *Yes* *or*

No

g) *Are the antibodies present* *Yes* *or*

No

APPENDIX VI: 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: 23rd September, 2016

TO: Prof. Guillaume Lettre,
University of Montreal,
Montreal Heart Institute, 5000 Belanger,

REF: MSU/DRPC/MUERC/000029/13

RE: Fetal Hemoglobin Expression; Deciphering its Role in Malaria Protection and also Exploiting it to Identify Potential Bio-Makers of Sickle Cell Disease. Proposal Reference Number: 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 23rd September, 2016 for a period of one (1) year.

Please note that authorization to conduct this study will automatically expire on 22nd September, 2017. If you plan to continue with the study beyond this date, please submit an application for continuation approval to the MUERC Secretariat by 23rd August, 2017.

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 23rd August, 2017.

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,

DIRECTORATE OF RESEARCH PUBLICATION & CONSULTANCIES 23 SEP 2016 MASENO UNIVERSITY

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

Cc: Chairman,
Maseno University Ethics Review Committee.

MASENO UNIVERSITY IS ISO 9001:2008 CERTIFIED

APPENDIX VII: Proposal Approval

MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: MSC/PH/00077/014

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 30th November, 2016

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR IMELDA AWUOR MBOYA — MSC/PH/00077/2014

The above named is registered in the Master of Science programme, in the School of Public Health and Community Development, Maseno University. This is to confirm that her research proposal titled "Fetal Haemoglobin Levels by HPLC and Betke Methods and Association with Antibody Responses to Plasmodium falciparum among Sickle Cell Patients in Bondo Sub County" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

01 DEC 2016

Prof. J.O. Agure
DEAN, SCHOOL OF GRADUATE STUDIES

Maseno University *ISO 9001:2008 Certified*