ASSOCIATIONS BETWEEN SDF-1 ALPHA POLYMORPHISMS AND MALARIAL OUTCOMES IN CHILDREN AT SIAYA COUNTY REFERAL HOSPITAL, WESTERN KENYA

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A thesis submitted in partial fulfillment of the requirements for the award of the Degree of Master of Science (Biotechnology) of Kenyatta University

November, 2015
DECLARATION

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

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DEDICATION

To my mother Dorcas A. Otieno and my late father, Johnson Otieno.

Special dedication to my wife Becky Achieng’, our children Sandra Oloo, Angelah Joan and Alexiah Waudi, finally to parents and children who have died due to severe malaria anemia.
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OPERATIONAL DEFINITIONS OF KEY CONCEPTS AND TERMS

**Candidate gene approach:** variation within a specific gene and their implications on expression and its role in disease outcomes

**Genome wide association study (GWAS):** scanning of genetic markers (SNP) across the entire genome of many individuals to identify genetic variation associated with particular disease outcomes matched for population ancestry and assessed for a disease or trait of interest. Correlations between variants and the trait are used to locate genetic risk factors.

**Whole genome sequencing (WGS):** Full genome sequencing provides raw data of all the bases in an individual's DNA to analyze and detect all disease-related genetic variants

**Haplotype** - A combination of alleles at multiple linked sites on a single chromosome that are transmitted together.

**Linkage disequilibrium (LD)** - This refers to the association of alleles at two or more sites on the same chromosome that are inherited together more often than expected by chance.

**Nonsynonymous SNP** - A SNP for which each allele encodes a different amino acid in the protein sequence.

**Polymorphism** - A form of genetic variation in which each allele occurs in at least 1% of the population.

**Single nucleotide polymorphism (SNP)** - Site within the genome that differs by a single nucleotide base across different individuals.
ACRONYMS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>CCL4</td>
<td>Chemokine (C-C) ligand-4</td>
</tr>
<tr>
<td>CXCL12</td>
<td>Chemokine (C-X-C) ligand-12</td>
</tr>
<tr>
<td>CM</td>
<td>Cerebral malaria</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>EIR</td>
<td>Entomological inoculation rate</td>
</tr>
<tr>
<td>Hb</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>Hct</td>
<td>Haematocrit</td>
</tr>
<tr>
<td>HDP</td>
<td>High density parasitaemia</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>MA</td>
<td>Malaria anaemia</td>
</tr>
<tr>
<td>MF</td>
<td>Maturation factor</td>
</tr>
<tr>
<td>MOH</td>
<td>Ministry of Health</td>
</tr>
<tr>
<td>MOP</td>
<td>Ministry of Planning</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PCM</td>
<td>Pigment containing monocytes</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pfHz</td>
<td>Plasmodium falciparum haemozoin</td>
</tr>
<tr>
<td>pRBC</td>
<td>Parasitized red blood cell</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation normal T-cell expressed and secreted</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RI</td>
<td>Reticulocyte index</td>
</tr>
<tr>
<td>RPI</td>
<td>Reticulocyte production index</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>Stromal cell derived factor-1α</td>
</tr>
<tr>
<td>SCRH</td>
<td>Siaya County Referal Hospital</td>
</tr>
<tr>
<td>SMA</td>
<td>Severe malaria anaemia</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzimidine</td>
</tr>
<tr>
<td>UTR</td>
<td>Un-translated region</td>
</tr>
<tr>
<td>UNM</td>
<td>University of New Mexico</td>
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<td>WHO</td>
<td>World Health Organization</td>
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ABSTRACT

In high Plasmodium transmission regions, malaria caused by Plasmodium falciparum is the leading cause of malaria-related morbidity and mortality among children under the age of 5 years. Severe malaria anaemia (SMA) is the most common cause of malaria burden in holoendemic P. falciparum transmission regions such. Although the mechanisms underlying development of SMA are poorly understood, direct and indirect lysis of erythrocytes, suppression of erythropoiesis, erythrophagocytosis, dyserthropoiesis and dysregulation in pro- and anti-inflammatory response characterize low haemoglobin levels in malaria. Children under the age of 5 years lack naturally-acquired malarial immunity thus innate immunity provides the children with the first line of defence against P. falciparum infections. The risk for progression of malaria infection is partly accounted for by host genetic variations in immune mediators. Stromal cell derived factor (SDF)-1α (CXCL12) is an important mediator of immune, inflammatory and haematopoietic responses. In human malaria, the plasma level of SDF-1α is elevated in children presenting with cerebral malaria and mild-to-moderate anaemia. Even though several studies indicate that SDF-1α genetic variation regulate outcomes of HIV-1 infection, hematopoiesis, and cancer, the role of genetic variability in SDF-1α in P. falciparum infections has not been explored. Genotyping for SDF-1α single nucleotide polymorphisms (SNP) in the promoter (C -1002T; rs2839686) and 3' UTR (A+801G; rs1801157), DNA was extracted from buccal swabs using the BuccAmp™ DNA extraction kit (Epicentre Biotechnologies). Genomic DNA was amplified using the GenomiPhenix DNA amplification kit (GE Healthcare, Life Sciences,Amersham), and then the SNPs were genotyped using a Taqman®5′ allelicdiscrimination Assay-By-Design method according to manufacturer’s instructions (Applied Biosystems). Outcomes of P.falciparum malaria (parasitaemia, high-density parasitaemia, SMA, reticulocyte production index, RPI) in children aged <3 years old (aparasitemic, n=199 and children presenting with P. falciparum non-SMA, n=510 or SMA, n=118) at Siaya County Reffaral, Hospital. Genotype frequencies at the A+801G loci, distribution of major allele A (0.951) and minor allele G (0.049) deviated from Hardy Weinberg Equilibrium (HWE) in the overall study cohort and in each of the study groups (P=0.048). C-1002T genotype frequencies varied significantly among the study groups (TT, 64.9%; CT, 20.1%; and CC, 15.0%; P=0.008). Distribution of major allele T (0.75) and minor allele C (0.25) showed departure from HWE in the overall study cohort and in each of the study groups (P<0.017 for all comparisons). Haplotype analyses identified a total of four haplotypes (AT, AC, GT and GC). Frequencies of haplotypes in overall cohort were (AT, 68.6%; AC, 28.1%; GT, 3.0%, and GC, 0.3%). Evaluation of associations between genotypes and malaria infection status and disease outcomes, indicated that AG genotype at the A+801G loci was significantly associated with about two-times higher odds of having parasitaemia (OR=1.889; P=0.014); but 52% lower odds of developing high-density parasitaemia (OR=0.480; P=0.006). Genotype association with suppression of erythropoiesis showed that AG (OR=1.544; P=0.057) and GG (OR=6.852; P=0.034) genotypes were associated with about one-and-a-half and seven times higher odds of having RPI<2.0, respectively. While at the C-1002T loci, homozygous CC genotype was associated with 43% lower odds of having parasitaemia (OR=0.568; P=0.001). Analysis of the associations of haplotypes showed that AC haplotype carriers were 26% less-likely of having parasitaemia (OR=0.740; P=0.034). GT haplotype carriage was associated with 55% less-likely of having high-density parasitaemia (OR=0.452; P=0.029). GT haplotype was associated with two-times likelihood of having suppression of erythropoiesis (OR=1.979; P=0.026). Taken together, the findings show that genotypic and haplotypic variation at the SDF1α A+801G and C-1002T loci are involved in regulating acquisition of malaria infection and erythropoiesis in children from Siaya region, western Kenya.
CHAPTER ONE

INTRODUCTION

1.1 Background information

Human malaria is caused by five protozoan parasites of the genus *Plasmodium*. These include: *Plasmodium falciparum*, *P. knowlesi*, *P. malariae*, *P. ovale* and *P. vivax* (Cox-Singh et al., 2008; Daneshvar et al., 2009). Among these parasites, *P. falciparum* is the most widely distributed and the most pathogenic, accounting for about 98% of malaria cases and mortality in the world (WHO, 2010). In the year 2010, approximately 216 million malaria incidences and 655,000 malaria related mortalities were reported (WHO, 2011). Most of the reported malaria cases and deaths occur in Africa among children under the age of five years and pregnant women (Breman et al., 2004).

The factors governing the development of clinical manifestations of malaria include the genetic composition of the host and parasite, host age and levels of acquired immunity, malaria endemicity, environmental factors, malnutrition and presence of co-infections (Kwiatkowski, 2005; Verra, et al., 2009). Signs and symptoms of malaria during human infection by *P. falciparum* evolves from an acute infection with or without fever, headaches, shivering, vomiting, nausea, coughs, diarrhoea, athralgia and joint pains to a chronic disease featuring life-threatening complications; hypoglycaemia, hyperlactaemia,
respiratory distress, cerebral malaria and severe malarial anaemia (SMA) (Marsh et al., 1995; WHO, 2000). In western Kenya, SMA with varying density of parasitaemia is the most common clinical manifestation of severe malaria (Bloland, et al., 1999; Ong'echa et al., 2006).

Stromal cell derived factor (SDF)-1α (CXC12) is a α-chemokine. It acts through utilization of two G-protein coupled receptors, mainly CXCR4 and CXCR7 (Burns et al., 2006; Cruz-Orengo et al., 2011). Production of SDF-1α is activated by TNF, lipopolysaccharides and IL-1 in addition to other pro-inflammatory stimuli, and regulates B- and T-cell maturation, and recruitment of macrophages (Rossi and Zlotnik, 2000; Welford et al., 2011). In human malaria, plasma levels of SDF-1α are elevated in children presenting with cerebral malaria (Shao et al., 2008; Gyan et al., 2009). Since SDF-1α induces erythroid cell formation and mobilization (Gibellini et al., 2000; Lataillade et al., 2000), perturbations in its production may result in dyserythropoiesis, which may enhance development of SMA. SDF1α gene is located on human chromosome 10 (Burns et al., 2006; Cruz-Orengo et al., 2011).

Several single nucleotide polymorphisms (SNP) in the SDF1α gene regulates gene expression and outcomes of infectious diseases. For instance, rs2297630 (8906 A/G) is associated with higher plasma levels of SDF-1α in HIV-1 infected individuals, while rs266085 (6201 A/C) and rs1801157 (12197A/G) are associated with progression of HIV-1 infection (Soriano et al., 2002; Kimura et al., 2005). Homozygosity at the SDF1α rs2297630 (8906A/A) loci
is associated with haematopoiesis and circulating levels of SDF-1α (Lataillade et al., 2000; Soriano et al., 2002). General view has been that a number of SDF-1α SNPs play a major role in the outcome of a number of diseases. Thus, the study investigated the association between SDF-1α single nucleotide polymorphisms (SNP) in the promoter (C -1002T; rs2839686), 3’ UTR (A+801G; rs1801157) and outcomes of P. falciparum malaria. In addition the study will consider genetic variants previously explored in other populations and deemed to be significantly associated with malaria outcomes in children from western Kenya.

1.2 Problem statement
Malaria associated morbidity and SMA mortality among infants and young children are common phenomena in western Kenya and other holoendemic P. falciparum transmission regions (Ochiel et al., 2005; Ong’echa et al., 2006). Since genetic variability in chemokine genes are associated with outcomes of SMA and reticulocyte production, it is probable that the erythropoietic response and development of severe anaemia in children exhibiting malaria is regulated at the gene level. It is therefore important to characterize SDF1α genotypic and haplotypic variation since they are known to regulate cell homing, trafficking and development of erythroid progenitor cells (Benboubker et al., 2001; Aboumrad et al., 2007). 3’ UTR (A+801G; rs1801157) whose a allele is regarded as a target of cis-acting factors, has been shown to up-regulate the expression of CXCL12 (Hirata et al., 2007).
In addition, studies on rs1801157 have shown association with susceptibility to blast invasion in acute myelogenous leukemia (Dommange et al., 2007) sporadic prostate cancer (Hirata et al., 2007) and breast cancer (Bodelon et al., 2013; Oliveira et al., 2013). Moreover, recent studies show that the promoter variant in SDF1α -1002C/T (rs2839686) is a significant marker to chemokine pathway signalling in rheumatoid arthritis (Nazarian et al., 2012). This study therefore characterized the genotypic and haplotypic associations between SDF-1α polymorphisms and malaria outcomes in infants and young children from a malaria holoendemic area of western Kenya.

1.3 Significance of the study

The determination of the association between genetic variation in SDF-1α gene and changes in circulating levels of these cytokines, chemokine, and clinical outcomes of malaria will enhance understanding of SMA pathogenesis in children. However, genetic variability at C-1002T in the SDF-1A has not been previously investigated in Plasmodium falciparum infections.

This study explored the role of SDF-1α genetic variants in conditioning susceptibility to SMA in children in a malaria holoendemic transmission area. Defining this molecular interaction may facilitate the design of therapeutic agents and/or intervention strategies that could reduce the morbidity and mortality associated with malaria. Ultimately, the results of this study will provide insights into the regulation of erythropoiesis and understanding of SMA in paediatric populations and possibly lead to the rational development...
of new management strategies and therapeutic agents in the treatment of malaria disease.

1.4. Null hypotheses

i. There is no differences in the frequencies of $SDF1α$ gene A+801G and C-1002T genotypes and haplotypes among a parasitemic children and those presenting with non-severe malaria anaemia or severe malaria anaemia at Siaya County Referral Hospital.

ii. There is no associations between genotypic and haplotypic variation at A+801G and C-1002T loci in the $SDF1α$ gene and parasitological and erythrocytic outcomes in children presenting with $P. falciparum$ infection at Siaya County Referral Hospital.

1.5 Objectives

1.5.1 General objective

To determine genotypic, allelic, haplotypic variations at the A+801G and C-1002T loci in the $SDF1α$ gene, association with malarial outcomes in children presenting with $P. falciparum$ infection at Siaya County Referral, Hospital.

1.5.2 Specific objectives

i. To establish the frequencies of $SDF1α$ genotypes, alleles, haplotypes at the A+801G & C-1002T loci in children without malaria, those
presenting with non-severe malaria anaemia and severe malaria anaemia at Siaya County Referral, Hospital.

ii. To examine the relationships between \( SDF1a \) genotypic, haplotypic variation at the A+801G & C-1002T loci with parasitological and erythrocytic outcomes in children presenting with \( P. falciparum \) infection at Siaya County Referral, Hospital.
CHAPTER TWO

LITERATURE REVIEW

2.1 Aetiology of malaria

Human malaria is caused by unicellular obligate intracellular protozoan parasites of the genus *Plasmodium* which is a complex and multi-stage organism. Five species of malaria parasites infect humans; *Plasmodium falciparum*, *P. knowlesi*, *P. malariae*, *P. ovale*, and *P. vivax* (Cox-Singh *et al.*, 2008; WHO, 2008a). *P. falciparum* is the most virulent and causes over 98% of malaria-related morbidity and mortality in the world (WHO, 2012). The main vectors for human malaria parasites in Kenya are *Anopheles funestus* and *A. gambiae* complex (WHO, 2011; WHO, 2012). Although *P. knowlesi*, *P. malariae*, *P. ovale*, and *P. vivax* only contribute a small percentage of infection globally, malaria disease resulting from *P. ovale* and *P. vivax* is characterized by a dormant liver stage (WHO, 2011; WHO, 2012).

2.2 Life cycle of *Plasmodium falciparum*

The survival and viability of malaria parasite need specific proteins, both in insects and mammals in the extracellular and intracellular environments as well as adaptation of the parasite to evade the host immune response (Florens *et al.*, 2002). The life cycle of *P. falciparum* malaria parasite involves two stages: the vector mosquito sexual phase and the human asexual phase. The life cycle figure 2.1 is initiated following the transfer of sporozoites during a
blood meal by an infected female *Anopheles* mosquito vector (Florens *et al.*, 2002). The sporozoites last in blood for 30 minutes from where they migrate to the liver and infect liver parenchymal cells hence producing approximately 5,000-10,000 merozoites (Mayer *et al.*, 2009). The sporozoites infect hepatocyte through a stick and slip mechanism(s) involving thrombospondin-related anonymous protein (TRAP) and actin, myosin, rhoptry and cell adhesion motor protein (Mayer *et al.*, 2009) and then the parasites undergo exoerythrocytic schizogony resulting in the release of mature merozoites from this schizogonic cycle. The liver stage merozoites rapidly (~60 sec.) infect the erythrocytes and undergo erythrocytic schizogony producing blood stage merozoites (Mayer *et al.*, 2009). The erythrocytic cycle lasts 5-6 days to form nucleated schizonts and trophozoites that release about 30,000 to 40,000 infective merozoites. In the first day of the erythrocytic cycle there is formation of trophozoites which then develops into schizonts containing between 5-36 infective merozoites (Mayer *et al.*, 2009).

As the parasite feeds and divides within the RBC, the erythrocyte bursts and releases merozoites into the blood stream where they re-infect the new RBCs and the cycle is repeated. Some of the merozoites transform into macro- and micro-gametes after about 5 cycles which further multiply to provide a new generation of sporozoites (Silvie *et al.*, 2008). At this point, the mature female mosquito may have a blood meal from the infected humans containing the male and female gametes and the male gamete fertilises the female gamete resulting in the formation of a zygote (Florens *et al.*, 2002).
The zygote develops into an ookinete which penetrates the mosquito epithelial lining in the mid-gut and transforms into an oocyst which ruptures and releases cysts that migrate to the salivary gland as sporozoites (Silvie et al., 2008). The sporozoites are injected during a mosquito’s blood meal and initiate another cycle (Silvie et al., 2008).

**Figure 2.1: Malaria parasite life cycle**  
 Lifecycle of *Plasmodium falciparum*  

(Adapted from CDC's web site for laboratory identification of parasites)  

[Link to CDC's web site](http://www.cdc.gov/malaria/about/biology, accessed on November 9, 2012).
2.3 Public health importance of *P. falciparum* malaria

Tropical and sub-tropical regions of Africa, Asia, south and central America are the most affected by malaria infections figure 2.2 (WHO, 2000; WHO, 2008b). Approximately, 3.3 billion people in the world are at risk of malaria infection (Daneshvar *et al.*, 2009; WHO, 2009) and 216 million are infected with *P. falciparum* resulting in about 655,000 deaths in the year 2009 - 2012 (WHO, 2010; WHO, 2012). Sub-Saharan Africa accounts for 85% of malaria cases and 91% of malaria-related deaths in the world with South East Asia accounting for 6% while Eastern Mediterranean region contributing 3%. Of all these deaths, 86% occur in infants and children <5 years in Africa, with the remaining percentage largely contributed by pregnant women, migrants and travellers to endemic regions (WHO, 2009; WHO, 2011). An estimated 1.4 to 5.7 million SMA cases lead to about a million deaths and contribute 53% of all malaria related mortality in Africa. In the year 1990, the mortality rate, for malaria under 5 years was 8.8%; these numbers did reduce to 5.7 % in 2010 and further decrease to 2.9% was noted in the year 2012. The low mortality and morbidity rates mark a step forward towards achieving a brighter economy and healthy living in developing countries (Hamel *et al.*, 2011; WHO, 2012).
2.3.1 Malaria cases in Kenya

Malaria is an important public health burden that hinders economic development and contributes to the high poverty levels prevalent in the endemic regions of coastal and western Kenya (Hamel et al., 2011). About 8 million outpatient malaria treatments are recorded in the country’s health facilities, that is, 20% of all admissions (DMC, 2009). Each year ~34,000 children under 5 years die due to malaria infections and related complications (MoH, 2009; WHO, 2010). Between 2003-2007, mortality rate of children and infants residing in Siaya district was estimated to have decreased from 16.7% to 9.3%. In 2008, there was a massive increase in child mortality at 45% under the age of 5 years old with malaria anemia and malnutrition being major contributors (Hamel et al., 2011).

In addition there was an up-turn of malaria parasitaemia in the blood smears in year 2007 through 2008 with less significant reduction (41%) in the year 2009 (Hamel et al., 2011), compared to the steady decrease experienced in the year 2004 to 2006. This did translate into one death of a child under five years every minute around the globe (WHO, 2012). Despite all the recent interventions, malaria still remains a great public health concern (Okiro et al., 2010; Hamel et al., 2011).

2.3.2 Malaria control strategy in Kenya

Due to the vulnerability of children under five years of age and expectant women, the Kenyan government through the Ministry of Health and other
research organizations did collaborate to come up with measures to fight malaria anaemia (WHO, 2005; MoH, 2010). The policies included malaria treatment through case management, vector control by use of insecticide treated nets (ITNs) and long-lasting insecticide-treated nets (LLIN) (WHO, 2004; WHO, 2006), malaria anemia management in pregnant women and young children and infants, educational approach to malaria and prepareness to act on the epidemic through the indoor residual spraying (IRS) campaigns (WHO, 2004; WHO, 2015).

Further, a number of measures have been put in place to control and defeat *P. falciparum* malaria (Noor et al., 2009; Gitonga et al., 2012), such as recommendations by WHO on use of artemisinin-based combination therapy (ACT) as a first line of treatment is in use, though some resistance has been reported in South East Asia (Dondorp et al., 2009; WHO, 2015). Currently, a vaccine RTS,S/AS01 approach towards the malaria disease is underway in phase three trials in seven endemic African countries, of which Kenya is included and could be in use in late 2015 providing additional hope and direction in malaria management (PATH, 2010). However, no effective vaccine is yet in the market (PATH, 2010) and none of the above developments in the fight of the parasite is 100% effective (WHO, 2012).
2.3.3 Challenges on malaria management

The emergence of resistance to anti-malarial drugs [chloroquine (CQ), sulfadoxine-pyrimethamine (SP) and mefloquine (MQ)] occurred historically in western Cambodia, at the border with Thailand (Roper et al., 2004). The resistance then spread westward to reach India in the 1970s and East Africa in the 1980s to then extend to the whole African continent by 1992. This situation led to millions of deaths (Marsh K. 1998; WHO, 2014). Anti-malarial drug resistance, as defined by WHO (WHO, 2015) was often documented decades after the events of emergence or spread, leading to a gap between the
information gathered by researchers and actions taken by programme managers and implementers. When revisiting the history of CQ and SP resistance, it is clear that the response was slow (delay in changing drug regimen) and that there was an inadequate assessment of the risk and of the cost of resistance. The adoption and use of ACT led to the expectation that control and elimination of malaria could be achieved in a relatively shorter period of time. Unfortunately the emergence of artemisinin and insecticide resistance renders this optimistic scenario unlikely (WHO, 2015).

2.4 *P. falciparum* malaria pathogenesis

A wide array of clinical outcomes due to *P. falciparum* malaria infection could involve malfunctioning of the immune system (WHO, 2010), which vary from minor to complicated life-threatening conditions. This includes fever, sweating, chills, headache, muscle aches, vomiting, diarrhoea, athragelea and joint pain to respiratory distress, metabolic acidosis, cerebral malaria (CM), kidney failure, hyperparasitaemia, SMA, and hyper-lactetamia (WHO, 2010). Manifestation of coma is largely related to CM (WHO, 2010). Determinants of the definitive outcome of the malaria pathophysiology depends on a number of factors, such as parasite virulence, multiplication rate, age of the host, host genetic makeup, endemic patterns and antigen functional variants (Abdalla and Pasvol, 2004).
2.4.1 Pathogenesis of severe malaria anaemia

If untreated, *P. falciparum* infection leads to progression of symptoms such as coma, low blood haemoglobin, severe breathing and low blood sugar to severe anemia which finally leads to death (Berkley et al., 2005; Bassat et al., 2009). The mechanisms governing development of SMA include direct and indirect lysis of RBCs, haemoglobinopathies, erythrophagocytosis, dyserythropoiesis, suppression of erythropoiesis, bacteraemia and HIV-1 co-infections, malnutrition of essential vitamins (Carter et al., 2005; Ong'eche et al., 2006; Calis et al., 2008; Davenport et al., 2010; Novelli et al., 2010; Were et al., 2011). The key factors being RBCs hemolysis and suppression of erythropoiesis which could be due to, dysregulation in the balance between pro- and anti-inflammatory cytokines, effector molecules and growth factors in children residing in endemic areas (Perkins et al., 2000; Were et al., 2009).

2.5 Inflammatory mediators and effector molecules in malaria pathogenesis

The balance between pro- and anti-inflammatory mediators must be modulated at all times of immune response to malaria infection for a better malaria outcome and control of parasitaemia (Winkler et al., 1998b). Tumor necrosis factor (TNF) -1α and interferons (IFN)-γ Th1 responses are known to control parasitaemia in children and adults (Kremsner et al., 1995). Uncontrolled production of these inflammatory molecules is associated with anaemia (Perkins et al., 2000; Lyke et al., 2004). Furthermore, over-production of TNF-α, IFN-γ and NO promotes development of malaria
anaemia, mainly through dyserythropoiesis, erythrophagocytosis and bone
marrow suppression (Clark et al., 2003). Nitric oxide (NO) suppresses TNF-α
which in turn suppresses the production of SDF-1α. This promotes severe
malaria through enhanced parasitaemia hence increased hemolysis of RBCs as
well as suppression of the progenitor cells development that could lead to
SMA (Clark and Cowden, 2003). NO is elevated in children with malaria
anaemia and can cause direct suppression of erythropoiesis (Clark and
Cowden, 2003). Studies on IL-1 have shown that immunoregulatory Th1
cytokine act as the first line of defence to malaria parasites (Dinarello, 2004).
Synergy between IL-1α, β isotypes and TNF-α do lead to elevated production
of IFN-γ and NO in murine malaria (Rockett et al., 1994). A previous study in
western Kenya showed elevated levels of IL-1β in children with non-SMA
compared to those with SMA (Ouma et al., 2008). In addition, previous
studies in Ugandan children, showed that increased plasma levels of IL-1β is
associated with CM (John et al., 2006; Prakash et al., 2006).

2.6. Genetic resistance to malaria
Various studies have attempted to identify factors that promote disease
development in only a fraction of the exposed population (Marquet et al.,
2008; Ong'eucha et al., 2011; Ouma et al., 2012). The significance played by
host genetics in disease development has been difficult to assess because of
several environmental factors and exposure rate, to a number of infectious
diseases (Aidoo et al., 2002; Kifude et al., 2007). A number of variations in
cytokine, chemokine, effector molecule and immune receptor, RBC membrane
surface antigen, Hb and enzyme genes confer resistance against malaria infection and disease outcomes (Verra et al., 2009; Lopez et al., 2010; Zhang et al., 2010; Ouma et al., 2012). Furthermore, selective pressure exerted by other infectious diseases have also led to spread of these polymorphisms in holoendemic regions in the world (Aidoo et al., 2002; Cowman and Crabb, 2006).

2.6.1 Red blood cell polymorphisms

Heterozygous and hemizygous glucose 6-phosphate dehydrogenase (G6PD) deficiency confer ~50% protection against development of *P. falciparum* infections (Ruwende et al., 1995; Mombo et al., 2003). The mechanism of resistance conferred by G6PD deficiency occurs through enhanced phagocytosis of infected RBCs before release of merozoites into the circulation (Mockenhaupt et al., 2003). Ovalocytosis which results from a 27-base pair deletion coding for membrane protein band-3, and variations in the complement receptor protein, duffy and Gerbich blood groups have been associated with SMA and CM (Cortes et al., 2004; Cortes et al., 2005).

Haemoglobin (Hb) S, C and E and impaired α-, β-globin production are common regulators of malarial outcomes in endemic regions of the world (Aidoo et al., 2002; Doolan et al., 2009). HbS is an autosomal recessive disorder that involves a change from Glu to Val in the amino acid sequence at position 6 of the β-globin chain and carriers of the sickle cell trait (HbAS) are
protected against development of high density parasitaemia, SMA, and CM (Kreuels et al., 2009; Verra et al., 2009). HbAC -CC, and HbAE, -EE benefit from partial protection to full protection, largely through sickling of the RBCs, faster clearance from the bloodstream by the spleen and killing by the WBCs and elimination before bursting and reinfecting other RBCs (Verra et al., 2007; Allison, 2009).

Homozygous α-thalassaemia state confers protection against SMA (Wellems and Fairhurst, 2005) while β-thalassaemia protects against severe malaria but lead to reduced life expectancy in the homozygous state (Williams et al., 2005b; Kreuels et al., 2009).

In summary it is thought that protection has been attributed to defective parasite growth or to enhanced removal of the parasitized RBCs. Hence it is suggested that enhanced phagocytosis of rings, in early intraerythrocytic form of the parasite, as an alternative explanation for protection in G6PD deficiency (Ruwende et al., 1995; Mombo et al., 2003). It has been shown that *P. falciparum* developed similarly in normal RBCs and in sickle trait, beta- and alpha-thalassemia trait, and HbH RBCs. Enhanced phagocytosis of ring-parasitized mutant RBCs may repre- sent the common mechanism for malaria protection in nonimmune individuals af- fected by widespread RBC mutations(Williams et al., 2005b ; Kreuels et al., 2009).
2.6.2 Cytokine gene variations and malaria outcomes

Previous studies show that *IFNA2* -173AT and *IFNA8* -884TA genotypes, and -173T/-884A (TA) haplotype were associated with increased susceptibility to SMA (Kempaiah *et al*., 2012). IL-18 -607 AA confers protection to SMA and haplotype construct of -137G and -607C (G/C) is associated with increased susceptibility to SMA and mortality in children from western Kenya (Anyona *et al*., 2011).

Variations in the *IL-1β* gene involving the +4845G/T and +3953C/T alleles are associated with increased susceptibility to malaria in parasitaemic children (Walley *et al*., 2004), while *IL-1β* -31C/T, -511A/G and haplotype construct of *IL-1β* -31C/-511A (CA) influences susceptibility to SMA in children from western Kenya (Ouma *et al*., 2008). Previous studies also show that *TNF-α* -308G/A transition and -376A allele are associated with susceptibility to CM among children from Kenya and Gambia (van Heel *et al*., 2002). In addition, *TNF-α* promoter variant -238G/A is associated with protection against malaria infections in Ghanaian children (McGuire *et al*., 1999; Hananantachai *et al*., 2007).

Additional genotypic and haplotypic studies among Thai adults showed that IL12Bpro-2′/3′ UTR-T influences susceptibility to severe malaria (Phawong *et al*., 2010). *IL-12Bpro* (rs17860508) polymorphic variant confers protection against CM in Malian children (Marquet *et al*., 2008). In addition, *IL-12A* (rs2243140) is associated with reduced risk of developing SMA, while *IL-
$I2RB$ (rs429774) is associated with protection from SMA (Zhang et al., 2010). Furthermore $IL12A$ (rs22431348) was associated with reduced risk of severe anaemia and of severe anaemia with any parasitaemia in children from western Kenya (Zhang et al., 2010). However, $IL12RB1$ (rs383483) was linked with susceptibility to high-density parasitaemia (HDP) (Zhang et al., 2010).

### 2.6.3 SDF-1alpha gene

The gene is located in chromosome 10q11.1 made up of 68 amino acid with two receptors CXCR4 and CXCR7 (Singh et al., 2012). $SDF-1\alpha$ is 14.94 kb, has 4 exons, and lacks the uniformed conserved amino acid chain Glutamic-Leucine-Arginine (Glu-Leu-Arg) motif as known with all alpha chemokines (Ratajczak et al., 2006; Ho et al., 2010). There are approximately 50 genes of the chemokines family in humans and 25 receptors that are grouped in four CXCR, CCR, XCR and Cx3R that bind them (Nomiyama et al., 2011). Failure of $SDF-1\alpha$ (CXCL12) binding to it is receptors (CXCR4 and CXCR7) results in development of an inflammatory disease condition - atherosclerosis - due to monocytosis and neutrophilia; a mechanism presumed to lead to development of severe malaria (Garnica et al., 2005; Massberg et al., 2006). Trafficking and localization properties of the CXCL12 is possible due to its expression in the bone marrow in addition to endothelial cells, smooth muscles and leukocytes (Zeiffer et al., 2004; Stellos et al., 2009). $SDF-1\alpha$ is considered a pro-inflammatory molecule associated with autoimmune diseases (Karin, 2010).
The pleiotropic nature of the human SDF-1α (CXCL12) still remains to be explored with the adaptability of its receptors CXCR4 and CXCR7 which serve as the natural receptors for CC- β and CXC -α chemokine ligands (Strieter et al., 2004; Thelen and Thelen, 2008). Mortier et al. investigated the action of the SDF-1α (CXCL12) gene, independently or in synergy with IFN-γ in regulating chemotaxis of leukocytes to sites of inflammation (Mortier et al., 2010). SDF-1α and SDF-1β are the major isotypes with the two playing a crucial role in the activation and signalling cascade (Ray et al., 2012), with a structural and functional classification (Mantovani et al., 2006; Nomiyama et al., 2010) with four alternative splice variants: SDF-1γ, SDF-1δ, SDF-1ε and SDF-1φ (Strieter et al., 2006; Janowski, 2009). CXCL12 exhibits diverse functions on inducement with IL-1β, IL-12, TNF-α, TGF-β2, IFN-γ, lipopolysaccharides and NO, including regulation of angiogenesis, cancer progression, chronic inflammation, and HIV infection outcomes (Kim et al., 2010; Lee and Jo, 2012; Zhuo et al., 2012). SDF-1α (CXCL12) is produced constitutively in the bone marrow (BM) and retains hematopoietic stem/progenitor cells in the BM microenvironment (Lapidot et al., 2005; Son et al., 2006; Rankin, 2012). SDF-1α suppress erythropoiesis by down-regulating BFU-E numbers and glycoporphin-A expression, and up-regulating CD95L (Gibellini et al., 2000). In murine malaria models, increased expression of SDF-1α promotes control of parasitaemia (Garnica et al., 2003; Garnica et al., 2005).
Further studies on the $SDF1\alpha$ rs1801157(801A/G)3’ UTR variability predicts CD34+ cell mobilization, survival and development of multiple cell lines (Brambilla et al., 2000; Benboubker et al., 2001; Dar et al., 2006). Additional studies show that a point mutation in the 3’ untranslated region of the $SDF1\alpha$ gene (801G/A) inhibits progression of HIV-1 infection to AIDS (Reiche et al., 2006). Furthermore, $SDF1\alpha$ gene (801G/A) is also a risk factor in the development of cancer (Gong et al., 2012). In the recent past, Genome wide association studies (GWAS) studies on cardiovascular disease manifestations and outcomes is directly linked to $SDF1\alpha$ functional variations at rs1801157, rs501120 and rs1746048 (Franceschini et al., 2011; Schunkert et al., 2011). In addition to the risk, locus 10q11 relates to $SDF1\alpha$ level transcript regulation (Mehta et al., 2011). Due to the ability of the β and α chemokine subclasses to modulate severe malaria outcome - MIP-1β promotes trafficking and homing of erythroid progenitor cells and counter-regulates the erythropoietic inhibitory activities of MIP-1α (Majka et al., 2000; Armah et al., 2007) - it is likely that MIP-1β protects against development of SMA through enhancing appropriate erythropoiesis.

Levels of β-chemokines, have also been shown to increase with malaria severity; MIP-1α and β levels increase with severity of the malaria disease (Ochiel et al., 2005), while levels of RANTES decreases under the same circumstances (Were et al., 2006). Promoter SNP in $CCL5$ gene at rs2107538 -403 G/A and -28 C/G has been found to be associated with coronary artery
disease, while the homozygous mutants -403A and -28 G are known to upregulate transcriptional factors hence increase in RANTES levels which are protective through delay of the HIV-1 progression to AIDS in the infected persons (McDermott et al., 2000; Simeoni et al., 2004). These studies on chemokines provide a basis for studying variations in SDF1a gene.

2.6.4 Effector molecule polymorphism

Heterozygous state at the -954G/C loci of the Nitric oxide synthase (NOS) promoter has been associated with protection against recurrent malarial attacks in Gabonese children (Kun et al., 2001). In addition, NOS2 promoter -1173 C/T SNP polymorphism is associated with ability to confer protection against CM and SMA (Hobbs et al., 2002) as well as development of severe malaria (Kun et al., 1998). Furthermore, it has been shown that microsatellite repeats in NOS2 (CCTTT)n variant play a major role in the production of NO and are associated with severe malaria outcomes in Gambian and Gabonese children (Kun et al., 2001; Burgner et al., 2003), thus implying that these polymorphisms play a greater role in determining disease course. A further study on the COX-2 transcripts in the autoimmune diseases has identified functional variants in rs20417 to be associated with resistance to aspirin while a -1195 G/A was associated with digestive system cancer (Dong et al., 2010). Other studies have also shown an association of the rs20417, rs689466 and rs2066826 with coronary cancer and the C minor allele of the rs20417 to be a associated with atherosclerosis plaque development (Rudock et al., 2009; Maguire et al., 2011).
CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

This thesis research was conducted at Siaya County Referal, Hospital which is located in Siaya County, western Kenya.

![Siaya map](image)

**Figure 3.1: Siaya map modified from (Ong`echa et al., 2006).**

The study area is located 67 km north of Kisumu city and lies between the longitude 33° 58’ east to 34° 33’ west and latitude 0° 26’ south to 0° 18’ degrees north. Siaya county is located in northeast of western Kenya, lies in an equatorial region at an altitude of 1140-1430m above sea level and receives
800-2000 mm of rain, a mean average of 1446 mm rainfall between years, 2003 and 2009 and temperature range of 15-30°C, annually (ROK, 2001; Hamel et al., 2011). It is bordered by Busia, Vihiga, Kakamega and Kisumu counties. It is approximately 1520 sq. km in size. It has 3 main geomorphological areas; dissected uplands, moderate lowlands and the Yala swamp. Siaya County is a P. falciparum holoendemic transmission region where residents experience perennial malaria exposures with entomological inoculation rate (EIR) less than 10 (Hamel et al., 2011). Peak malaria transmission in this area follows the seasonal rains occurring during and after the long rains in April to August and short rains in November to January (Hamel et al., 2011).

The population of Siaya according to the 2009 population census was 842,304 comprising of approximately 398,385 males and 443,361 females with infants and children under 5 years mortality rates of 17.6% and 25.7%, respectively (MoP, 2009; Kenya Census Report, 2009). Residents of the study area are culturally homogenous and belong to the Luo ethnic group accounting for over 96% of the population (Kenya Census Report, 2009). Under 5 year mortality rate reduced from 24.1% in 2003 to 13.7% in 2007 but a steady increase was realised in 2008 at 21.2% and into 2/3 of the year 2009 with malaria anaemia being a major contributor (Hamel et al., 2011). Malaria prevalence is 55% in Siaya County (Okiro et al., 2010).
3.2 Study design
This study was conducted as part of an on-going prospective study investigating the genetic and immunological basis of malarial outcomes in infants and young children. The current study used a cross-sectional design to determine the associations between $SDF1\alpha$ haplotypic and genotypic variations and malarial outcomes.

3.3 Study population
Children aged between 3 and 36 months presenting with acute malaria were stratified into two groups: SMA (Hb<5.0 g/dL and any density asexual $P. falciparum$ parasitaemia), and non-SMA (Hb≥5.0 g/dL and any density asexual $P. falciparum$ parasitaemia). Determination of the sample size was based on the formula (Creative Research Systems, 2003):

$$SS = \frac{Z^2 \times (p) \times (1-p)}{c^2}$$

Where,

SS = sample size

$Z = Z$ value (1.96 at 95% confidence level)

$P = \text{proportion of 7C haplotype (0.614 ) (Awandare et al., 2009)}$

$c = \text{confidence interval}$

Hence,

$$[(1.96)^2 \times (0.61) \times (1-0.61)]/(0.05 \times 0.05) = 366$$

With a 10% error allowance (i.e., 37), a total of 827 study participants were recruited: 118 were SMA, 199 were aaparasiteamic, while 510 were non-SMA controls.
### 3.4 Inclusion and Exclusion criteria

Male and female children of:

i. Age between 3 and 36 months.

ii. Resident in study area for at least the last six months.

iii. Presenting with asexual *P. falciparum* malaria parasitaemia and haemoglobin <5.0 g/dl.

iv. Parent/guardian willing and able to sign consent form.

v. Able to keep schedule and study appointments.

vi. Distance to the hospital ≤15km.

vii. Able to provide two contacts familiar with the child’s whereabouts during the study period.

#### Exclusion criteria

i. Children with CM (rare in this holoendemic area).

ii. History of any hook-worm, bacteraemia and HIV-1 related symptoms such as oral thrush.

iii. Clinical evidence of acute respiratory infection.

iv. Prior hospitalization, and reported use of anti-malarial therapy two weeks prior to enrolment.

v. Intent to relocate during the study period.

vi. Children whose parents or legal guardians failed to provide consent to enrol into the study or withdrew their consent after enrollment.
3.4.1 Patient management

Management of the children presenting with malaria commenced immediately after collection of the blood sample through administration of anti-malarial following the Ministry of Health (MoH)-Kenya guidelines. The treatment included the use of oral artemether/lumefantrine (Coartem®) for uncomplicated malaria and intravenous quinine for severe malaria. In addition, supportive care including hematinsics and blood transfusions were provided according to the MoH guidelines (MoH, 2009; MoH, 2010).

3.5 Clinical and laboratory evaluation

Beckman Coulter AcT diff 2™ (Beckman-Counter Corporation, FL, USA) was used to determine full haemograms which included erythrocytic indices [hematocrit (HCT), red blood cells (RBCs), mean corpuscular volume (MCV), MCH, MCHC, RDW], leucocytic indices [(total white blood cell count, (WBCs) monocytes, and granulocytes] and thrombocytic indices [platelet count, platelet distribution width, (PDW) and mean platelet volume (MPV)].

3.5.1 Determination of malaria parasitaemia

Determination of parasitaemia was conducted by microscopic examination of 3% Giemsa-stained thin and thick blood smears. *P. falciparum* parasites were counted against 300 WBC and parasitaemia (/µL) approximated using coulter analyzer generated total WBC count. Cheek buccal swabs were also collected and stored for DNA extraction. All samples were stored frozen at a stable temperature of -80°C until analysis. To mark out severe anemia caused by
malaria against other pro- anemia co-infections such as bacteremia, G6PD deficiency, sickle-cell status and HIV-1 were determined.

3.5.2 HIV-1 Screening

Parents and/or guardians of the study participants received pre- and post-test counselling for HIV/AIDS testing. HIV-1 sero-reactivity was determined using Unigold (Trinity Biotech Plc., Bray, Ireland) and Determine (Abbott Laboratories, Tokyo, Japan) in line with the National guidelines for HIV diagnosis (NASCOP, 2010). Proviral DNA PCR was then performed on all concordant positive and discordant results. Briefly, blood samples were collected on FTA cards and stored dried at -80°C. DNA was extracted from these cards according to Gentra System DNA extraction protocol. A housekeeping gene hG3PDH was used to confirm the presence of DNA extracted through PCR reaction, PCR products were resolved using a 2% agarose gel (Otieno et al., 2006). In summary, a house-keeping gene for the PCR testing was done through use of hG3PDH forward primer (5’-TGA AGG TCG GAG TCA ACG GAT TTG GT-3’) and reverse primer (5’-CAT GTG GGC CAT GAG GTC CAC CAC-3’) with the initial denaturation temperature at 94°C for 2 min, denaturation temperature at 94°C for 45 sec, annealing at 55 °C for 45 sec, extension at 72°C for 2 min for 30 cycles, a final extension at 72°C for 7 min. and finally stored at 4 °C until required.
To determine the HIV status a PCR was performed by the HIV-1 gp40 (5′-TCT TAG GAG CAG GAA GCA CTA TC-3′), gp41 (5′-AAC GAC AAA GGT GAG TAT CCC TGC CT-3′), gp46 (5′-ACA ATT ATT GTC TGG TAT AGT GCA ACA-3′) and lastly gp47 (5′-TTA AAC CTA TCA AGC CTC CTA CTA TCA-3′) primers due to the conservancy nature of gp41 molecule which anchors the gp120 into the membrane of the virion with a cycling condition of initial denaturation of 95°C for 5 min, denaturation temperature at 95°C for 45 sec, annealing at 55°C for 45 sec, extension at 72°C for 1 min for 35 cycles, a final extension at 72°C for 10 min. The PCR products were then resolved through electrophoresis on a 2% ethidium bromide-stained agarose gels and viewed under UV high performance trans-illuminator (UVP, Upland, USA) at 302 nm. HIV-1 PCR positive was defined by the presence of the 460 bp fragment.

3.5.3 Bacteremia screening

Bacteremia was determined using standard microbial cultivation methods (Were et al., 2011). Approximately, 1.0 ml of venipuncture blood was collected aseptically into sterile paediatric isolator microbial tubes (Wampole Laboratories, NJ, USA) for bacterial cultures. The blood samples were inoculated directly onto chocolate agar plates and incubated for 18 hours at 37°C in 5% CO₂, followed by subculture for 18 to 24 hours in an inverted position. If no growth was obtained, sub-cultures were incubated for a further 96 hours. Plates were observed daily for signs of microbial growth and/or
contaminations. Colonial characteristics, appearances and biochemical tests, gram staining, serology and API biochemical galleries (bioMe´rieux, Louvres, France) were used to confirm the existence of blood-borne bacterial pathogens.

3.5.4 Sickle cell status screening

Sickle-cell status was determined by alkaline cellulose acetate electrophoresis on Titan III plates (Helena BioSciences, Sunderland, UK). In summary, haemolysates prepared from blood samples or Hemo AFSC controls were dispensed onto the acetate paper, and haemoglobin variants were separated by electrophoresis with an alkaline buffer at pH 8.6. The plates were then stained using Ponceau S stain, and Hb types were scored using the Hemo AFSC control.

3.5.5 Screening for G6PD deficiency

G6PD deficiencies were determined by a fluorescent mark test following the manufacturer's methods (Trinity Biotech Plc., Bray, Ireland). In summary, blood was haemolyzed and marked onto a filter paper. Assay solution containing glucose-6-phosphate and oxidized NADP (NADP+) was added, and samples were energized with UV light at 340nm. Depending on the occurrence or non-appearance of fluorescence emissions, the samples were scored as standard (high emission, this is score for normal sample),
intermediary (moderate emission, this for moderate deficiency), or lacking (no emission, abnormal sample G6PD deficient).

3.5.6 DNA extraction for genotyping of the SDF1α C-1002T and A+801G

Genomic DNA was extracted from buccal swabs using the Buccal Amp™ DNA extraction kit (Epicentre Biotechnologies, Madison, USA) and thereafter subjected to amplification using GenomiPhi® system (GE Healthcare, Piscataway, NJ, USA) to obtain sufficient quantities of DNA for genetic analyses.

3.6 Genotyping

SDF1α gene variants rs2839686 (C-1002T) (ABI assay identification number; C_15833403_10); and rs2839685(A+801G) (ABI assay identification number; C_15833412_20), were genotyped using the high through-put TaqMan® 5’ allelic discrimination Assay-By-Design method on a Step One Plus Real Time PCR system. Total volume for the Taqman PCR assay was 5 μL with the following amplification protocol 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 17 seconds and annealing and extension at 60°C for 1 minute. Following the above amplification, the genotype of each study individual was assigned automatically by measuring the allelic specific fluorescence on the ABI-PRISM 7900 HT sequence detection systems using the SDS 2.1 software for allelic discrimination (Applied Biosystems, CA, USA).
3.7 Data analysis

Kruskal-Wallis tests tests were used to compare differences in medians for continuous variables such as parasitaemia, age, and haematologic measures. Chi-square ($\chi^2$) tests were used to compare differences in genotype, allele, and haplotype proportions across the study groups (aparasitemic, non-SMA and SMA children). In cases of significant Kruskal-Wallis tests, post-hoc corrections for multiple comparisons in non-parametric ANOVA were done through Dunn’s tests performed to identify the groups that differed significantly. Hardy-Weinberg equilibrium (HWE) was calculated using a $\chi^2$ goodness-of-fit test, and haplotype construction was implemented through use of HPlus software (version 2.5). Binomial logistic regression models were used to examine associations between haplotypes and malarial outcomes controlling for the potential confounding effects of age, gender, bacteraemia, G6PD deficiency, HIV-1, and sickle cell status. Statistical significances for all analyses were set at $P<0.05$. 
CHAPTER FOUR

RESULTS

4.1. Demographic and clinical characteristics of the study participants

The demographic and clinical characteristics of the study participants are summarised in table 4.1. A total of eight-hundred and twenty seven children of both gender and age less than three years old were enrolled and categorized into three groups based on parasitaemia and Hb levels as follows: 1) aparasitaemic \( (n=199) \); 2) non-SMA \( (\text{Hb} \geq 5.0 \text{g/dL}, \ n=510) \); and 3) SMA \( (\text{Hb} < 5.0 \text{g/dL}, \ n=118) \). Gender distribution was similar across the study groups \( (P=0.104) \) but age differed significantly across the study groups with the SMA group having younger children than the aparasitaemic and non-SMA \( (P=0.043) \) groups. The admission axillary temperature was not significantly different across the groups \( (P=0.582) \).

Analyses of hematological indices, indicated that children presenting with SMA had significantly lower Hb levels \( (P<0.0001) \), hematocrit \( (P<0.0001) \), erythrocyte \( (P<0.0001) \) and absolute erythrocyte numbers \( (P<0.0001) \) relative to non-SMA and aparasitaemic group. In addition, the frequencies of suppression of erythropoiesis \( (\text{RPI}<2.0) \) was higher in the SMA group \( (P<0.0001) \).
The parasitological outcomes of parasitaemia was not significantly associated between the SMA and non-SMA groups ($P=0.311$). Moreover, the proportions of children presenting with high-density parasitaemia (HDP; parasites $\geq 10,000/\mu \text{L}$) were comparable between the SMA and non-SMA groups ($P=0.385$).

Genotyping of the red blood cell variants indicated lower frequencies of the sickle cell trait ($P<0.0001$) and glucose-6-phosphate (G6PD) deficiency ($P<0.0001$) in children presenting with SMA. However, the frequencies of the homozygous ($aa/aa$), heterozygous (-$\alpha^{3.7}$/aa) and homozygous mutant variant (-$\alpha^{3.7}$/-$\alpha^{3.7}$) $\alpha$-thalassemia variants was similar across the groups ($P=0.715$).

Evaluation of co-infections indicated higher frequencies of HIV-positive ($P<0.0001$) and comparable bacteremia ($P=0.309$) in the SMA group relative to the non-SMA and aparasitemic children.
Table 4.1. Demographic and clinical characteristics of the study participants

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Aparasitemic, n=199</th>
<th>Non-SMA, n=510</th>
<th>SMA, n=118</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>98 (49.2)</td>
<td>205 (50.0)</td>
<td>68 (57.6)</td>
<td>0.104</td>
</tr>
<tr>
<td>Female</td>
<td>101 (50.8)</td>
<td>205 (50.0)</td>
<td>50 (42.4)</td>
<td></td>
</tr>
<tr>
<td>Age, mos.</td>
<td>9.1 (9.7)</td>
<td>9.8 (8.8)</td>
<td>8.0 (7.3)</td>
<td>0.043</td>
</tr>
<tr>
<td>Admission temperature, °C</td>
<td>37.0 (1.6)</td>
<td>37.6 (1.7)</td>
<td>37.6 (1.5)</td>
<td>0.582</td>
</tr>
<tr>
<td><strong>Hematological Parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>10.1 (4.8)</td>
<td>6.4 (2.3)</td>
<td>4.3 (1.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>31.4 (14.3)</td>
<td>20.8 (8.4)</td>
<td>13.2 (3.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RBCs, × 10^6/µL</td>
<td>4.6 (1.8)</td>
<td>3.2 (1.4)</td>
<td>1.8 (0.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ARN, × 10^9/L</td>
<td>51 (72)</td>
<td>67 (86)</td>
<td>32 (49)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RPI&lt;2, n (%)</td>
<td>-</td>
<td>172 (33.7)</td>
<td>73 (61.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Parasitological Indices</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parasites, /µL</td>
<td>32604 (71223)</td>
<td>29242 (76068)</td>
<td>311</td>
<td></td>
</tr>
<tr>
<td>HDP (≥10,000 parasites/µL), n (%)</td>
<td>373 (73)</td>
<td>83 (70)</td>
<td>0.385</td>
<td></td>
</tr>
<tr>
<td><strong>Genetic Variants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sickle cell trait, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbAA</td>
<td>6 (60)</td>
<td>2 (20)</td>
<td>2 (20)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HbAS</td>
<td>29 (26.1)</td>
<td>74 (66.7)</td>
<td>8 (7.2)</td>
<td></td>
</tr>
<tr>
<td>HbSS</td>
<td>111 (22.9)</td>
<td>433 (61.7)</td>
<td>108 (15.4)</td>
<td></td>
</tr>
<tr>
<td>G6PD normal, n (%)</td>
<td>8 (21.1)</td>
<td>29 (76.3)</td>
<td>1 (2.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>G6PD heterozygotes</td>
<td>26 (27.7)</td>
<td>63 (65.6)</td>
<td>7 (7.3)</td>
<td></td>
</tr>
<tr>
<td>G6PD deficient</td>
<td>146 (27.7)</td>
<td>391 (60.8)</td>
<td>106 (16.5)</td>
<td></td>
</tr>
<tr>
<td>α^-Thalassemia, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αα/αα</td>
<td>77 (23.5)</td>
<td>199 (60.9)</td>
<td>51 (15.6)</td>
<td>0.715</td>
</tr>
<tr>
<td>-α^-/αα</td>
<td>78 (26.6)</td>
<td>174 (36.8)</td>
<td>36 (31.9)</td>
<td></td>
</tr>
<tr>
<td>-α^-/-α^-</td>
<td>72 (22.0)</td>
<td>100 (21.1)</td>
<td>26 (23.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Co-infections</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-1 positive, n (%)</td>
<td>18 (37.5)</td>
<td>10 (20.8)</td>
<td>20 (41.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Bacteremia, n (%)</td>
<td>25 (28.7)</td>
<td>50 (57.5)</td>
<td>12 (13.8)</td>
<td>0.309</td>
</tr>
</tbody>
</table>

Data represented as median, (interquartile range; IQR) unless indicated. The study participants were categorised into aparasitemic (n=199), non-severe malaria anaemia (non-SMA, n=510) and SMA (n=118) groups. SMA was defined using the WHO criteria as haemoglobin (Hb) concentrations less than 5.0 g/dL, with any density parasitaemia. Statistical data analysis was conducted using the the Kruskal-Wallis test for comparing differences in continuous variables and Chi-square tests for comparing distribution of of proportions across the groups. α-statistical significance was determined by Mann-Whitney U test. Abbreviations: RBCs, red blood cells (erythrocytes); MPS, malaria parasites; HDP, high density parasitaemia; G6PD, Glucose-6- phosphate dehydrogenase deficiency; RPI, reticulocyte production index.
4.2. *SDF-1α* genotypic and allelic distribution in the study population

Genotypic and allelic distribution of the *SDF-1α* gene at the A+801G and C-1002T loci are shown in table 4.2. The genotypic frequencies at the A+801G loci in all the study participants were: AA (90.8%), AG (8.6%) and GG (0.6%). Although the frequencies of the A+801G genotypes were not significantly different across the study groups (*P*=0.136), the pattern of the distribution paralleled that observed for the overall study cohort. The frequencies of the AA, AG and GG genotypes in the SMA (90.7%, 8.5% and 0.8%); non-SMA (89.6%, 9.8% and 0.6%), and aparasitemic (94.0%, 5.5% and 0.5%), respectively were distributed across the study cohorts.

Although A allele was dominant in the overall study cohort and across the study groups (*P*=0.048), the frequencies of the A and G alleles was similar in the overall cohort (0.951 and 0.049); SMA (0.949 and 0.051); non-SMA (0.945 and 0.055); and aparasitemic (0.967 and 0.033) groups.

Analysis of the C-1002T loci indicated that the TT, CT and TT genotype frequencies differed significantly across the groups (*P*=0.008) with the TT genotype dominating in the SMA (TT, 62.7%; CT, 23.7%; and CC, 13.6%), non-SMA (TT, 66.1%; CT, 20.6%; and CC, 13.3%), and aparasitemic (TT, 63.3%; CT, 16.6%; and CC, 20.1%) study groups including the overall study cohort (TT, 64.9%; CT, 20.1%; and CC, 15.0%), respectively.
The C-1002T allele distribution also differed significantly across the groups \((P=0.017)\). The T allele was the most frequent in all the study groups relative to the C allele. The distribution of the frequencies of the T and C alleles was similar in the overall cohort (0.75 and 0.25); SMA (0.75 and 0.25); non-SMA (0.76 and 0.24); and aparasitemic (0.72 and 0.28) groups, respectively.

### Table 4.2. Genotypic and allelic distribution of SDF-1α polymorphisms in the study participants

<table>
<thead>
<tr>
<th>Genotype (dbSNP ref.)</th>
<th>Aparasitemic (n=199)</th>
<th>Non-SMA (n=510)</th>
<th>SMA (n=118)</th>
<th>Total (n=827)</th>
<th>(P^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA, n (%)</td>
<td>187 (94.0)</td>
<td>457 (89.6)</td>
<td>107 (90.7)</td>
<td>751 (90.8)</td>
<td></td>
</tr>
<tr>
<td>AG, n (%)</td>
<td>11 (5.5)</td>
<td>50 (9.8)</td>
<td>10 (8.5)</td>
<td>71 (8.6)</td>
<td>0.136</td>
</tr>
<tr>
<td>GG, n (%)</td>
<td>1 (0.5)</td>
<td>3 (0.6)</td>
<td>1 (0.8)</td>
<td>5 (0.6)</td>
<td></td>
</tr>
<tr>
<td>Allele (G) frequency</td>
<td>0.033</td>
<td>0.055</td>
<td>0.051</td>
<td>0.049</td>
<td>0.048</td>
</tr>
<tr>
<td>C-1002T (rs2839686)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>126 (63.3)</td>
<td>337 (66.1)</td>
<td>74 (62.7)</td>
<td>537 (64.9)</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>33 (16.6)</td>
<td>105 (20.6)</td>
<td>28 (23.7)</td>
<td>166 (20.1)</td>
<td>0.008</td>
</tr>
<tr>
<td>CC</td>
<td>40 (20.1)</td>
<td>68 (13.3)</td>
<td>16 (13.6)</td>
<td>124 (15.0)</td>
<td></td>
</tr>
<tr>
<td>Allele (C) frequency</td>
<td>0.28</td>
<td>0.24</td>
<td>0.25</td>
<td>0.25</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Data shown represent number \((n)\) and proportions \((\%)\) of the SDF1α genotype frequencies at the C-1002T and A+801G loci. Aparasitemic (Giemsa-stained smear negative, \(\leq 0\) \(P. falciparum\) parasites/\(\mu\)L); SMA, severe malaria anaemia; Hb, hemoglobin \((\text{Hb}<5.0\ \text{g/dL})\) and Non-SMA, \(\text{Hb}>5.0\ \text{g/dL}.\) Minor allele frequencies \((G)\) at the A+801G and \((C)\) at the C-1002T loci are shown. \(P^*\), statistical differences in the genotype frequencies across the aparasitaemic, non-SMA and SMA groups were performed using the Pearson's \(\chi^2\) tests. Values in bold denoting statistical significance at \(P\)-values indicated.
4.3. Association of SDF1α genotypes with parasitaemia and high density parasitaemia

The association between the SDF-1α A+801G and C-1002T genotypes and presence of parasitaemia and high-density parasitaemia are shown on table 4.3. Analysis of the association of the A+801G genotypes with presence or absence of parasitaemia, and high-density parasitaemia in the study groups indicated that the AG genotype was significantly associated with about two-times higher odds of having parasitaemia (OR=1.889; 95% CI = 1.140-3.129; P=0.014); but 52% lower odds of developing high-density parasitaemia (OR=0.480; 95% CI=0.284-0.812; P=0.006) in children with malaria in reference to the AA genotype. However, the mutant GG genotype was not significantly associated with parasitaemia (OR=1.533; 95% CI=0.309-7.598; P=0.601) and high-density parasitaemia (OR=0.898; 95% CI=0.175-4.611; P=0.898) in reference to the AA genotype.

At the C-1002T loci, the heterozygous CT genotype was neither significantly associated with the presence of parasitaemia (OR=1.889; 95% CI=0.864-1.567; P=0.306) nor high-density parasitaemia (OR=1.225; 95% CI=0.882-1.702; P=0.225) in reference to the TT genotype. Importantly, the homozygous CC genotype was significantly associated with 43% lower odds of having parasitaemia (OR=0.568; 95% CI=0.406-0.794; P=0.001) but not with high-density parasitaemia (OR=1.052; 95% CI=0.694-1.594; P=0.810) relative to the reference TT genotype.
Table 4.3. Association between SDF-1α genotypes and parasitaemia and high-density parasitaemia

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Parasitaemia</th>
<th>HDP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>A+801G (rs1801157)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>1.889</td>
<td>1.140-3.129</td>
</tr>
<tr>
<td>GG</td>
<td>1.533</td>
<td>0.309-7.598</td>
</tr>
<tr>
<td>C-1002T (rs2839686)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>1.189</td>
<td>0.864-1.567</td>
</tr>
<tr>
<td>CC</td>
<td>0.568</td>
<td>0.406-0.794</td>
</tr>
</tbody>
</table>

Data shown represent odds ratios (OR) and 95% confidence interval (CI). To determine the association between the SDF1α loci and parasitaemia or high-density parasitaemia (HDP, *P. falciparum* parasitaemia >0 parasites/uL) binary logistic regression modelling controlling for age, gender, HIV-1, α-thalassemia, G6PD deficiency, sickle-cell trait status and bacteremia was performed. The homozygous most-frequent (wild-type, ancestral) genotypes in the population (homozygous ancestral AA and TT genotype) was entered into the model as the reference (Ref) and parasitaemia and HDP as dependent variables.
4.4. Association between *SDF-1α* genotypes and severe malarial anemia and suppression of erythropoiesis

Associations of the *SDF-1α* A+801G and C-1002T polymorphisms and severe malaria anaemia and suppression of erythropoiesis are shown on table 4.4. The AG (OR=0.969; 95% CI=0.635-1.541; *P*=0.963 and OR=0.705; 95% CI=0.409-1.218; *P*=0.210) and GG (OR=0.374; 95% CI=0.056-2.476; *P*=0.308 and OR=0.591; 95% CI=0.113-3.099; *P*=0.534) genotypes were, respectively, not significantly associated with SMA using both the WHO (Hb<5.0 g/dL) and the modified definition of SMA (Hb<6.0 g/dL, with any density parasitaemia) in reference to the AA genotype.

Likewise, at the C-1002T loci, the CT (OR=1.276; 95% CI=0.883-1.844; *P*=0.194 and OR=0.941; 95% CI=0.682-1.297; *P*=0.708) and CC (OR=0.893; 95% CI=0.542-1.470; *P*=0.659 and OR=0.798; 95% CI=0.526-1.212; *P*=0.290) genotypes were, respectively, not significantly associated with SMA using both the WHO (Hb<5.0 g/dL) and the modified definitions of SMA (Hb<6.0 g/dL, with any density parasitaemia) compared to the reference TT genotype.

Analysis of the association between the *SDF-1α* A+801G and C-1002T polymorphisms and suppression of erythropoiesis revealed the AG (OR=1.544; 95% CI=0.987-2.415; *P*=0.057) and GG (OR=6.852; 95% CI=1.153-40.705; *P*=0.034) genotypes were associated with about one-and-a-
half and seven times higher odds of having erythropoietic suppression in reference to the AA genotype.

With regards to the C-1002T loci, no significant associations were noted for the CT (OR=1.012; 95% CI=0.735-1.392; \( P=0.944 \)) nor CC (OR=1.164; 95% CI=0.779-1.741; \( P=0.459 \)) genotypes and suppression of erythropoiesis in reference to the TT genotype (Table 4.4).
Table 4.4. Association between SDF1α genotypes and severe malarial anemia and suppression of erythropoiesis

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>SMA (Hb&lt;6.0 g/dL)</th>
<th></th>
<th>SMA (Hb&lt;5.0 g/dL)</th>
<th></th>
<th>RPI&lt;2.0</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
<td>P</td>
<td>OR</td>
<td>95% CI</td>
<td>P</td>
</tr>
<tr>
<td>A+801G (rs1801157)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>Ref</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>0.969</td>
<td>0.635-1.541</td>
<td>0.963</td>
<td>0.705</td>
<td>0.409-1.218</td>
<td>0.210</td>
</tr>
<tr>
<td>GG</td>
<td>0.591</td>
<td>0.113-3.099</td>
<td>0.534</td>
<td>0.374</td>
<td>0.056-2.476</td>
<td>0.308</td>
</tr>
<tr>
<td>C-1002T (rs2839686)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>Ref</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>0.941</td>
<td>0.682-1.297</td>
<td>0.708</td>
<td>1.276</td>
<td>0.883-1.844</td>
<td>0.194</td>
</tr>
<tr>
<td>CC</td>
<td>0.798</td>
<td>0.526-1.212</td>
<td>0.290</td>
<td>0.893</td>
<td>0.542-1.474</td>
<td>0.659</td>
</tr>
</tbody>
</table>

Data shown are odds ratio (OR) and the 95% confidence intervals (CI). Associations of the A+801G and C-1002T genotypes with SMA and suppression of erythropoiesis was determined using binary logistic regression analyses, in a model controlling for age, gender, HIV-1 infection, α⁺ thalassemia, G6PD deficiency, sickle-cell trait status and the presence of bacteraemia infections. The homozygous most-frequent (wild-type, ancestral) genotypes (AA at the A+801G and TT at the C-1002T loci) were used as references (Ref) in the analyses. SMA, severe malarial anemia (WHO definition, Hb<5.0 g/dL and modified definition, Hb<6.0 g/dL). Suppression of erythropoiesis was defined by reticulocyte production index (RPI) <2.0. Significant P-values are shown in bold.
4.5. Distribution of $SDF-1\alpha$ functional haplotype in the study participants

A summary of the haplotypes constructed from the $SDF-1\alpha$ A+801G and C-1002T are shown on table 4.5. The construction yielded a total of four haplotypes (AT, AC, GT and GC), but none of the haplotypes distribution differed significantly across the study groups ($P>0.05$). The most frequent haplotype was AT in the overall study cohort (70.3%), aparasitemic (68.6%), non-SMA (71.0%) and SMA (70.3%) groups ($P=0.676$). About a quarter (28.1%, 23.5%, 24.6%, 24.8%) of the individuals in the overall study population, aparasitemic, non-SMA and SMA carried the AC haplotype, respectively ($P=0.195$). The frequencies of the GT ($P=0.107$) and GC ($P=0.153$) haplotypes were, however, lower in the total study cohort (4.7% and 0.2%), aparasitaemic (3% and 0.3%), non-SMA (5.4% and 0.1%) and SMA (4.2% and 0.9%), respectively.
Table 4.5 SDF-1α haplotype distribution in the study population

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Aparasitemic n=199</th>
<th>Non-SMA n=510</th>
<th>SMA n=118</th>
<th>Total n=827</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT</td>
<td>136 (68.6)</td>
<td>362 (71.0)</td>
<td>83 (70.3)</td>
<td>581 (70.3)</td>
<td>0.676</td>
</tr>
<tr>
<td>AC</td>
<td>56 (28.1)</td>
<td>120 (23.5)</td>
<td>29 (24.6)</td>
<td>205 (24.8)</td>
<td>0.195</td>
</tr>
<tr>
<td>GT</td>
<td>6 (3.0)</td>
<td>27 (5.4)</td>
<td>5 (4.2)</td>
<td>39 (4.7)</td>
<td>0.153</td>
</tr>
<tr>
<td>GC</td>
<td>1 (0.3)</td>
<td>1 (0.1)</td>
<td>1 (0.9)</td>
<td>2 (0.2)</td>
<td>0.107</td>
</tr>
</tbody>
</table>

Data shown represents number (n) and proportions (%) of the SDF-1α haplotypes constructed from the C-1002T and A+801G polymorphisms. Statistical differences in haplotype frequencies across the study groups was examined using the Pearson's χ² tests. SMA, severe malarial anaemia was defined according to the WHO criteria of haemoglobin less than 5.0 g/dL with parasite density of any density.

4.6. Association between SDF-1α haplotypes and parasitological outcomes

The association of the haplotypes with parasitaemia and high-density parasitaemia are shown on table 4.6. Although carriage of the most frequent haplotype, AT was not significantly associated with parasitaemia (OR=1.169; 95% CI=0.894-1.529; P=0.253) or high-density parasitaemia (OR=1.092; 95% CI=0.809-1.475; P=0.565), carriage of the AC haplotype was significantly associated with 26% likelihood of having parasitaemia (OR=0.740; 95% CI=0.560-0.977; P=0.034) but not of developing high-density parasitaemia (OR=1.061; 95% CI=0.773-1.456; P=0.714).
The GT haplotype carriage was associated with about two-times likelihood of having parasitaemia (OR=1.876; 95% CI=0.962-3.658; \( P=0.065 \)) and were significantly, 55% less-likely of having high-density parasitaemia (OR=0.452; 95% CI=0.221-0.922; \( P=0.029 \)). However, carriage of the GC haplotype was not significantly associated with parasitaemia (OR=0.690; 95% CI=0.071-6.746; \( P=0.750 \)) and high-density parasitaemia (OR=6.550; 95% CI=0.585-73.36; \( P=0.127 \)).

Table 4.6. Association between SDF1 mRNA haplotypes and parasitological outcomes

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Parasitaemia</th>
<th>HDP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>AT</td>
<td>1.169</td>
<td>0.894-1.529</td>
</tr>
<tr>
<td>AC</td>
<td>0.740</td>
<td>0.560-0.977</td>
</tr>
<tr>
<td>GT</td>
<td>1.876</td>
<td>0.962-3.658</td>
</tr>
<tr>
<td>GC</td>
<td>0.690</td>
<td>0.071-6.746</td>
</tr>
</tbody>
</table>

Data shown are odds (OR) and the 95% confidence intervals (CI). Binary logistic regression analysis for association of SDF1α haplotypes with parasitaemia (presence of >0 \( P. falciparum \) parasites/\( \mu \)L) and high density parasitaemia (≥10,000 parasites/\( \mu \)L HDP) controlling for age, gender, HIV-1 infection, \( \alpha^+ \) thalassaemia, G6PD deficiency, sickle-cell trait status and the presence of bacteraemia infections. The bolden \( P \)-values were significant.
4.7. Association between \( SDF-1\alpha \) haplotypes and severe malarial anemia and suppression of erythropoiesis

Associations of the \( SDF-1\alpha \) haplotypes and SMA and suppression of erythropoiesis are shown on table 4.7. No significant associations were noted between carriage of the AT (OR=0.985; 95% CI=0.682-1.421; \( P=0.934 \)), AC (OR=1.129; 95% CI=0.800-1.594; \( P=0.489 \)), GC (OR=6.636; 95% CI=0.553-79.592; \( P=0.135 \)) and GT (OR=0.536; 95% CI=0.249-1.153; \( P=0.111 \)) haplotypes and SMA using the WHO definition. Likewise, no significant associations were found for the AT (OR=0.854; 95% CI=0.625-1.168; \( P=0.324 \)), AC (OR=1.169; 95% CI=0.873-1.566; \( P=0.295 \)), GC (OR=3.451; 95% CI=0.280-42.475; \( P=0.334 \)) and GT (OR=0.862; 95% CI=0.477-1.557; \( P=0.622 \)) haplotypes with SMA using the modified definition.

Although the AT (OR=1.106; 95% CI=0.814-1.502; \( P=0.520 \)), AC (OR=0.788; 95% CI=0.591-1.051; \( P=0.104 \)) and GC (OR=0.377; 95% CI=0.032-4.482; \( P=0.440 \)) haplotypes were not significantly associated with suppression of erythropoiesis, the GT haplotype was significantly associated with two-times likelihood of having suppression of erythropoiesis (OR=1.979; 95% CI=1.085-3.609; \( P=0.026 \)).
Table 4.7. Association between SDF1α haplotypes and severe malarial anemia and suppression of erythropoiesis

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>SMA (Hb&lt;6.0 g/dL)</th>
<th></th>
<th>SMA (Hb&lt;5.0 g/dL)</th>
<th></th>
<th>RPI&lt;2.0</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
<td>P</td>
<td>OR</td>
<td>95% CI</td>
<td>P</td>
</tr>
<tr>
<td>AC</td>
<td>0.854</td>
<td>0.625-1.168</td>
<td>0.324</td>
<td>0.985</td>
<td>0.682-1.421</td>
<td>0.934</td>
</tr>
<tr>
<td>AT</td>
<td>1.169</td>
<td>0.873-1.566</td>
<td>0.295</td>
<td>1.129</td>
<td>0.800-1.594</td>
<td>0.489</td>
</tr>
<tr>
<td>GC</td>
<td>3.451</td>
<td>0.280-42.475</td>
<td>0.334</td>
<td>6.636</td>
<td>0.553-79.592</td>
<td>0.135</td>
</tr>
<tr>
<td>GT</td>
<td>0.862</td>
<td>0.477-1.557</td>
<td>0.622</td>
<td>0.536</td>
<td>0.249-1.153</td>
<td>0.111</td>
</tr>
</tbody>
</table>

Data shown are odds (OR) and the 95% confidence intervals (CI). Binary logistic regression analysis for association of SDF-1α haplotypes with parasitaemia (presence of >0 *P. falciparum* parasites/µL) and high density parasitaemia (≥10,000 parasites/µL HDP). The bolden *P*-values were significant.
CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Even though up-scaling of insecticide treated bednets and residual in-door spraying, malaria remains a major cause of infectious disease burden in the world (WHO, 2010). Most of the malaria-associated morbidity and mortality is borne by infants and young children below the age of five years in sub-Saharan Africa (WHO, 2014). Severe malaria anaemia is the most common cause of malaria-disease burden in pediatric populations residing in rural African settings where availability and accessibility to quality health care services is largely lacking (Taylor et al., 2006; WHO, 2014).

A number of factors including parasite and host genetic constitution determine whether an individual infected with *P. falciparum* malaria remains asymptomatic or becomes symptomatic and progresses to malaria disease state leading to development of severe disease syndromes such as SMA and high levels of parasitaemia (Mackinnon et al., 2005, Kwiatkowski et al., 2005, Hedrick, 2011).

The AA (90.8%), genotype and the A (0.951) allele at the A+801G locus showed at least 90% dominance in all groups of study participants. However, dominance of the TT (64.9%) genotype and T (0.75) allele at the C-1002T
locus, was only about 63-66%, with the minor allele (C) of approximately 25% in all the study groups. These distributions are consistent with the Hapmap populations from Africa. Hence it possible to assume based on this pediatric study findings that there have been selective pressure to enhance survival in this holoendemic malaria region. This is the first study to my knowledge showing association of SDF-1α gene variants, genotypes /haplotypes distribution in Western Kenya with SMA prone for P. falciparum malaria infections.

Even though this study did not find any significant association of SDF-1α gene genotypes/haplotypes with SMA, for the first time the study reports that heterozygosity (AG) at the A+801G locus and recessive homozygosity (CC) at the C-1002T loci are associated with parasitaemia and high density parasitaemia. While, the AC haplotype carriage has reduced odds for having malaria, and GT haplotype carriage presenting reduced odds for developing high density parasitaemia.

Immune and inflammatory cytokine genes regulate the magnitude and course of the host response to P. falciparum infections (Ouma et al., 2010, Ong'eche et al., 2011; Perkins et al., 2011; Were et al., 2011), however, the role of chemokine gene variations in regulating the host response to malaria has previously not been examined. This study, therefore delineated variation in the SDF1α gene at the A+801G and C-1002T loci and their association with clinical measures of P. falciparum malaria (presence of parasitaemia, high-
density parasitaemia, SMA, and suppression of erythropoiesis) in children at Siaya County Referal Hospital.

The prevalence of the A+801G variants observed in the Siaya, Luo population (88% AA genotype and 0.951 for the A allele) is similar to that observed for the HapMap populations: Yoruba from Ibadan, Nigeria (92% for AA genotype and 0.923 for the A allele); Luhya from Webuye, Kenya (88% for AA genotype and 0.878 for the A allele); and Masai from Kinyawa, Kenya (91% for AA genotype and 0.909 for the A allele) (International-HapMap-Consortium, 2010). Unlike the A+801G variation, the dominance of the TT genotype and T allele at the C-1002T locus, was only about 63-66%, with the minor allele (C) of approximately 25% in all the study groups. These distribution is consistent with the HapMap populations from Africa: Yoruba from Ibadan, Nigeria (65% for TT genotype and 0.649 for the T allele); Luhya from Webuye, Kenya (69% for TT genotype and 0.693 for the T allele); and Masai from Kinyawa, Kenya (3.2% for TT genotype and 0.151 for the T allele) (International-HapMap-Consortium, 2010). Overall, it appears that similar genetic mechanisms are acting upon the A+801G and C-100T loci (or their haploblocks) across the indigenous populations of sub-Saharan Africa. Consequently, the significant departure from HWE at the A+801G and C-1002T loci (in all the study groups including the overall cohort), suggests possible selection for the mutant G and C alleles in this population.
It is possible that the suggested selection pressure at the A+801G and C-1002T loci are driven by frequent bombardment of the human genome with *P. falciparum* infections, that arose and evolved with the agricultural revolution in Africa (Kwiatkowski, 2005; Hedrick, 2011), compared to the other human malaria parasites (*P. knowlesi, P. malariae, P. ovale,* and *P. vivax*) (Cox-Singh et al., 2008; WHO, 2014). This is supported by findings showing significant associations between heterozygosity (AG) at the A+801G locus and recessive homozygosity (CC) at the C-1002T loci with parasitaemia and high density parasitaemia. While AG carriage is associated with nearly two-times odds for having malaria infection, it seems to reduce development of high-density parasitaemia by 52%. Moreover, the CC genotype at the C-1002T is associated with 43% reduced odds for having malaria infection. This is further supported by A+801G and C-1002T haplotype analysis illustrating that AC haplotype carriage has 26% reduced odds for having malaria, and GT haplotype carriage presenting 55% reduced odds for developing high density parasitaemia.

This result is supported by murine studies showing that SDF-1α protects against *P. chabaudi* malaria infection by inhibiting NOS2 expression and NO production (Garnica et al., 2002; Garnica et al., 2003) and also control parasitaemia in *P. berghei*-infected murines through establishment of favourable environment for spleen dendritic cells for malaria parasites elimination (Garnica et al., 2005). Additional studies, indicate that *SDF1α* genetic variation regulates outcomes in the context of HIV-1 infection,
hematopoiesis, and cancer (Benboubker et al., 2001; Zhu et al., 2014). Further studies on SDF-1α have also show that it has protective effects against autoimmune diabetes (Aspord et al., 2004; Aboumrad et al., 2007). Altogether, the two SDF1α loci (A+801G and C-1002T or associated haploblocks) may be undergoing differential selection for malaria protection and/or disease.

Although no significant associations were noted for A+801G and C-1002T genotypes and haplotypes with SMA using both the WHO (Hb<5.0 g/dl) and modified (Hb<6.0 g/dl) definitions, the GG genotype at A+801G locus and GT haplotype were associated with nearly seven- and two-times increased odds of developing RPI<2.0 (suppression of erythropoiesis) in children with malaria. These observations are consistent with in vitro studies showing that SDF-1α reduces formation of erythroid blast-forming units (BFU-E) (Bassini et al., 2000); and murine studies showing that SDF-1α suppresses erythropoiesis (Benboubker et al., 2001; Ben Nasr et al., 2011).

In addition, a possible effect of genetic variation in the SDF1α gene was revealed by in vitro studies demonstrating that the rs1801157 in the SDF1α gene is associated with CD34+ haematopoietic progenitor cell mobilization (Feng et al., 1996; Muller et al., 2001; Benboubker et al., 2001). It therefore, appears that the genetic variants A+801G and C-1002T in the SDF1α gene may regulate the erythrocytic outcomes of malaria through regulating erythropoiesis.
5.2 Conclusions and recommendations

In conclusion, findings of this study indicate that variation in the SDF-1α gene, at least in part, genetically modulates parasitological and erythrocytic outcomes of malaria in paediatric populations living in high P. falciparum transmission regions, such as western Kenya. These results demonstrate that:

i. Although variation at C-1002T in the SDF-1α promoter and UTR region A +801G appears to protect against acquisition of P. falciparum infection, this variants may not affect malaria disease outcomes once an individual becomes infected.

ii. SDF-1α mRNA expression is suppressed in children with SMA. This could be attributed to variation at the SDF-1α functional region, which may impact on appropriate erythropoiesis.

5.3 Recommendations

i. Homozygous individuals for the SDF1α genotype GG and CC had a reduced risk of developing SMA hence could act as an important biomarker in malaria disease scientific research.

ii. In addition, heterozygous individuals (AG genotype) have an association with parasitaemia and HDP. As well it showed increased risk of developing suppression of erythropoiesis this genotype will also act as biomarker hence results will assist medics on drug trials and
healthcare providers to tailor individual child therapy due to genotyping/analysis.

iii. AC haplotype carriage has reduced odds for having malaria, and GT haplotype carriage presenting reduced odds for developing high density parasitaemia hence both are important biomarkers in malaria. These results will assist healthcare providers redirect the limited resources to those in agent need and increased risk hence efficiency and reduced child mortality.

5.4 Gaps for Future Research

i. A longitudinal study should be designed to exhaustively and extensively study *SDF-1α* gene, since this were only two SNPs with a small sample size.

ii. Genome wide association studies (GWAS) to identify more genotypic, allelic and haplotypic blocks that control the *SDF-1α* pathway in malaria infection in holoendemic regions.

iii. Further studies will be crucial in understanding how *SDF-1α* gene pathway interacts and the regulation mechanisms of gene expression during malaria disease outcomes.

For the first time its reported in this study findings that although *SDF1α* gene variants A+801G and C-1002T appear to protect against acquisition of *P. falciparum* infection, they may not affect malaria disease outcomes once an individual becomes infected.
REFERENCES


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PATH. (2010) PATH Malaria Vaccine Initiative. The PATH *Malaria Vaccine Initiative (fact sheet).*


APPENDICES

Appendix I: Ethical Approval - KEMRI

KENYA MEDICAL RESEARCH INSTITUTE

This is to inform that during the 150th meeting of the KEMRI/ERC meeting held on the 14th of June, 2011, the Committee conducted the annual review and approved.

Future plans are to continue enrolment

This approval is valid from today June 14, 2011 through to June 14, 2012. Please note that authorization to conduct this study will automatically expire on June 14, 2012.

If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the ERC secretariat by April 14, 2012.

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SSC and ERC for review prior to initiation.

Yours sincerely,

Caroline Kimani,
FOH; Secretary
KEMRI/ETHICS REVIEW COMMITTEE

In Search of Better Health
Appendix II: Ethical Approval - UNM

KENYA MEDICAL RESEARCH INSTITUTE
PO. Box 54810-00200, NAIROBI, Kenya
Tel: (254) (020) 2722641, 2723368, 0722-205901, 0733-408003; Fax: (254) (020) 2720030
E-mail: director@kemri.org  info@kemri.org  Website: www.kemri.org

KEMRI/RES/7/3/1 October 28, 2013

TO: PROF. DOUGLAS J. PERKINS, (PRINCIPAL INVESTIGATOR)

ATTN: JOHN MICHAEL ONG’ECHA

THROUGH: DR. STEPHEN MUNGA
ACTING DIRECTOR, CGHR
KISUMU

Dear Sir,

RE: SSC PROTOCOL No. 696: RESUBMISSION (REQUEST FOR ANNUAL RENEWAL):
GENETIC BASIS OF SEVERE MALARIAL ANEMIA.

Reference is made to your letter dated 22nd October, 2013. The ERC Secretariat acknowledges receipt of 5 copies of abstracts and 5 copies of manuscripts developed during the period under review on October 24, 2013.

This is to inform you that the Ethics Review Committee (ERC) reviewed the document listed above and approved the application for another year.

This approval is valid from today, 28th October 2013 through to 27th October 2014. Please note that authorization to conduct this study will automatically expire on 27th October 2014.

If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the ERC secretariat by 15th September 2014.

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SSC and ERC for review prior to initiation.

Yours faithfully,

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

In Search of Better Health